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Localization of putative transcription initiation site on the cloned rDNA fragment of Tetrahymena pyriformis

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ABSTRACT

A DNA fragment (1.4 Kb) which codes for 5' region of 35S ribosomal precursor RNA (pre-rRNA) in Tetrahymena pyriformis was cloned with pBR322. The fragment was cleaved from the central part of the palindromic rDNA with restriction endonuclease KpnI and HindIII, and ligated to the larger moiety of pBR322 DNA-HindIII-BamHI fragment together with λDNA-KpnI-BamHI fragment through trimolecular ligation. The analysis of R-loop formed between KpnI-linearized recombinant plasmid and 35S pre-rRNA revealed a DNA:RNA hybrid region of 465±30 base pairs in length. Considering the contraction of DNA:RNA hybrids relative to DNA duplexes (Philippsen et al., J. Mol. Biol., 123, 387-404, 1978), the size of the hybrid region was corrected to about 490 base pairs. Alternatively, the size of DNA which was protected against nuclease S1 due to hybrid formation with 35S pre-rRNA was estimated to be 490 nucleotides long. These data indicate that the transcription initiation site is localized at about 490 base pairs from the HindIII site of the cloned rDNA fragment.

INTRODUCTION

In macronucleus of Tetrahymena, the genes coding for ribosomal 17S and 25S RNAs (rDNA) exist as free palindromic molecules with a molecular weight of about 13 x 10^6 daltons (1,2). These rDNAs are packaged in a number of extrachromosomal nucleoli and synthesize 35S RNA as the primary precursor to cytoplasmic ribosomal RNAs (3). The majority of 35S RNA have been shown to be the primary transcript of this gene since they possess triphosphate residues at the 5' termini (3). It has been shown that the activity of these genes is under physiological control; at the logarithmic phase these genes are active while at the stationary phase or when cells are starved they are relatively inactive in the synthesis of 35S pre-rRNA (4,5).

Stimulated by these facts, we have been interested in studying the mechanism controlling the activity of these genes through the following two approaches. On the one hand, we have worked out the procedure to isolate extrachromosomal nucleoli in which free rDNA resides in its native functional

state (6). Along this line studies have been performed to examine the kinds, amount and distribution of the proteins on the rDNA and the conformation of rDNA in nucleolar chromatin. As an alternative approach in the search for the DNA sequences and cellular factors controlling the transcription, we have been trying to construct a faithful *in vitro* transcription system which mimics the events *in vivo*. Several reports along this line, but in other species, have recently appeared (7-9). In this paper we report the cloning of a *Tetrahymena* rDNA fragment, on which the putative transcription initiation site has been localized.

MATERIALS AND METHODS

Cloning

rDNA-KpnI-HindIII (0.98 x 10^6 d, termed K-H fragment), pBR322 DNA-HindIII -BamHI (2.51 x 10^6 d) and λDNA-KpnI-BamHI (2.26 x 10^6 d) fragments (see Fig. 1) were obtained by digesting respective DNA molecule with appropriate restriction enzymes. In each case the digested DNA fragments were separated by agarose gel electrophoresis and the fragments were eluted from a corresponding band. The three kinds of DNA fragments were mixed together and the ligation was performed with T4 ligase in a standard reaction mixture. Transformation of E.coli K12 LE392 was performed according to Norgard *et al.*(10) and the transformants were selected by the use of L-agar plates containing 50 μg/ml of ampicillin. All the ampicillin resistant colonies were picked up and screened for the presence of inserted K-H fragment hy the "mini-prep" procedure according to Klein *et al.*(11). One of the clones, termed pTpr14, was selected and analyzed in detail.

