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IN VITRO TRANSCRIPTION OF CLONED YEAST RIBOSOMAL DNA BY YEAST RNA POLYMERASE A

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SUMMARY : A hybrid plasmid, containing yeast ribosomal DNA, was transcribed by purified yeast RNA polymerase A in the presence of the stimulation factor P37. The transcription was not random. The yeast sequences were copied highly asymetrically, as shown by competition hybridization with yeast ribosomal RNA. Using the same DNA, truncated by different restriction enzymes, it appeared, however, that the *in vitro* preferential initiation region was located upstream of the presumptive *in vivo* initiation site for the 37S pre-rRNA.

INTRODUCTION

Transcription studies with the elementary system constituted of deproteinized DNA and purified RNA polymerase have failed to detect any *in vitro* specificity of eucaryotic RNA polymerases, with the exception of yeast RNA polymerase A, for which the matter has given rise to some controversy. Using total genomic yeast DNA and partially purified yeast RNA polymerase A, Hollenberg concluded that rRNA genes were quasi randomly transcribed (1) whereas Cramer *et al.* observed with yeast RNA polymerase A, and not with RNA polymerase B, a slightly asymmetric transcription of a yeast DNA preparation enriched in rRNA genes (2). Van Keulen *et al.* reported that purified RNA polymerase A from *Saccharomyces carlsbergensis* was capable of highly selective and asymmetric transcription of the ribosomal genes provided high molecular weight DNA was used as template (3, 4). Holland *et al.* also reported a preferential transcription of ribosomal genes by yeast RNA polymerase A as compared to RNA polymerase B or *E. coli* RNA polymerase, the selectivity being dependent on Mg^{2+} ions and on the method of enzyme purification (5). However, this speci-

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fic transcription of ribosomal genes could not be reproduced when recombinant plasmid containing the ribosomal transcription unit was used as template (6).

Recently, the *in vivo* initiation site for the transcription of the 37S precursor rRNA has been precisely mapped (7, 8). Therefore, it is now possible to investigate whether the *in vitro* transcripts are correctly initiated. In this paper, we describe the results obtained for the transcription of cloned ribosomal DNA by purified yeast RNA polymerase A in the presence of the stimulation factor P_{37} (9,10).

MATERIALS AND METHODS

Yeast RNA polymerase A (10, 11) and P_{37} factor (9) were purified as previously described. Yeast ribosomal RNA was prepared according to Wilson *et al.* (12). Plasmid pJHC35, containing more than one repetitive unit of yeast ribosomal DNA (13) was prepared essentially as reported by Guerry *et al.* (14). Agarose was purchased from Seakem and nitrocellulose filters (SM 113) from Sartorius.

Electrophoresis and transfer of DNA : The electrophoretic separation of the major fragments of pJHC35 DNA digested by BamH1 and EcoR1 was achieved on 1.1 % agarose vertical slab gel in the presence of ethidium bromide (8 µg/ml). After denaturation, the DNA was transferred from agarose gel to a nitrocellulose sheet by a modified Southern procedure (15). The nitrocellulose filter was dried at 85°C for 2 hours, then cut in 4 mm wide strips containing the restriction fragments equivalent to 0.5 µg of full-length DNA.

Transcription and hybridizations : Transcription experiments were performed in 100 µl mixtures containing 70 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM each of ATP, GTP and CTP, 0.05 mM [α-^{32}P] UTP (2 µCi/nmole), 6 µg/ml of P_{37} protein, 50 µg/ml of DNA and 5 µg/ml of RNA polymerase A. After a 15 min incubation period at 30°C, the reactions were stopped by addition of 0.2 % SDS. For each nitrocellulose strip, 10 to 30 µl of transcription mixture (about 0.1 nmole of cRNA) were then added to 1 ml of a sterile solution containing 50 % formamide, 0.6 M NaCl, 0.18 M sodium phosphate pH 6.3 and 6 mM EDTA. After hybridization (performed in heat-sealed plastic bags for 48 hours at 40°C with shaking) the nitrocellulose strips were rinsed 6 times with a 6XSSC buffer, treated for 30 min at 37°C with RNase A (5 µg/ml), washed again with 2XSSC buffer and dried. The hybridized RNA was then revealed by autoradiography.

