Yeast RNA polymerase I binds preferentially to A+T-rich linkers in rDNA

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ABSTRACT

Restriction fragments of yeast rDNA retained by purified RNA polymerases on nitrocellulose filters were analysed by gel electrophoresis. The EcoRI fragment B was preferentially retained by RNA polymerase I, but not by RNA polymerase III. The in vivo initiation sites for both polymerases are located within this fragment. Further analysis indicated that the preferred binding site for RNA polymerase I is a highly AT-rich region rather than a true promoter. The reported selective in vitro transcription of rDNA by purified yeast RNA polymerase I could then be explained by this preferential binding.

INTRODUCTION

Significant progress in the understanding of DNA sequences involved in control of eukaryotic transcription has been made after the development of cell-free systems capable of faithful initiation of transcription (1-4). However, studies of more elementary systems may be necessary to elucidate what role the RNA polymerase plays in the recognition and binding to specific DNA sequences. The problem is that most eukaryotic RNA polymerases seem to be unable to recognize control regions, and transcribe deproteinized DNA randomly, unless factors are supplied by more or less crude cell extracts. In this respect, yeast RNA polymerase I may be an exception. Holland et al. (5) reported that purified yeast RNA polymerase I showed preferential transcription of rDNA when purified yeast DNA was used as template. With DNA of high molecular weight from Saccharomyces carlsbergensis, van Keulen and Retel (6) found highly selective and asymmetric transcription of ribosomal genes by homologous RNA polymerase I. However, Tekamp et al. (7) found that specific transcription of ribosomal genes could not be repro-
duced with a recombinant plasmid containing yeast rDNA as template. On the other hand, Sawadogo et al. (8) recently reported a non-random transcription of an rDNA containing recombinant plasmid. Here, RNA polymerase I seemed to initiate asymmetric transcription upstream of the mapped in vivo initiation site.

The repeating unit of yeast rDNA contains a large region transcribed by RNA polymerase I into a 35S precursor rRNA and a smaller gene for 5S RNA transcribed in the opposite direction by RNA polymerase III (9). According to Cramer et al. (10) both these genes are flanked with regions rich in A-T basepairs. Apparently, two possible explanations for the observed selectivity of transcription in vitro, therefore exist. The polymerase might recognize a control region in rDNA. Alternatively, it might bind stronger to AT-rich DNA and thus give rise to a pseudo-selectivity in vitro.

In the present work, we have studied the binding of yeast RNA polymerase I to different regions of yeast rDNA. We find that the enzyme preferentially binds to the AT-rich regions flanking the 35S rRNA gene. However, this seems to be due to a more general preferential binding to AT-rich DNA, since the RNA polymerase also interacts strongly with mitochondrial DNA.

MATERIALS AND METHODS

Nuclear and total DNA and the γ-DNA fraction were prepared from Saccharomyces cerevisiae essentially as described (11,12). The γ-DNA fraction contains approximately 80% rDNA (12) and was used as a source of rDNA. The recombinant plasmids pOSG7, pOSG86 and pOSG42 were constructed in our laboratory. They contain respectively the EcoRI fragment A, B and C of yeast rDNA inserted into the EcoRI site of pBR325. Plasmid DNA was isolated by standard methods (13). RNA polymerase I, II and III were purified from a haploid wild type strain of Saccharomyces cerevisiae by a procedure developed in our laboratory (to be published elsewhere). All three polymerases contained complete sets of subunits as shown for RNA polymerase I in Fig.1a. Restriction enzyme PvuII, HpaII and TaqI were from Boehringer-
Mannheim. EcoRI was prepared by standard methods. Nuclease S₁ and agarose type II were from Sigma. 6-[³H]-uracil used for labeling yeast DNA (14) was purchased from The Radiochemical Centre, Amersham. Nitrocellulose sheets were from Millipore.

**Restriction and nuclease S₁ treatment.**

Digestions with restriction enzymes were performed under standard conditions (13). The single-stranded protruding ends generated by restriction enzyme EcoRI, TaqI and HpaII were digested with nuclease S₁ at 37°C for 30 min under standard conditions (15). To facilitate the shift to lower pH, a moderate buffer capacity (10 mM Tris-HCl pH 7.4) was used under restriction. Nuclease S₁ treatment was terminated by increasing the pH to 7.9 by addition of 0.6M Tris-HCl pH 9.0 and by complexing Zn ions by EDTA.