Determination of the Tss value of cloned rDNA fragment

Tss, the temperature at which the DNA is irreversibly denatured to single strands, was determined by agarose gel assay developed by Rosbash *et al.* (12). About 5 μg of pTpr14 DNA digested with HindIII and KpnI was dissolved in 100 μl of 70% deionized formamide-0.1 M Pipes (pH 7.8)-0.01 M EDTA and aliquots of 7 μl each were taken in siliconized test tubes. They were incubated at various temperatures for 3 min. At the end of incubation, 10 μl of ice-cold quench buffer (8 mM Tris-HCl (pH 7.5)-0.8 mM EDTA-20 % glycerol containing bromophenol blue and xylene cyanol) was added while quick mixing and the samples were immediately placed in a dry ice-ethanol bath. The samples were applied to 1 % agarose gel and electrophoresed at 3 V/cm overnight at 4°C.

Isolation of 35S pre-rRNA

RNA was extracted by the SDS-hot phenol procedure (13) from a crude nucleolar preparation (described elsewhere) and fractionated by electrophoresis on 2.4 % polyacrylamide gel containing 10 %(v/v) glycerol (14). The gel was frozen in dry ice-ethanol and the band of 35S pre-rRNA was cut out. The RNA was eluted from gel slices and precipitated with ethanol in the presence of 50 µg/ml of E.coli tRNA.

R-loop analysis

The 35S pre-rRNA and pTpr14 DNA linearized by KpnI (or BamHI) were precipitated together with ethanol. The pelleted nucleic acids were dried *in vacuo* and dissolved in 10 µl of R-loop buffer (70 % v/v deionized formamide, 0.1 M Pipes, pH 7.8, 10 mM EDTA, 0.002 %(v/v) diethylpyrocarbonate). The hybridization mixture was sealed in a 10 µl capillary tube and incubated for 2 hrs at 44.5°C. After the reaction, the mixture was treated with glyoxal as described by Kaback *et al.* (15). An aliquot was spread for electronmicroscopy by the cytochrome C procedure according to Davis *et al.* (16). Col EI DNA (4.2×10^6 d) was added as a size marker. The nucleic acids were picked up with a Parlodion-coated grid and the grids were rotary-shadowed with Pt-Pd at an angle of 7°. Observations were performed with a JEM-100CX electronmicroscope with an acceleration voltage of 80 KV. Contour length measurements were carried out with a Numonics Calculator.

Nuclease S1 protection mapping

This was performed according to Berk and Sharp (17). The cloned rDNA fragment (K-H fragment) was cut out from pTpr14 DNA with HindIII and KpnI and labeled with [γ-^{32}P]ATP and polynucleotide kinase. About 0.01 µg of the labeled rDNA fragment and 0.1 µg of purified 35S RNA (or cytoplasmic 17S RNA) were coprecipitated. 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 2.5 hrs. At the end of incubation, 200 µl of ice-cold S1 buffer containing 0.25 M NaCl -0.03 M CH_3COONa (pH 4.6)-1 mM $ZnSO_4$ was added and the sample was quickly chilled at 0°C. Then 60 units of nuclease S1 was added and the mixture was incubated at 37°C for 30 min. After extraction with phenol, the radioactive material was precipitated with 2 volumes of ethanol and dissolved in 5 µl of 80 % deionized formamide-10 mM NaOH-1 mM EDTA-0.05 % bromophenol blue-0.05 % xylene cyanol. After heating at 90°C for 2 min, the samples were run on a 5 % acrylamide-8.3 M urea gel. The gel was dried and autoradiograghed with a Fuji Rx-s film and a DuPont Cronex Lightening Plus Screen at -80°C.

Enzymes and radioisotopes

Restriction enzymes were obtained from Takara Shuzo Co., Bethesda Research Laboratories, Inc. and New England Biolabs Inc.. [γ-^{32}P]ATP was purchased from Radiochemical Centre, Amersham. Polynucleotide kinase was obtained from Boehlinger-Mannheim. Nuclease S1 was from Sigma. Alkaline phosphatase was from Worthington Biochemical Corp.

Physical containment for cloning experiments

The cloning experiments were performed under P2 level conditions according to the "Guidelines for Recombinant DNA Experiments" of the Ministry of Education, Science and Culture, Japan.

