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**Efficient and selective initiation by yeast RNA polymerase B in a dinucleotide-primed reaction**

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Bernard Lescure\*, Valérie Williamson\*\* and André Sentenac\*

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\*Service de Biochimie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, B.P. no 2, 91190 Gif-sur-Yvette, France, and \*\*Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

Yeast RNA polymerase B catalyzes an efficient abortive initiation on double-stranded DNA templates using the appropriate combination of primer and substrate. The specificity of initiation was investigated using a recombinant plasmid (pJD14 DNA) containing the structural gene for yeast alcohol dehydrogenase I (ADHI). The combination of the dinucleotide UpA and UTP was 10 fold more efficient with pJD14 DNA than with the vector pBR322 DNA to direct the synthesis of the trinucleotide UpApU. Under these conditions, stable enzyme - DNA complexes were formed and could be retained on nitro-cellulose filters. Using the UpA - primed system and a short pulse of RNA synthesis, transcription complexes were located on the yeast part of pJD14 DNA as evidenced by agarose gel electrophoresis. Southern hybridization of the pulsed RNA was restricted to a region, within the yeast DNA fragment, upstream to the initial region of the ADHI gene.

INTRODUCTION

Three structurally distinct nuclear RNA polymerases, with distinct transcriptive functions, have been identified in eukaryotic cells (1-2). However, the molecular mechanisms which regulate gene expression are poorly understood. *In vitro* transcription studies with eukaryotic RNA polymerases have encountered two main difficulties. The first one was the low specific activity of the purified enzymes on double stranded, linear or supercoiled templates, compared with that of the bacterial RNA polymerase (2). It was generally assumed that this low activity reflected a deficiency in RNA chain initiation (2-3). The second problem was the lack of specificity of transcription in the absence of additional cellular components (1,2,4,5). This general situation has led to an inten-

sive search for stimulatory components which would increase the initiation efficiency and/or direct a specific transcription. The finding that RNA polymerase C accurately transcribes *in vitro* various 5S and tRNA genes within the chromatin (5) encouraged the development of soluble reconstituted systems. Several laboratories reported independently the specific transcription, by RNA polymerase C, of several viral or 5S genes when complemented with a crude soluble cell extract (6-10). An identical reconstituted system has recently been used to accurately transcribe viral and cloned cellular genes by RNA polymerase B (11,12). It is clear that several cellular factors are playing an essential role in the regulation of transcription. An important step in their purification was made by the purification of one of the proteins required for the transcription of *Xenopus* 5S genes (13).

Yeast RNA polymerase B is probably the best characterized eukaryotic type B RNA polymerase (3,14,15). Recently, the cloning of several yeast genes provided defined templates for transcription specificity studies (16-19). Here we first present data showing that purified yeast RNA polymerase B is able to catalyse a very efficient abortive initiation on a double-stranded template. This observation prompted us to reconsider the problem of the specificity of chain initiation by a purified eukaryotic RNA polymerase B. In the present paper, we show that yeast RNA polymerase B initiates selectively within the yeast part of a recombinant plasmid carrying the structural gene for yeast alcohol dehydrogenase I (ADHI).

### MATERIAL AND METHODS

1. Enzymes and DNA purification. Yeast RNA polymerase B was purified as previously described (15) from a protease strain of *S. cerevisiae* (PEP 4) kindly provided by E.W.Jones. Using this strain, the purified enzyme was at least 80 % in the unproteolyzed BI form (15). *E.coli* RNA polymerase was purified from *E.coli* MRE 600 cells as described by Humphries et al. (20). Plasmid pJD14 containing the structural gene for yeast alcohol dehydrogenase I was derived from the original clone of

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Williamson et al. (18). Plasmid DNA was prepared essentially as reported by Guerry et al. (21).

2. Synthesis of di- and trinucleotides. Synthesis of trinucleosides diphosphate or dinucleosides diphosphate by RNA polymerase B was monitored by chromatography. Incubation mixtures contained in 50  $\mu$ l : 70 mM Tris-HCl pH 8, 2.5 mM  $MnCl_2$ , 20 mM ammonium sulfate, 0.5 mM DTT, 0.2 mM of a dinucleoside monophosphate or nucleoside monophosphate as indicated, 5  $\mu$ M ( $\alpha^{32}P$ ) UTP (1000 cpm/pmol), 0.5  $\mu$ g of yeast RNA polymerase B and 1  $\mu$ g of DNA as indicated. After 30 min incubation at 30°C; the reaction was stopped by adding 10  $\mu$ l of 0.1 M EDTA. Aliquots of 5  $\mu$ l were spotted on polyethyleneimine sheets (Macherey - Nagel CEL 300 PEI). Ascending chromatography was performed with 1 M formic acid adjusted to pH 4.3 with pyridin. The sheets were dried and subjected to autoradiography. For quantification of the results, the chromatogram was cut into strips and the radioactivity of the residual UTP and of the product trinucleotide was estimated by liquid scintillation.