Competition hybridization on filter with ribosomal RNA : The ribosomal DNA sequences of one nitrocellulose strip were first saturated by hybridizing with a large excess of purified unlabelled yeast ribosomal RNA. After extensive washing, the strip was then hybridized, at the same time as an untreated control strip, to ^{32}P -labelled cRNA under the conditions described above.

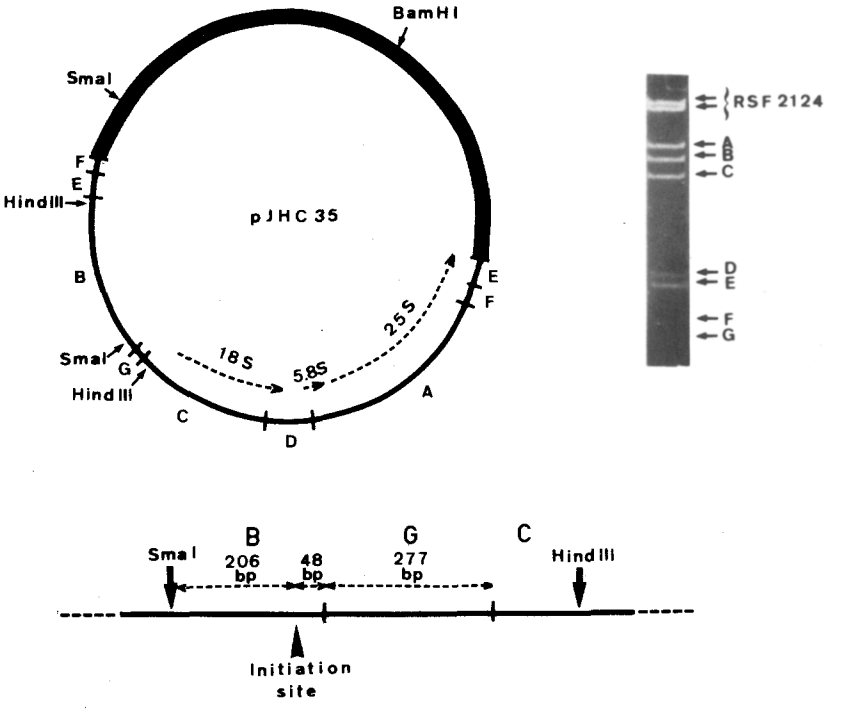

Figure 1 : Restriction map of plasmid pJHC35.

Bars indicate EcoR1 restriction sites, dashed lines represent the localization of the rRNA genes, arrows indicate direction of transcription. Heavy line : plasmid DNA (RSF 2124). Light line : yeast DNA insert. The *in vivo* initiation site for the 37S pre-rRNA (7,8) is indicated on the expanded map of EcoR1-B, G and C region.
An agarose gel pattern of (BamH1+EcoR1) restriction fragments of plasmid pJHC35 DNA is shown.

Gel electrophoretic analysis of the transcription complexes : The transcription was performed as described above, except for the UTP and CTP concentrations which were decreased to 5 µM in order to lower the reaction rate. The specific radioactivity of [α-^{32}P] UTP was 60 µCi/nmole. Transcription was initiated by the addition of the nucleotides after a 10 min preincubation period of pJHC35 DNA (BamH1 digested) with RNA polymerase A, and stopped 10 min later by addition of 6 mM EDTA. The DNA with is transcription complexes was then purified by gel filtration on a small Sephadex-G50 column, digested by EcoR1 and loaded on top of a 1.2 % agarose gel. After electrophoresis, the gel was coloured with ethidium bromide, photographed to localize the DNA fragments, then dried and autoradiographed.

RESULTS AND DISCUSSION

As shown in figure 1, plasmid pJHC35 contains, inserted into the bacterial plasmid RSF2124, a fragment of yeast DNA

resulting from the partial digestion of yeast ribosomal DNA by EcoR1 (13). The bacterial and yeast DNA regions have approximately the same length (molecular weight 7.4 and 6.8 x 10^6 respectively). The inserted fragment represents more than one repetitive unit of ribosomal sequences. The plasmid DNA was linearized by BamH1 digestion, which cuts the plasmid approximately in the middle of the bacterial region. When linear plasmid DNA was used as template for purified yeast RNA polymerase A, optimal transcription was obtained with a mixture of Mg^{2+} and Mn^{2+}, in the presence of P_{37} stimulation factor (9,10).