**Filter binding assay and electrophoretic analysis of retained fragments.**

The filter binding assay of Hinkle and Chamberlin (16) was used. Nitrocellulose filters were treated with 0.4 M KOH before use to reduce background adsorption (17). Complexes between RNA polymerase and nuclease S₁ treated nuclear DNA or restriction fragments were formed at 30°C for 15 min in a volume of 200 - 400 µl binding buffer (10 mM Tris-HCl (pH 7.9, 30°C), 6 mM MgCl₂, 1 mM EDTA, 4% glycerol, 1 mM DTT) and a total of 15µg bovine serum albumine and the indicated amounts of DNA, RNA polymerase and sodium chloride. Immediately before filtration, the mixture was diluted with 0.25 - 1.0 ml pre-warmed binding buffer. For gel electrophoretic analysis, the retained DNA was eluted by three portions of 15µl elution buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.2% SDS, or 0.2% Triton X-100 for analysis of smaller fragments). DNA not retained by the nitrocellulose filter was precipitated with ethanol and dissolved in 10 mM Tris-HCl, 1 mM EDTA. Electrophoresis was performed in 1.2 or 2.5% agarose gels in a Tris-Acetate buffer. The gel was stained with ethidium bromide (1 µg/ml) for 60 min and photographed using Polaroid Land Film type 55. Densitometric tracings of the negative film were done in a Gilford spectrophotometer.
Fractionation of retained DNA in an Ag⁺/Cs₂SO₄-density gradient.

Binding and filtration were performed as above using nuclease S₁-treated total yeast DNA labeled with tritium. Retained labeled DNA was eluted from the filter by soaking in elution buffer. 150 μg unlabeled total yeast DNA of the same origin as the labeled DNA was added and the DNA was precipitated with ethanol. The precipitate was dissolved in 0.025 M Na₂SO₄, and AgClO₄ and Cs₂SO₄ were added as described (12). The DNA was fractionated by centrifugation at 35,000 rpm in Beckman rotor 50 for 60 hrs. Fractions of 190 μl were collected and absorbance at 260 nm as well as acid precipitable radioactivity were measured in each fraction.

RESULTS

The possibility that the reported selective transcription of rDNA by yeast RNA polymerase I is due to a stronger binding of the enzyme to regions upstream of the 35 S precursor RNA gene was studied by analyzing the binding to restriction fragments of rDNA. When yeast rDNA is digested with EcoRI, seven fragments are generated as illustrated in Fig.1b.(18,19). Of the three largest fragments, fragment B contains the in vivo initiation site for both RNA polymerase I and III (20,21,22), while fragments A and C presumably are without any control regions for the polymerases. The retention of these fragments by RNA polymerase I on nitrocellulose filters was analyzed by gel electrophoresis. As shown in Fig.2, RNA polymerase I clearly retained fragment B more efficiently than it retained fragment A and C. The relative preference for fragment B increased as the polymerase:DNA ratio was lowered (Fig.2a,b). The real difference in retention is probably even greater than appears from Fig.2 as the darkening of the film from which the scan was made, is logarithmically dependent on the intensity of the DNA fluorescence (23).

When a similar experiment was done with RNA polymerase III, which bound more strongly to nuclease S₁-treated yeast DNA than RNA polymerase I did, the three largest EcoRI fragments were retained with the same efficiency. The preference for fragment B is therefore a particular property of RNA polymerase...
I. RNA polymerase II bound the fragments too weakly to allow similar analysis.

We also analysed binding of RNA polymerase I to PvuII digested rDNA and found that the smallest PvuII fragment, which maps in the same region as the EcoRI fragment B (Fig.1b), was preferentially retained by the enzyme. Hence, the polymerase seems to bind preferentially to internal regions common to the EcoRI fragment B and the smallest PvuII fragment.

Fig.1 Subunit pattern of RNA polymerase I (a) and restriction map of rDNA, the EcoRI fragment B and pOSG86 (b).
a: SDS-polyacrylamide gel electrophoretic analysis of RNA polymerase I. 10% polyacrylamide slab gels were prepared according to Laemmli (24).
b: Restriction map. Bars indicate restriction sites for 1: EcoRI, 2: PvuII, 3: TaqI and 4: HpaII. In rDNA the seven EcoRI fragments are named A – G and the initiation sites for RNA polymerases I and III are indicated. In the extended map of the EcoRI fragment B, the AT-rich regions reported by Cramer et al. (10), are indicated by hatched areas. The parts of fragment B that have been sequenced (20), are underlined. In the recombinant plasmid pOSG86 the inserted fragment B is marked with a dotted area, and the inactivated chloramphenicol gene by cross-hatched areas. The restriction sites for EcoRI, TaqI and HpaII were taken from (9,20). The PvuII restriction sites were mapped in our laboratory. The PvuII site in fragment B was also found in the reported sequence (20).
The preferential binding could not be due to different contents of nicks in the EcoRI fragments generated from isolated γ-DNA because the same preference for fragment B was found using restriction fragments derived from plasmids pOSG7, pOSG86 and pOSG42.