3. Isolation of complexes between yeast RNA polymerase B and plasmid DNA. Complexes between RNA polymerase B and pJD14 DNA or pBR322 DNA were isolated by the nitrocellulose filter technique (22,23). The reaction was performed in 100  $\mu$ l of a mixture containing : 70 mM Tris-HCl pH 8, 2.5 mM  $MnCl_2$ , 20 mM ammonium sulfate, 0.5 mM DTT, 5  $\mu$ g of yeast RNA polymerase B and 5  $\mu$ g of plasmid DNA. When indicated, the dinucleotide and the ribonucleoside triphosphate were added at the final concentration of 0.2 mM and 5  $\mu$ M, respectively. After 5 min of incubation at 30°C, EDTA and heparin were added at the respective concentrations of 10 mM and 20  $\mu$ g/ml. The incubation was continued for 15 min at 0°C, then the reaction mixture was filtrated through a nitrocellulose filter (pore size 2.5  $\mu$ ). After three washing in 0.2 M NaCl, the DNA retained on the filter was eluted by an incubation of 30 min at 50°C in 300  $\mu$ l of a mixture containing 0.2 M NaCl and 0.5 % sodium lauryl sulfate. The eluted DNA was ethanol precipitated and identified by electrophoresis on a 0.7 % agarose gel in 40 mM Tris, 20 mM sodium acetate, pH 8.2

4. Isolation by gel electrophoresis of the ternary transcription complexes. The method used was essentially the one described by Chelm and Geiduschek (24). Incubation were done in 50  $\mu$ l of the mixture used for the synthesis of trinucleotides. The amount of enzyme and DNA was 1  $\mu$ g and 5  $\mu$ g respectively. After a 2 min preincubation at 30°C in the presence of 0.2 mM UpA and 5  $\mu$ M ( $\alpha^{32}$ P) UTP (10000 cpm/pmol), ATP, GTP and CTP were added at the final concentration of 5  $\mu$ M. After 40 sec synthesis at 30°C the reaction was stopped by adding 10  $\mu$ l of 0.1 M EDTA. A control reaction, in absence of dinucleotide, was run for 40 sec with 0.5 mM each of ATP, GTP, CTP and 5  $\mu$ M ( $\alpha^{32}$ P) UTP, after a 2 min preincubation of enzyme and DNA at 30°C. Ternary complexes were isolated by filtration through G50 Sephadex column. When indicated, the DNA template was digested for 30 min with restriction enzymes. DNA fragments were separated by electrophoresis on agarose gel. The gel was stained with ethidium bromide to identify the DNA bands, dried under vacuum at 37°C and subjected to autoradiography to localize the transcription complexes.

5. Southern hybridization of the RNA synthesized *in vitro*. BamHI + HinfI pJD14 DNA restriction fragments were transferred from agarose gel to nitrocellulose filter by the method of Southern (25) using 20 X SSC as recommended by Nagamine et al. (26). Ternary complexes, isolated as described above, were treated by EcoRI endonuclease to remove negative superturns in order to prevent the annealing of the short RNA products with the supercoiled DNA. The ternary complexes were then dissociated with 0.5 % SDS (w/v) and the RNA was directly hybridized at 40°C for 24 h to the blotted DNA fragments in a mixture containing 5 X SCP (SCP is 0.1 M NaCl, 0.06 M Na<sub>2</sub> HPO<sub>4</sub>, 1 mM EDTA adjusted to pH 6.2 with HCl), 50 % formamide and 0.2 % SDS (w/v). Nitrocellulose filters were then washed three times with 0.2 M NaCl, dried and subjected to autoradiography.

6. Products. Restriction endonucleases were purchased from Boehringer, dinucleotides, nucleotides and agarose were from Sigma, ( $\alpha^{32}$ P) UTP (2000 Ci/mM) from Amersham, acrylamide and bisacrylamide from Serva, nitrocellulose membranes from Millipore or Schleicher and Schull (BA 85). All other chemicals

were analytical grade products.