RESULTS

Cloning of KpnI-HindIII fragment of rDNA

According to Niles and Jain (18), the transcription initiation site for 35S pre-rRNA was mapped in a rDNA fragment flanked by the KpnI and the HindIII site, termed K-H fragment in this paper (Fig. 1a). We have confirmed these

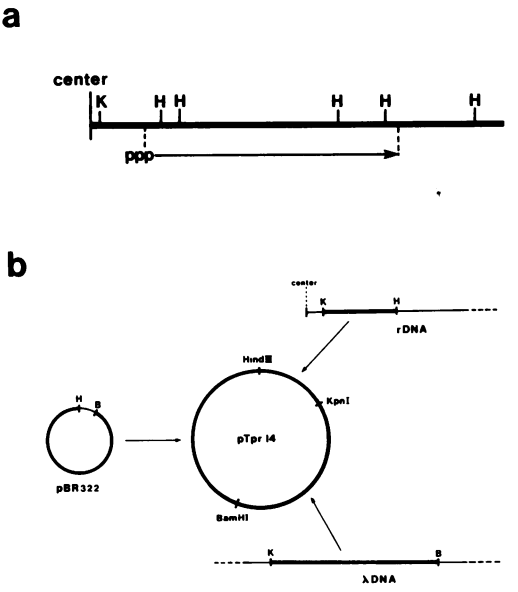

Fig. 1.a. KpnI and HindIII sites on half the palindrome of Tetrahymena pyriformis rDNA. Transcribed region and the direction of transcription are also indicated. Drawn according to Niles and Jain (19).
b. Strategy for cloning K-H fragment.

restriction sites for HindIII (19) and KpnI (data not shown) in our batch of Tetrahymena pyriformis GL. The cloning strategy is shown in Fig. 1b. rDNA-KpnI-HindIII fragments (0.98 x 10^6 d) and pBR322-HindIII-BamHI fragments (2.51 x 10^6 d, containing Amp region) were isolated by agarose gel electrophoresis. In order to ligate the KpnI site to plasmid vector pBR322 which lacks a KpnI site, the KpnI-BamHI fragments (2.26 x 10^6 d) derived from λDNA were used as an intermediate linker (Fig. 1b). The three kinds of DNA fragments were mixed together and ligation reaction was performed as illustrated in Fig. 1b. On transformation and subsequent selection, eight ampicillin resistant clones appeared and each, upon digestion by HindIII and KpnI, gave three kinds of DNA fragments used in ligation reaction in a 1:1:1 molar retio. The cleavage pattern of some of the clones are shown in Fig. 2a. One of them (termed pTpr14) was arbitrarily selected and used for further analyses. From Fig. 2b, the cloned K-H fragment in pTpr14 has a size of about 1400 base pairs. The length of K-H fragment was also determined by electronmicroscopy. In accordance with the result of gel electrophoresis, contour length measurement gave a value of 1387 ± 67 base pairs (data not shown).