At first sight, no preferential transcription of the ribosomal genes by yeast RNA polymerase A could be detected since both pJHC35 DNA and the parental plasmid RSF2124 were transcribed with the same efficiency. The rate of transcription of the different regions of pJHC35 DNA was investigated by hybridization of the RNA synthesized to different restriction fragments of the plasmid separated by agarose gel electrophoresis and transferred to nitrocellulose filters. The hybridizations were performed with filters containing the major DNA fragments resulting from the digestion of pJHC35 by BamH1 and EcoR1. The two largest fragments correspond to the bacterial DNA, the other three, named respectively EcoR1-A,B and C being yeast ribosomal fragments (see figure 1). As illustrated in figure 2, hybridization took place with all these fragments, indicating that all the regions of the plasmid had been transcribed. The hybridization to the yeast sequences was however sligthly more efficient, especially to the EcoR1-A and C fragments. These two fragments code respectively for the 25S and 18S ribosomal RNAs. This led us to investigate the asymmetry of the RNA synthesized from these regions by competitive hybridization with ribosomal RNA. To avoid any RNA-RNA annealing in the presence of a large excess of competitor RNA, the DNA bound to nitrocellulose filter was first hybridized with cold yeast ribosomal RNA, then, after washing the filter, with the *in vitro* labelled RNA. Under these conditions, only the RNA chains copied from the non-coding strand could hybridize and be visualized on the autoradiogram. In fact, while hybridization to the bacterial fragments and to the yeast EcoR1-B fragment were unaffected, almost no hybridization

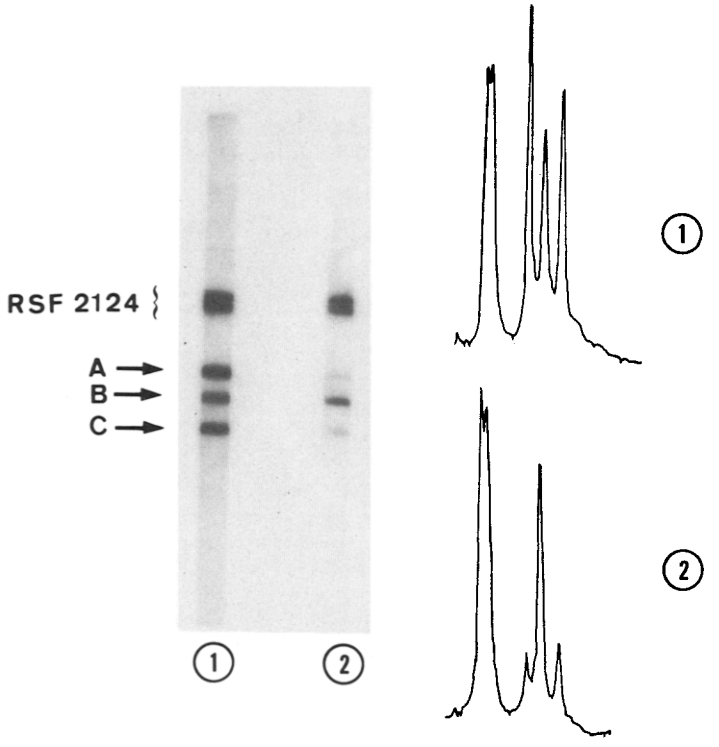

Figure 2 : Southern hybridization of RNA synthesized *in vitro* to (BamH1+EcoR1) restriction fragments of pJHC35. Competition with yeast ribosomal RNA.

RNA synthesized on BamH1 treated pJHC35 DNA by yeast RNA polymerase A with P_{37} factor was hybridized on nitrocellulose filters where the 5 major fragments resulting from the digestion of the plasmid by BamH1 and EcoR1 had been transferred as described under Materials and Methods. The hybridized RNA was then revealed by autoradiography. 1) Control experiment without competitor rRNA. 2) Competitive hybridization with cold yeast ribosomal RNA (see Materials and Methods). The scanning of the autoradiograms is shown.

occurred to the EcoR1-A and C fragments (figure 2), indicating that these DNA sequences had been transcribed highly asymmetrically *in vitro* by RNA polymerase A, the strand transcribed being the same as that copied *in vivo*. This result suggested that the RNA chains were initiated at a limited number of sites on the yeast DNA insert, since random initiation sites would statistically be distributed on both strands and lead to symmetric transcription. In a first approach to localize these preferential initiation regions, the plasmid pJHC35 was transcribed under conditions that allowed only very short RNA

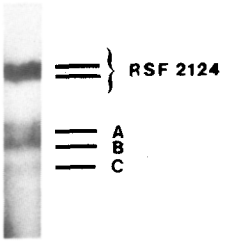

Figure 3 : Analysis of nascent transcription complexes by agarose gel electrophoresis.