## RESULTS

1. Efficient synthesis of di and trinucleotides by yeast RNA polymerase B. Using a dinucleotide as primer and one nucleoside triphosphate as substrate, yeast RNA polymerase B actively catalyses the synthesis of a trinucleotide. The results presented in Fig.1 shows, as an example, the formation of GpApU, using ( $\alpha^{32}\text{P}$ ) UTP and GpA as primer. This was one of the best combination for a complex DNA like calf thymus DNA. With single-stranded calf thymus DNA as template, after 30 min of incubation, up to 40 % of input UTP was incorporated into GpApU which was well resolved from the substrate by polyethyleneimine chromatography (Fig.1, lane 2). The synthesis was strictly DNA-dependent (Fig.1, lane 1) and was inhibited by  $\alpha$ -amanitin at 25  $\mu\text{g}/\text{ml}$  (result not shown). It is long known that the activity of yeast RNA polymerase B is drastically reduced on a native template (3). However, unexpectedly, the enzyme catalyzed the efficient synthesis of the same trinucleotide on native calf thymus DNA (Fig.1, lane 3), even after

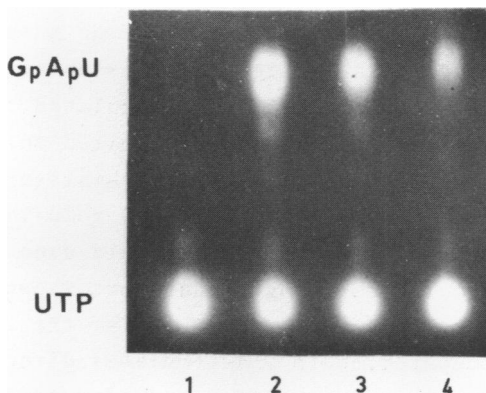


Figure 1: trinucleotide synthesis by yeast RNA polymerase B  
GpApU synthesis was done as described under Material and Methods. Mixture 1 to 4 contained (1) no DNA (2) denatured calf thymus DNA, (3) native calf thymus DNA, (4) native calf thymus DNA treated by  $\text{S}_1$  nuclease.

treatment of the template by  $S_1$  nuclease to remove single-stranded gaps (Fig.1, lane 4). The formation of the trinucleotide GpApU on the three different templates was quantified and compared with the extent of RNA synthesis with the four nucleoside triphosphates. We found that there was no direct correlation between the efficiency of RNA chain initiation, as estimated by the formation of GpApU, and the extent of RNA synthesis. The synthesis of GpApU was 100, 66 and 35 pmol/h/ $\mu$ g of RNA polymerase with the denatured, native and  $S_1$ -treated template, respectively, whereas RNA synthesis was 2950, 320 and 110 pmol of UMP incorporated per hour per  $\mu$ g of RNA polymerase on the corresponding templates. These results prompted us to investigate the efficiency and the specificity of RNA chain initiation by yeast RNA polymerase B on a defined template harboring an active yeast gene.

2. pJD14 DNA and pBR322 DNA - directed synthesis of trinucleotides. The structural gene for yeast alcohol dehydrogenase I (ADHI) has been cloned in pBR322 DNA at the BamHI site (18) (Fig.2A). The purified recombinant plasmid DNA (pJD14 DNA) and pBR322 DNA vector were used to direct the synthesis of trinucleotides by yeast RNA polymerase B. With one dinucleotide as primer and UTP as substrate, pJD14 DNA directed the synthesis of various trinucleotides (Table 1). One of them, UpApU was synthesized at a remarkably high rate. Assuming that all the enzyme molecules are active, it was calculated that every molecule of RNA polymerase B had reinitiated an average of 50 times in 30 min. In comparison, the synthesis of that particular trinucleotide with pBR322 DNA was 10 fold lower. The difference between the two templates could even be accentuated by increasing the ionic strength (data not shown). When the primer dinucleotide was replaced by one of the four ribonucleoside monophosphate, both DNA templates directed the synthesis of a dinucleotide diphosphate, provided the primer was a purine. Again in that case, a difference was seen between the two templates since the rate of synthesis of the dinucleotide pApU was 4 times higher with pJD14 DNA than with pBR322. Assuming that the ability of the enzyme to synthesize a di or a trinucleotide depended on the selection of the correct