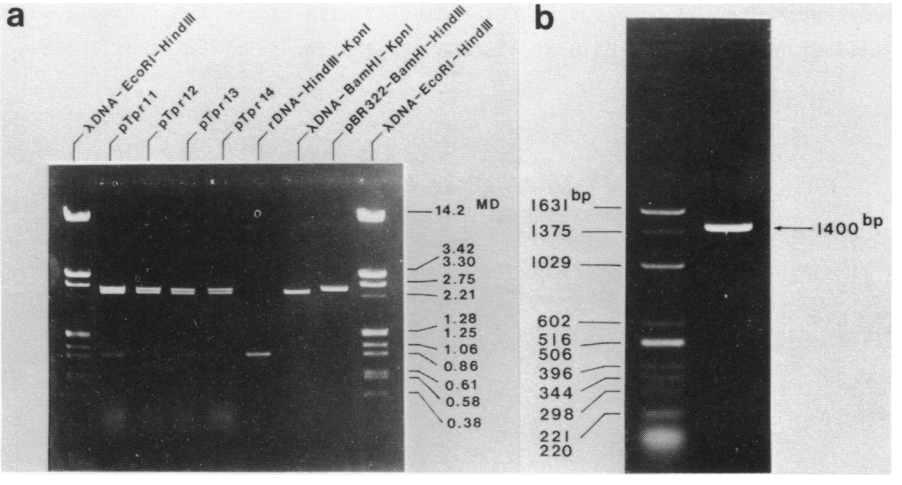

Fig. 2.a. Cleavage patterns of recombinant DNAs doubly digested with HindIII and KpnI. DNA fragments were electrophoresed on a 0.6 % agarose gel.
b. Size determination of K-H fragment. The fragment was electrophoresed on a 1.4 % agarose gel. Marker DNA fragments on the left were the mixture of HinfI, HinfI-BamHI and HinfI-HindIII digest of pBR322 DNA.

Determination of Tss of the K-H fragment

As a prerequisite for performing R-loop analysis or nuclease S1 protection mapping, we next determined the Tss of the cloned K-H fragment. This was carried out by the agarose gel assay according to Rosbash et al.(12). From the result shown in Fig. 3, the Tss of the K-H fragment was determined to be 42-43°C.

R-loop analysis

In order to demonstrate that the K-H fragment contains the transcription initiation site, and further to estimate the size of the transcribed region, a R-loop analysis was performed using 35S pre-rRNA. 35S pre-rRNA was isolated from the nucleolar fraction prepared by the recently developed procedure (Higashinakagawa et al., manuscript in preparation) and purified by acrylamide gel electrophoresis (Fig. 4). A faint band just below the 35S RNA band was observed in the gel of Fig. 4 (see arrow). This minor RNA species was also detected by a Niles, designated Y, and considered to be a processing intermediate (3). Due to poor resolution this minor RNA species was enevitably included in cutting out the 35S RNA band and in subsequent purification steps. In the presence of 50 μg/ml E.coli tRNA, 35S RNA remained intact throughout the purification procedure. According to Niles and Jain (18), a R-loop structure of the size of about 400 base pairs (3.9 % of half palindrome) was expected to occur near the HindIII site of the K-H fragment with a tail of unhybridized chain of 35S RNA originating from the HindIII site.

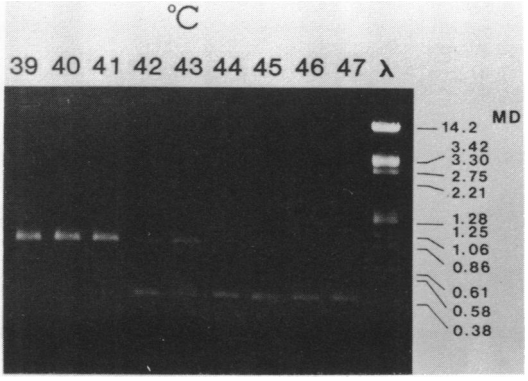

Fig. 3. Determination of Tss of K-H fragment. K-H fragments were incubated in the hybridization buffer at various temperatures indicated at the top of the gel. The mixture was chilled at -80°C and electrophoresed on a 1 % gel at 4°C. λDNA-HindIII-EcoRI was coelectrophoresed (designated λ at the far right side of the gel).

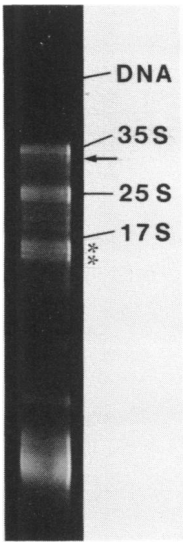

Fig. 4. Electrophoresis profile of RNA extracted from isolated nucleoli. Electrophoresis was carried out on a 2.4 % acrylamide gel containing 10 %(v/v) glycerol (14). The gel was stained with ethidium bromide. Note the faint band (arrow) below 35S band. The two RNA bands (marked by *) below 17S RNA are the cleavage products of 25S rRNA (21).