BamH1 digested pJHC35 DNA was transcribed by RNA polymerase A with P_{37} factor at low CTP and UTP concentration in order to synthesize short RNA chains as described under Materials and Methods. After digestion by EcoR1, the DNA fragments with their nascent transcription complexes were separated by electrophoresis on 1.2 % agarose gel. The localization of the DNA fragments, as revealed by ethidium bromide coloration, is indicated in front of the autoradiogram of the gel.

chains to be synthesized, then digested with EcoR1, and the DNA fragments with their labelled nascent transcription complexes were separated by electrophoresis. As shown in figure 3, transcription complexes localized in the yeast DNA were initiated mostly in the EcoR1-B fragment. Some transcription complexes were also found at the level of the A fragment but almost none at the level of the C fragment whose size, however, is almost identical to that of fragment B. Hence, a selectivity was detected, not only at the level of the strand being copied, but also in the selection of the initiation region.

Recent studies on yeast ribosomal RNA have mapped the 5' end of the 37S precursor rRNA inside the EcoR1-B fragment, 48 nucleotides upstream from the EcoR1 site (7, 8). This prompted us to investigate whether the above-mentioned preferential *in vitro* initiation site in B fragment was located at the correct place. For this purpose, the template was truncated, before transcription, with two different restriction enzymes, whose cleavage sites are located on each side of the *in vivo* initiation site (see figure 1). HindIII cuts the plasmid after the initiation site, at the very beginning of EcoR1-C fragment. With HindIII digested pJHC35 DNA, the transcription of the region corresponding to the EcoR1-C fragment decreased drastically, as expected. The amount of

Table 1 : Analysis of the RNA synthesized on truncated pJHC35 DNA

Template	Amount of hybridized RNA (arbitrary units)			
	bacterial fragments	EcoR1-A fragment	EcoR1-B fragment	EcoR1-C fragment
BamH1 digested pJHC35	1	0.53	0.19	0.21
BamH1 - HindIII digested pJHC35	1	0.24	0.16	0.06
SmaI digested pJHC35	1	0.26	0.16	0.05

Plasmid pJHC35 DNA was digested by different restriction enzymes as indicated. The RNA synthesized on these different templates was hybridized to the (EcoR1 + BamH1) fragments of the plasmid as described in figure 2. The amount of RNA hybridized to each fragment was then quantified by measuring the height of the corresponding peaks on the scanning of the autoradiogram. For standardization, the height of the two peaks corresponding to the bacterial fragments was summed and taken as one.

RNA hybridizing to the EcoR1-A fragment also decreased, to half the control value (table 1). These results can only be accounted for if the transcription of EcoR1-C region has been initiated upstream of the HindIII site, in the EcoRI-G or in the EcoRI-B region. Furthermore, this was also the case for half of the transcripts of fragment A, implying that RNA chains in excess of 2000 nucleotides long had been synthesized. However, similar results were obtained with SmaI digested pJHC35 DNA as template (table 1). Therefore, the *in vitro* initiation site(s) is located upstream of the SmaI site, i.e. more than 200 bases upstream of the *in vivo* initiation site of the 37S precursor rRNA (7,8). On these grounds, the *in vitro* transcription appears to be non-accurate, although it cannot be completely ruled out that *in vitro* initiation does in fact take place downstream of the SmaI site but requires the presence of adjacent sequences on the 5' side.

The asymmetric and clearly non-random transcription that we observed here explains in part the results from other groups who concluded, on the basis of hybridization experiments, to the highly specific transcription of ribosomal genes by purified RNA polymerase A (3-5). The present

data are also similar to the hybridization results obtained on acid-treated nuclei by Tekamp et al. (6). Additional factors are probably necessary for accurate transcription. Recently, a soluble reconstituted transcription system was developed by Grummt(16) and shown to achieve the accurate transcription of mouse ribosomal DNA. It is likely that multiple factors are required in this process, some of them being probably chromatin components.

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