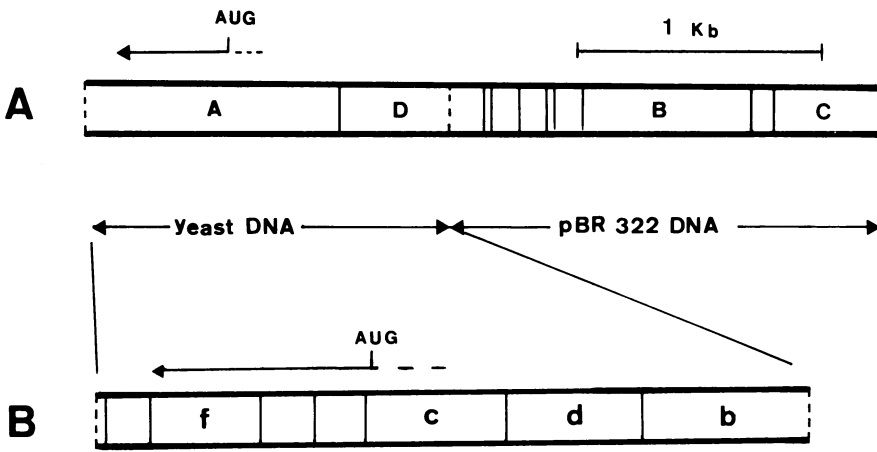


Figure 2: Physical map of pJD14 DNA

- A/ Mapping of BamHI and AvaII cleavage sites on pJD14 DNA. Vertical solid and dotted lines represent AvaII and BamHI sites respectively. The region coding for the structural gene of yeast alcohol dehydrogenase I is shown with the location of the initiation codon (B.Hall, personal communication).
- B/ Location of the HinFI cleavage sites within the yeast BamHI fragment.

sequence on the template, these data suggested a selectivity of yeast RNA polymerase B for initiating on the yeast moiety of pJD14 DNA.

### 3. RNA polymerase B binding to pJD14 DNA and pBR322 DNA.

Complexes were formed between RNA polymerase B and pJD14 DNA or pBR322 DNA, then analyzed by the nitrocellulose binding assay (22,23). When the enzyme was incubated for 5 min with the template in the absence of primer and substrate, no complexes were detected (Fig.3, lane 5). However, when the complexes were formed in the presence of UpA and UTP, a significant amount of pJD14 DNA, amounting to 2 - 5% of input, was recovered on the filter (Fig.3, lane 4). The yield of stable complexes could be increased by a short incubation of 15 sec in the presence of all four nucleoside triphosphates at low concentration to allow for a limited chain elongation at the UpA-primed site(s). Under these conditions, the amount of pJD14

Dinucleotide or trinucleotide synthesis directed by the recombinant plasmid pJD14 DNA or the vector pBR322		
pmoles/h/ $\mu$ g		
<i>primer</i>	<i>pJD14 DNA</i>	<i>pBR322 DNA</i>
ApA	16	8
ApG	13	16
ApC	7	4
ApU	3	2
GpA	17	18
GpG	1	4
GpC	2	4
GpU	17	17
CpA	15	16
CpG	7	2
CpC	1	2
CpU	2	3
UpA	190	21
UpG	26	11
UpC	14	7
UpU	0	0
pA	13	3
pG	8	7
pC	0	0
pU	0	0

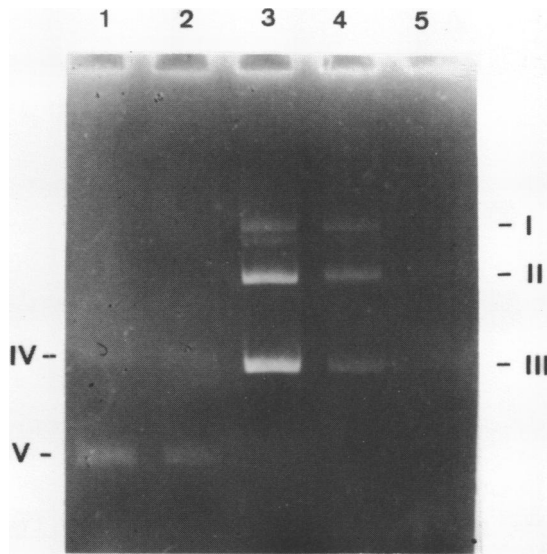
Table 1 : pJD14 and pBR322 - directed synthesis of trinucleotides and dinucleotides.