The observed R-loop shown in Fig. 5a possessed structures fulfilling the above expectations. Further detailed analyses were performed with R-loop formed between pTpr14-KpnI and 35S pre-rRNA. Only molecules having R-loop and unhybridized tail of RNA chain were subjected to contour length measurements. The distribution of contour lengths of each segment, i.e., vector segment (A), R-loop segment (B) and the rest of K-H fragment (C), is shown in Fig. 5b. The size of DNA:RNA hybrid in the R-loop revealed a bimodal distribution with peaks at 465 ± 30 base pairs and 348 ± 33 base pairs, respectively. The segment (C) did not show any bimodal distribution although when the segment (C) from molecules having larger or smaller R-loops is poltted separately, the distribution of the size of the former segment (C) tends to skew to the shorter side of the histogram, and vise versa (data not shown). As mentioned, the 35S RNA preparation used in the R-loop experiment contained minor RNA species slightly smaller than 35S RNA (Fig. 4). Therefore, in estimating the size of the transcribed region, we take the value from the longer R-loop structures which is 465 ± 30 base pairs. Since it has been reported that the contour length of DNA:RNA hybrids is shorter by about 4.3 % than that of DNA duplexes (21), the observed value was corrected to 486 ± 31 base pairs. This means that the transcription initiation site is localized at about 490 base pairs from the HindIII site of the K-H fragment.

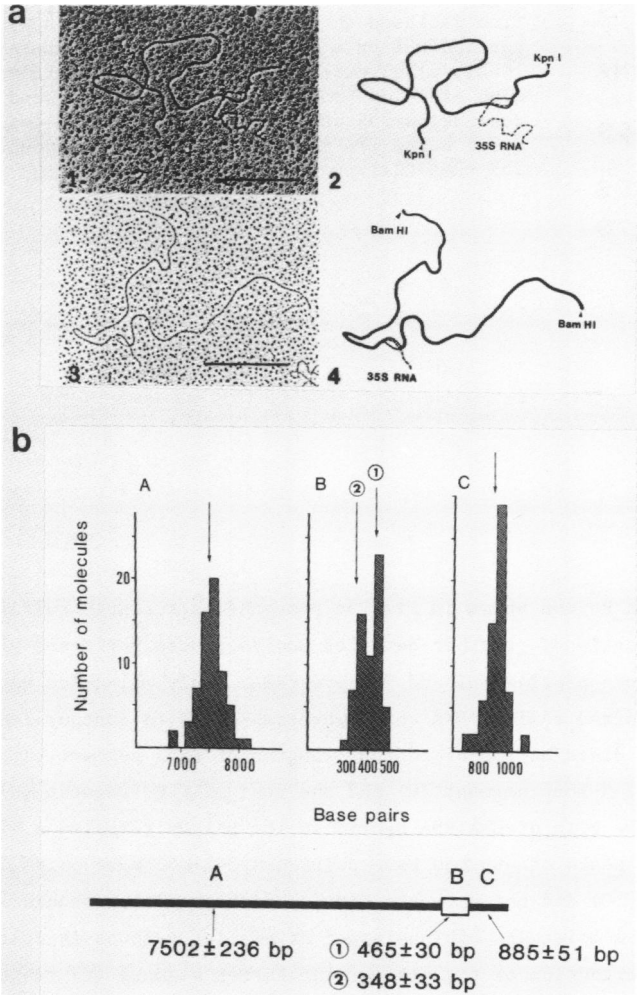

Fig. 5.a. R-loop between linearized pTpr14 and 35S pre-rRNA. 1 and 2, R-loop between pTpr14-KpnI and 35S RNA. Spreading is from 50 % formamide to 18 % formamide (hypophase). 3 and 4, R-loop between pTpr14-BamHI and 35S RNA. Spreading is from 50 % formamide to distilled H_2O. The bar represents 0.5 µm.

b. Size distribution of contour lengths of each segment (n=63). R-loop reaction was carried out between pTpr14-KpnI and 35S RNA. Calibration of the contour length was performed with Col EI DNA (4.2×10^6 daltons) as a standard.