The Eco El fragment B has two distinguishing features. Firstly, the 5'end of the 35 S precursor rRNA has been mapped 48 basepairs from one end of the EcoRI fragment B (21, 22, Fig.1b). It therefore probably contains control regions used by the enzyme in vivo. In addition, it is significantly enriched in A-T basepairs compared to the other fragments. This has been shown by denaturation mapping (10) and by two-dimensional electrophoresis of EcoRI digested rDNA (Gabrielsen, unpublished results) using the method of Fisher and Lerman (25). Several parts of fragment B have also been sequenced and shown to contain regions highly enriched in A-T basepairs (20). The preferential binding could therefore either reflect a recognition property of the enzyme for control regions in rDNA or be

![Figure 2: Retention of EcoRI and nuclease S1 digested γ-DNA by RNA polymerase I.](image)

2 μg of EcoRI and nuclease S1 digested γ-DNA were retained on nitrocellulose filters by 0.4μg (a) or 2.0μg (b) RNA polymerase I in the presence of 50 mM NaCl and analyzed by gel electrophoresis as described in Materials and Methods. Densitometric scans of a negative photograph of the stained gel are shown. Only the three largest EcoRI fragments are included. A sample of the digested γ-DNA was run separately as a reference (c).
due to the formation of stronger complexes in regions rich in A-T basepairs. In the latter case, one would expect a regular increase in the efficiency of retention of DNA with increasing A+T content. To investigate this, we analysed the distribution of RNA polymerase I retained total DNA in a Ag⁺/Cs₂SO₄ density gradient where the DNA is fractionated into γ, α, and β fractions according to A+T content (12). The result is shown in Fig.3. Throughout the nuclear fractions, the distribution of the retained labeled DNA followed the profile of the unlabeled carrier DNA in spite of the differences in A+T contents. However, the labeled DNA in the β-band, containing mitochondrial DNA, was present in large excess. No preferential retention in this area was seen with labeled nuclear DNA isolated from cells grown in the presence of ethidium bromide. Hence, RNA polymerase I apparently binds preferentially to mitochondrial DNA, a DNA exceptionally rich in A-T basepairs (26). The A+T-content therefore probably influences the stability of the binary complexes provided that regions sufficiently enriched in A-T basepairs take part in the complex.

Fig. 3 Analysis of total yeast DNA retained by RNA polymerase I in Ag⁺/Cs₂SO₄ density gradient. 20 µg nuclease S₁ treated total yeast [³H]-DNA was incubated with 3 µg RNA polymerase I and analysed in a Ag⁺/Cs₂SO₄ density gradient as described in Materials and Methods. (O): [³H]-DNA retained by RNA polymerase I. (●): A₂₆₀ nm of carrier DNA.
If this were the case, several subfragments of the EcoRI fragment B could be expected to be preferentially retained by RNA polymerase I. Cramer et al. (10) found AT-rich regions to be distributed over several parts of fragment B (Fig.1b). The sequence data also show high content of A-T basepairs in various parts of fragment B (20). On the other hand, if the preferential retention of fragment B were due to the formation of a stronger complex with a control region, only one subfragment would be expected to be preferentially retained. The recombinant plasmid pOSG86 was digested with TaqI, EcoRI and nuclease S1. This gave three subfragments of the inserted fragment B, apart from a small fragment of 21 basepairs at one end (Fig.1b), in addition to vector fragments. As shown in Fig.4, the second largest insert fragment, which maps outside the initiation region (Fig.1b), was retained with similar efficiency as the largest insert fragment containing the in vivo initiation site and upstream sequences. Compared with vector fragments, both these larger insert fragments were retained with higher efficiency. This observation of a preferential

![Fig. 4 Retention by RNA polymerase I of the recombinant plasmid pOSG86 digested with TaqI, EcoRI and nuclease S1. 2.0 μg of pOSG86 digested with TaqI, EcoRI and nuclease S1 were incubated with RNA polymerase I and analysed as described in the legend to Fig.2. Fragments of the inserted yeast DNA are marked (▼). a.: Fragments retained by RNA polymerase I. b.: pOSG86 digested with TaqI, EcoRI and nuclease S1 (reference). c: Fragments not retained by RNA polymerase I.](image-url)
binding to more than one fragment is not compatible with a recognition of control sequences through the formation of more stable complexes adjacent to coding sequences. Similar results were also found with HpaII digested pOSG86 where fragments in the same region as found with analysis of TaqI digested plasmid, were preferentially retained (results not shown).