Incubations were conducted as described under Material and Methods with the indicated template and primer.

retained on the filter increased to 10 - 15 % of the input (Fig.3, lane 3). In contrast, under similar conditions, the amount of pBR322 DNA retained on the nitrocellulose filter remained at the level of experimental background (Fig.3, lanes 1 and 2). These results confirmed our previous observation that UpA primes the initiation of transcription within the yeast DNA.

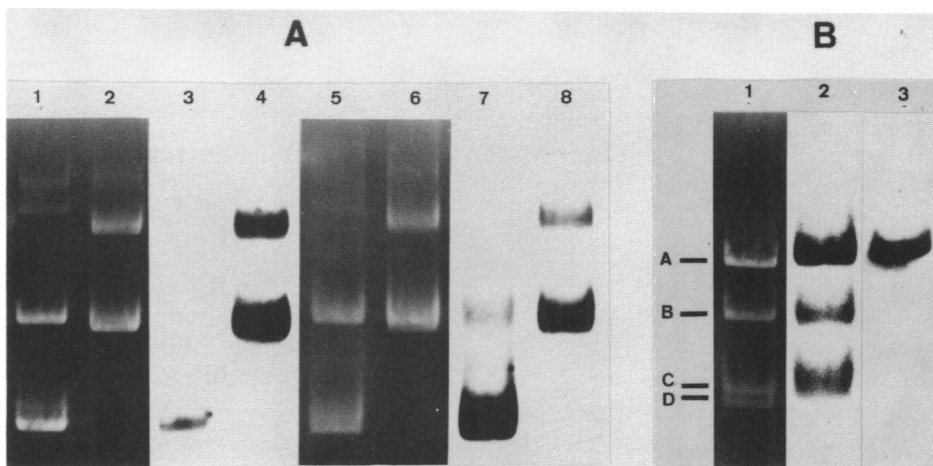
4. Analysis by gel electrophoresis of the ternary transcription complexes. The method developed by Chelm and Geiduschek (24) for analysing ternary transcription complexes by agarose gel electrophoresis was used to visualize the transcription of





**Figure 3: Yeast RNA polymerase B binding to plasmid DNA.** Stable enzyme DNA complexes were isolated by retention on nitrocellulose filters and agarose gel electrophoresis as described under Material and Methods. (1): Complexes were formed with pBR322 DNA at 30°C for 5 min in presence of 0.2 mM UpA and 5μM UTP, followed by a 15 sec pulse of transcription in the presence of all four nucleoside triphosphates at the final concentration of 5μM; (2) pBR322 DNA, 5 min of incubation with UpA and UTP; (3): pJD14 DNA, 5 min of incubation with UpA and UTP, 15 sec of RNA synthesis; (4) pJD14 DNA, 5 min of incubation with UpA and UTP; (5) pJD14 DNA, 5 min of incubation without primer or nucleoside triphosphate. The migration position for the different allomorphic forms of plasmid DNA are shown: (I), relaxed dimer pJD14 DNA; (II), supercoiled dimer and relaxed monomer of pJD14 DNA; (III), supercoiled monomer of pJD14 DNA; (IV), relaxed monomer of pBR322 DNA; (V), supercoiled monomer of pBR322 DNA.

pJD14 DNA or pBR322 DNA by yeast RNA polymerase B. After a short pulse of RNA synthesis, using the UpA - primed system, ternary complexes were isolated by gel filtration then subjected to agarose gel electrophoresis. The autoradiogram revealed that the recombinant plasmid pJD14 was a much better template than the vector pBR322 (Fig.4A, lanes 1-4). (The two radioactive bands observed with pJD14 DNA correspond to the monomer and dimer form I of the recombinant DNA). On the other hand, in a simi-



**Figure 4 : Agarose gel electrophoresis of ternary transcription complexes.**

A/ Ternary complexes were formed and isolated as described under Material and Methods using pBR322 DNA or pJD14 DNA as template. Electrophoretic separation was carried out in a 0.7 % agarose gel. Lane (1) and (3) show the results of ethidium bromide fluorescence and autoradiography respectively, when pBR322 was used as template ; in lane (2) and (4) the template was pJD14 DNA. Lanes (5) to (8) show the corresponding experiment using *E. coli* RNA polymerase. Complexes were formed at the same ratio of enzyme to DNA under similar experimental conditions excepted that the ionic conditions and nucleotide concentrations were : 70 mM Tris-HCl pH 8, 5 mM MgCl<sub>2</sub>, 50 mM NaCl, 0.5 mM ATP, GTP and CTP, and 5 $\mu$ M ( $\alpha$ -<sup>32</sup>P) UTP (10<sup>4</sup> cpm/pmol).