S1 protection mapping

As an alternative way to localize the transcription initiation site, the nuclease S1 protection mapping was performed between K-H fragment and 35S pre-rRNA according to Berk and Sharp (17). As shown in Fig. 6, lane b, the major protected fragment was about 490 nucleotides in length. Upon longer exposure of the gel, however, the bands of shorter length (the second strongest band is about 380 nucleotides long) with detectable intensity appeared (Fig. 6, labe c). These shorter fragments seem to correspond to the smaller R-loop structures discussed in the previous section, since the difference between longer and shorter fragments is almost the same in both cases (117 nucleotides

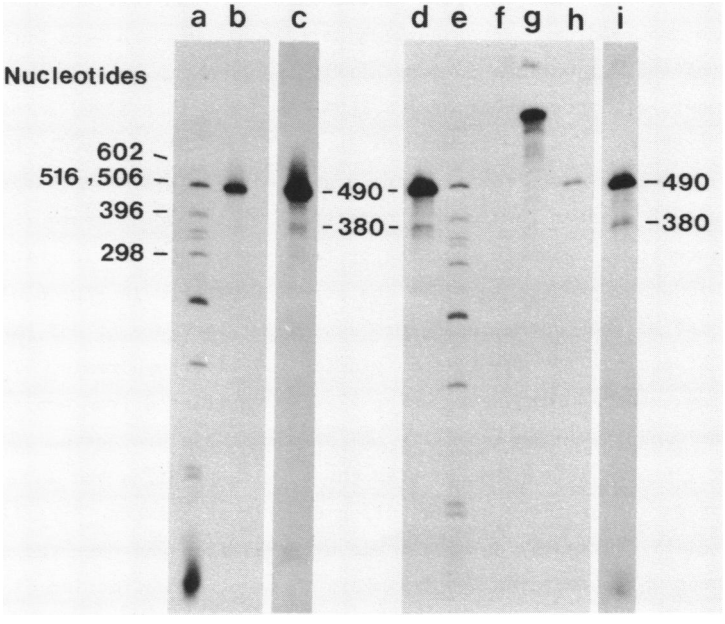

Fig. 6. Size determination of S1 nuclease resistant hybrids formed between K-H fragment and 35S pre-rRNA or cytoplasmic 17S rRNA. K-H fragment labeled at the HindIII site was hybridized with 35S pre-rRNA (lanes b,c and d) or cytoplasmic 17 rRNA (lanes h and i), followed by treatment with S1 nuclease. Lanes a,b and c represent the autoradiogram from the same gel but with different exposure time; a and b for 26 hours and c for 7 days. Lanes d, e, f, g, h and i are also from the same gel with different exposure time; d, e, f, g and h for 20 hours and i for 30 days. Lane f shows the K-H fragment incubated under the same hybridization conditions but without 35S pre-rRNA or 17S rRNA, followed by S1 treatment. Lane g shows the K-H fragment incubated under the same hybridization conditions but without S1 nuclease treatment. Lanes a and e represent size marker; mixture of three kinds of [^{32}P]pBR322 DNA digests (pBR322 DNA digested with HinfI, HinfI-BamHI and HinfI-HindIII).

in R-loop and 110 nucleotides in S1 experiment). Considering the shortening of the contour length of the DNA:RNA hybrid mentioned above, these data can be interpreted to reflect the same degree of hybridization of the transcribed region with 35S pre-rRNA. The difference in the ratio of longer and shorter hybridized (or protected) fragments between R-loop (Fig. 5) and S1 mapping experiment (Fig. 6) may be due to the type of the two experiments. In R-loop analysis we have measured 63 molecules which have distinctive R-loop structures and constructed the histogram shown in Fig. 5. While in S1 mapping experiment the ratio of protected longer and shorter fragments reflects the number of all the hybridized molecules. Therefore it is not unreasonable even if the ratio in two experiments did not coincide with each other.