**DISCUSSION**

In the present work, we have tried to find out whether the reported selective *in vitro* transcription of rDNA by yeast RNA polymerase I is due to an intrinsic capability of the enzyme to recognize control regions in rDNA. Our approach has been to study the binding step to see whether a recognition takes place through the formation of stronger binary complexes to regions adjacent to the coding sequence than to other regions in rDNA. While little is known of how eukaryotic RNA polymerases recognize control regions in DNA, recognition of promoters in bacterial and phage DNA usually takes place through the formation of highly stable binary complexes in promoter regions (reviewed in 27).

We find that RNA polymerase I binds preferentially to the EcoRI fragment B of rDNA. This fragment contains the *in vivo* initiation sites for both RNA polymerase I and III. RNA polymerase I binds relatively weakly to duplex DNA in general and also transcribes nuclease S1 treated DNA poorly (15,28). Preferential binding to a particular region in DNA would therefore be expected to lead to preferential transcription of adjacent regions provided that productive complexes are formed.

We believe that the reported selective *in vitro* transcription of rDNA by RNA polymerase I, is due to a preferential binding to highly AT-rich sequences found in the EcoRI fragment B of rDNA and not due to recognition by the enzyme of *in vivo* control regions. This hypothesis is based on the following evidence:

1. The preferentially retained fragment B differs from fragment A and C not only by being the fragment containing *in vivo* control regions, but also by being significantly more AT-rich. This is seen from the denaturation mapping of Cramer et
al. (10) and was confirmed by gelelectrophoretic analysis of melting properties (Gabrielsen, unpublished results).

2. Stronger complexes are formed with mitochondrial DNA than with nuclear DNA (Fig. 3). Yeast mitochondrial DNA is one of the most AT-rich functional DNAs in nature. The mole percentage of A+T is 82% and about half of the DNA contains less than 5% G-C basepairs (26).

3. When smaller subfragments of the AT-rich EcoRI fragment B of rDNA were analysed, we observed preferential retention not only of the subfragment containing the in vivo initiation site, but another noncontiguous AT-rich fragment as well (Fig. 4).

4. Efficient initiation of transcription from complexes with AT-tracks is expected as Sentenac et al. (29) found that the affinity for the initiating nucleotide ATP is about 100-fold higher with d(A-T)$_n$ than with native calf thymus DNA as template. They also reported that RNA polymerase I was very active in RNA synthesis with d(A-T)$_n$ as template (30).

RNA polymerase I has been reported to transcribe rDNA not only selectively, but asymmetrically as well (6, 8). This could also be explained by a stronger interaction with highly AT-rich regions in fragment B provided that the adenine and thymine bases were asymmetrically distributed in the binding site. Three almost pure poly(dA)-poly(dT) tracks, more than 15 basepairs long, can be found in the reported sequences (20), two of which upon binding would direct the polymerase into the 35 S coding region. Information about the sequence of the AT-rich region closest to the in vivo initiation site (Fig. 1b) is lacking so far.

One would expect singlestranded regions in AT-tracks to be exposed more frequently than in more GC-rich DNA, generating a potential stronger binding site for RNA polymerases in general. However, RNA polymerase II which strongly prefers singlestranded DNA as template (31) and also binds duplex DNA very weakly (Gabrielsen, unpublished results), transcribes d(A-T)$_n$ less efficiently than it transcribes d(G-C)$_n$ (31). RNA polymerase III, which transcribes d(A-T)$_n$ about four times more efficiently than it transcribes native calf thymus DNA (29), showed no preferential binding to the AT-rich fragment B, and it seems
to transcribe all regions of rDNA with similar efficiency (Gabrielsen and Jonassen, unpublished results). The strong interaction with AT-rich DNA therefore appears to be an intrinsic property of RNA polymerase I and not only a secondary effect of DNA structure. Probably, this interaction plays part in polymerase binding in vivo, but is not sufficient for a precise positioning of the enzyme at the in vivo initiation site.

REFERENCES