B/ Ternary complexes obtained with pJD14 DNA were cleaved by BamHI and AvaII restriction enzymes (Fig.1A). Electrophoretic separation was carried out in a 1.2 % agarose gel. Lane (1) shows the ethidium bromide fluorescence of the four largest fragments, lanes (2) shows the autoradiography of ternary complexes obtained without priming, lane (3) shows the ternary complexes formed under the UpA primed conditions.

lar experiment with *E. coli* RNA polymerase, ternary complexes were formed with about the same efficiency on both DNA (Fig.4A, lanes 5-8). In order to identify the DNA region in which RNA synthesis was initiated, ternary complexes formed on pJD14 DNA were treated by BamHI and AvaII restriction enzymes which yield four major DNA fragments (Fig.2A), two originating from pBR322 (bands B and C) and two from yeast DNA (bands A and D). Ternary complexes were stable during this treatment and could be ana-

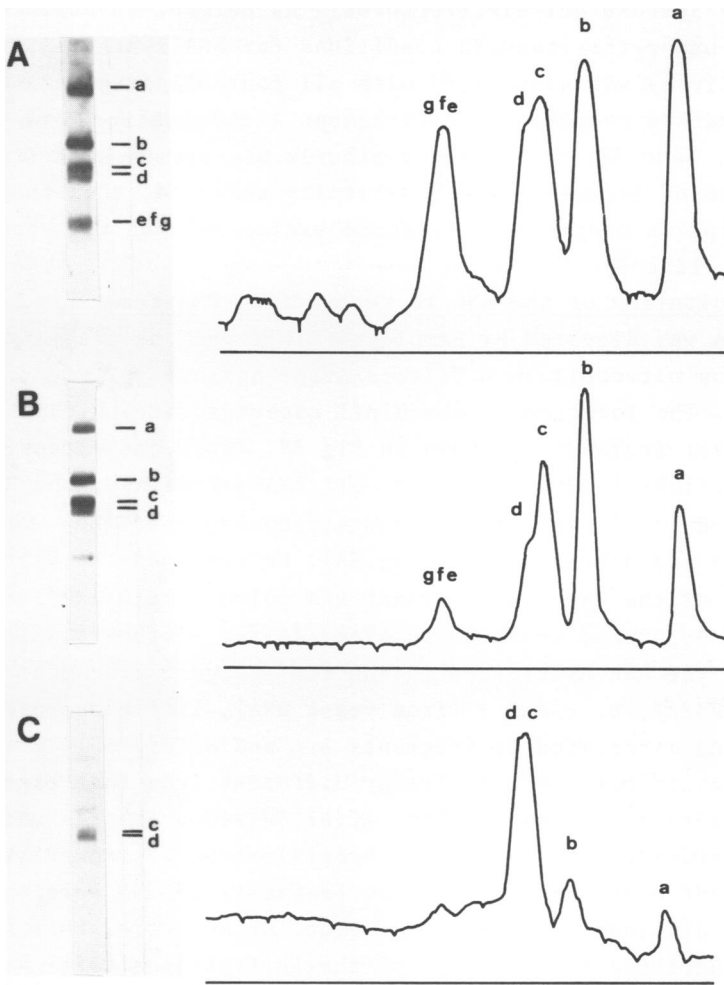
lyzed by agarose gel electrophoresis as before. In absence of primer, under the standard conditions for RNA synthesis, the radioactivity was associated with all four DNA fragments but with a marked preference for fragment A originating from yeast (Fig.4B, lane 2). When RNA synthesis was primed with UpA, the selection of fragment A was drastically enhanced, then the transcription complexes were found exclusively on this fragment (Fig.4B, lane 3).