S1 protection mapping with cytoplasmic 17S rRNA (extracted from total ribosome and separated on an acrylamide gel) also gave a weak but detectable signal of 490 nucleotides in length (Fig. 6, lane h). In this hybridization experiment, a larger amount of 17S rRNA was added to a fixed amount of labeled DNA fragment than the experiment in which 35S RNA was used. However, the signal is much weaker (compare lane d and h in Fig. 6). In this case also, the bands of shorter length were detectable on longer exposure (Fig. 6, lane i). Since it is unlikely that the 5' terminus of 35S RNA is retained in 17S rRNA throughout processing (18), the signals detected in Fig. 6, lane h and i may be due to the contaminating processing intermediate of 35S RNA which migrates at around 17S region in acrylamide gel electrophoresis. This point is now being examined further by using 17S rRNA extracted from separated small ribosome subunits. The inability of the comparable protection of K-H fragment by 17S rRNA is consistent with the results of Niles and Jain (18), showing that in the Southern hybridization experiment between [^{32}P] 17S rRNA and rDNA-HindIII fragments, no detectable hybridization was observed with HindIII B fragment which contains K-H fragments. One other estimation of the length of the transcribed region of the K-H fragment was performed by reverse transcription mapping based upon our preliminary sequencing data. This also mapps the 5' terminus of 35S RNA at about 500 base pairs from the HindIII site of the K-H fragment (data not shown).

DISCUSSION

As Niles and Jain (18) showed by Southern blotting, the rDNA fragment flanked by KpnI and HindIII (K-H fragment) was found to contain the transcription initiation site. We have confirmed and extended this point in a different way using cloned K-H fragments, and showed that the K-H fragment consists

of about 500 base pairs long transcribed region and of 5' upstream region of 900 base pairs in length. The most crucial point in the present experiment is obviously whether the 35S pre-rRNA we isolated is really a primary transcript. According to Niles (3), the majority of 35S RNA they isolated were found to possess triphosphate residues at the 5' termini. Therefore, it is highly probable that the 35S RNA used in the present experiment also constitutes the primary transcript. However, it would be safer to say that the initiation site shown in the present study is a putative one until the 35S RNA is finally proved to be a primary transcript by any means. In order to prove this, we are now looking at whether 35S RNA can be capped by capping enzyme in vitro as has been shown in other cases (22, 23).

The difference in the size of the transcribed region in the K-H fragment between R-loop analysis and S1 protection may be due to the shortening (by about 4 %) of the contour length of DNA:RNA hybrids as compared with DNA duplexes (21), but not due to branch migration since the R-loop formed was stabilized by a glyoxal treatment (15). Therefore, we think it reasonable to take the value of about 500 base pairs as the size of the transcribed region of the K-H fragment.

The introduction of the KpnI site into vector by use of the λDNA-KpnI-BamHI fragment in the cloning of K-H fragment has, from a different point of view, led to a new plasmid vehicle in which the KpnI site can be used as a cloning site. Since the KpnI site is $\overset{5'\cdots GGTAC\overset{\blacktriangledown}{C}\cdots 3'}{3'\cdots C\underset{\blacktriangle}{C}ATGG\cdots 5'}$ and thus has 3' protruding end, this site could be used in cloning experiments using dG:dC tailing with terminal transferase. An inserted fragment can be recovered by KpnI cleavage as in the case of the PstI site in pBR322. Recently, we have eliminated the dispensable portion from pTpr14 leaving the KpnI site and Amp region, and obtained a derivative of pTpr14 with much smaller molecular weight (2.3×10^6 d) (manuscript in preparation).

The more exact determination of the transcription initiation site and the length of the transcribed region at the nucleotide level should be carried out by running an appropriate fragment produced by S1 protection or reverse transcription with sequencing ladder. Comparison of the 5' upstream sequences between various organisms will specify the sequences corresponding to the procaryotic promoter if it resides in the upstream region. Experiments along these lines are now in progress. Furthermore, in vitro transcription studies using cloned rDNA (pTpr14), coupled with the in vitro mutation of the cloned DNA, will specify the nucleotide sequences and cellular factors responsible for correct transcription initiation and its regulation, which has been suc-

cessful in some other systems. It is our interest to see how these findings relate to the functional state of the rDNA in nucleolus or in chromatin.

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