#### 5. Hybridization of the RNA to restriction DNA fragments.

pJD14 DNA was digested by BamHI and HinfI and the fragments blotted on nitrocellulose filters after agarose gel electrophoresis. The location of the HinfI cleavage sites within the BamHI yeast fragment is shown in Fig.2B. After the extensive transcription of pJD14 DNA by *E.coli* RNA polymerase, the RNA hybridized to all the DNA fragments, showing that they were available for hybridization (Fig.5A). We compared the hybridization of the RNA made by yeast RNA polymerase B under the unprimed or primed conditions. After 40 sec synthesis without priming, the RNA hybridized to the four largest fragments *a*, (from pBR322), *b*, *c* and *d* (from yeast DNA), little radioactivity being associated to fragments *e*, *f* and *g* (Fig.5B). This hybridization pattern was already different from that expected for a random transcription. After 40 sec synthesis under the UpA priming conditions, the hybridization was restricted to fragment *c* or/and *d*. These two fragments of DNA were too close to distinguish them on the blot. At any rate, this result confirmed the location of the initiation site(s) within the yeast DNA fragment, upstream to the initial region of the ADHI gene.

#### DISCUSSION

The efficiency of the abortive initiation reaction catalyzed by yeast RNA polymerase B on double-stranded DNA template was totally unexpected. If one assumes that the trinucleotide synthesis reflects the ability of the enzyme to initiate *de novo* an RNA chain, then chain initiation does not appear to be the limiting step of transcription. This observation con-



**Figure 5 : Hybridization of cRNA to BamHI-HinfI pJD14 DNA fragments**

- A/ cRNA obtained after a transcription of pJD14 DNA during 20 min at 37°C by *E.coli* RNA polymerase was hybridized to the blotted BamHI-HinfI DNA fragments as described in Material and Methods. Scanning of the autoradiogram is shown.
- B/ Hybridization of cRNA obtained after a transcription of pJD14 DNA during 40 sec at 30°C by yeast RNA polymerase B without priming.
- C/ Hybridization of cRNA obtained under UpA primed conditions, after 40 sec transcription at 30°C by yeast RNA polymerase B. The DNA fragments originating from pBR322 are fragments a, e and g. The other fragments (b, c, d, f) originate from the yeast DNA insert as indicated in Fig.2B.

trasts with the extremely low number of RNA chains initiated by yeast RNA polymerase B (3,14) or the mammalian RNA polymerase B (27,28) on linear or supercoiled DNA template. However, in this case, the end-labelled RNA was collected by acid precipitation, under conditions which would promote the loss of small RNA chains. The possible formation of abortive RNA products during the standard transcription reaction will be investigated. Whatever the answer is, the trinucleotide synthesis made possible the study of RNA chain initiation, as for *E.coli* RNA polymerase (29,30). The template selected was a recombinant plasmid carrying the structural gene for yeast ADHI. This gene was cloned by transformation of a yeast mutant (18). End mapping of the *in vivo* transcript showed that the initiation of transcription occurred within the cloned DNA fragment which therefore contains all the required control regions (J.Bennetzen and B.Hall, manuscript in preparation).

After selecting the most active combination of primer and substrate, three approaches were followed to study the enzyme-DNA interaction and in each case a marked selectivity could be demonstrated. First, under the conditions for UpApU synthesis, with UpA as primer and UTP as substrate, a complex of enzyme B and pJD14 DNA could be isolated on a nitrocellulose filter. The yield of the complex increased after a short pulse of RNA synthesis. In contrast, practically no complex could be detected with pBR322. The fact that there was no stable complex between enzyme B and pJD14 DNA in absence of primer and substrate is in keeping with several similar observations (31,32). The second approach was to visualize directly the transcription complexes, as first shown for *E.coli* RNA polymerase (24). The transcription complexes resisted the various purification treatments and electrophoresis and could be detected upon autoradiography. Radioactive complexes were preferentially formed on the allomorphic form I of DNA, confirming that superhelicity of the DNA greatly favours enzyme-DNA binding and RNA chain initiation (33). When the location of the transcription complexes was analyzed in more detail, by fragmenting the DNA with restriction enzymes, it was found that the UpA-primed RNA synthesis occurred on the yeast frag-

ment carrying the ADHI gene. The third approach was Southern hybridization of the dinucleotide - primed RNA to blotted DNA fragments. The RNA was found to hybridize almost exclusively to two contiguous yeast DNA fragments located upstream to the ADHI gene.

At this point it is clear that yeast RNA polymerase B can selectively transcribe yeast DNA in a dinucleotide - primed reaction. We observed the formation of very short stutter products at low substrate concentration (B.Lescure, unpublished result). Therefore, it will be possible to analyze the RNA product. The DNA sequence of the ADHI gene has been determined (J.Bennetzen and B.Hall, manuscript in preparation). This will facilitate the mapping of the *in vitro* initiation site(s).

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