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Electron microscopic analysis of transcription of a ribosomal RNA operon of E. coli

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ABSTRACT

Transcription in vitro of the E. coli ribosomal RNA operon, rrnE, was analysed by electron microscopy. The transcription initiation sites of the two rrnE promoters in tandem, P_1 and P_2, were mapped and the transcription from both sites was compared. The first and the second transcription initiation site are about equally used when all nucleotides are present at 200 μM. Lowering the concentration of the second promoter's start nucleotide CTP to 3 μM reduces the use of the P_2 site sharply. At all CTP concentrations used the nascent RNA chains from P_1 are in the average longer than those from P_2 after a fixed transcription time. Most probably, this difference is caused by a longer average interval before formation of the productive complex with the second promoter.

INTRODUCTION

In E. coli there are probably seven ribosomal RNA operons per chromosome (1,2,3). The promoter regions of five rRNA operons have been sequenced (4,5,6) and found to be non-identical. Interestingly, each of the operons contains two promoters in tandem (5,7,8) about 100 base pairs apart, as transcription experiments in vitro have revealed. The transcript from the first promoter starts with ATP (in rrnA, B, E and X) or GTP (in rrnD) whereas the transcript from the second promoter starts with CTP in all five of these operons (5,6,8). Both the first and the second promoter are used in vivo (9,10).

Under optimal conditions, initiation of rRNA transcription is very efficient in vivo as well as in vitro. This feature is inherent to the ribosomal RNA promoters proper, since it is also observed in a purified system containing no other proteins than RNA polymerase. In addition, however, regulatory factors may stimulate the synthesis of rRNA even further (11, and for

references see Ref. 12). One can only speculate about the function of the tandem arrangement of two initiation sites. It has been suggested that it determines the strength of the rRNA promoters (13,14,15); it may also have a role in regulation of rRNA transcription (8).

In previous papers (16,17) we have described the interaction between RNA polymerase and rRNA promoters. The promoters of the rrnE operon behave differently towards heparin; stable complexes with the first rrnE promoter are transformed to unstable complexes in the presence of heparin, whereas those with the second rrnE promoter are not (17). About 60% of the complexes formed between RNA polymerase and a restriction fragment containing both rrnE promoters, appeared to be heparin-sensitive. This result implies that about 60% of the stable complexes contain one RNA polymerase molecule, only at the first promoter (single P_1 complexes). The heparin-resistant complexes may contain an RNA polymerase molecule only at the second promoter (single P_2 complexes), or at both promoters (double complexes).

In the present paper we analysed transcription of the rrnE operon by electron microscopy according to the method of Brack (18). This allowed us to map the two transcription initiation sites of the rrnE operon, to estimate the relative use of both sites under various conditions and to investigate whether both sites on one fragment are used simultaneously.

MATERIALS AND METHODS

Materials.

Nucleoside triphosphates were purchased from Boehringer. Formamide puriss. p.a. (Fluka) was deionised with Amberlite MB-1 (Serva) (1 g/25 ml) and stored at -20°C. Other chemicals were as described in Ref. 16. RNA polymerase was isolated with the method of Burgess and Jendrisak (19), including the steps for obtaining fully sigma-saturated RNA polymerase. Phage λmetA20 (20) was grown by thermal induction of the lysogen, strain NO 1821[AB 2569 (λcI857S7, λmetA20)]. Separation from helper phage and DNA extraction was as described by Miller (21). Phage DNA was stored at 4°C in 10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA. Digestion of λmetA20 DNA with EcoRI was carried out at 37°C in a buffer

containing 10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 1 mM DTT; 50 mM NaCl. Purification of the 1.4 kb EcoRI fragment (16) from λmetA20 DNA, containing the promoters of the rrnE operon, was as described earlier (16).

Transcription in vitro and R-loop formation.

To visualize the initiation sites of the rrnE operon we used the method of Brack (18). The 1.4 kb rrnE fragment (50 µg/ml) was incubated with E. coli RNA polymerase (50 µg/ml) in a buffer containing 20 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 0.2 mM DTT; 50 mM KCl; 0.2 mM each of ATP, UTP, GTP and various CTP concentrations. The reaction volume was 12 µl. Transcription was started by the addition of RNA polymerase and was at 37°C for 30-45 sec. To stop the reaction 190 µl R-loop mix, consisting of 140 µl formamide, 40 µl 0.5 M Piperazine-N,N'-bis(2-ethanesulfonic acid)(Pipes), pH 7.8; 50 mM EDTA; 2 M NaCl and 10 µl 0.1 M Tris-HCl, pH 8.5; 0.01 M EDTA; 3 M NaCl, was added. The DNA concentration in the R-loop mixture was 2.5 µg/ml. R-loop formation was for 30-60 minutes at 55°C.

Electron microscopy.

To 50 µl R-loop mixture, 2 µl 0.1% cytochrome c were added, or to 25 µl R-loop mixture, 25 µl of the following solution were added: 120 mM Tris-HCl, pH 8.0; 50% (v/v) formamide; 8 mM EDTA; 0.008% cytochrome c. Further treatments and measurements were as reported (22). The rest of the R-loop mixture was frozen in liquid nitrogen.

Statistics.

To assess the significance of the shift in promoter use at decreasing CTP concentrations we applied a distribution-free test for ordered alternatives according to Jonckheere, Terpstra (see Ref. 23). The same test was used to analyse the transcript measurements.

Other methods.

All other methods were as described in Ref. 17.

RESULTS AND DISCUSSION

Formation of transcription R-loops.

The 1.4 kb EcoRI fragment from λmetA20 DNA (Fig. 1) was used as the template for transcription in vitro. The fragment contains

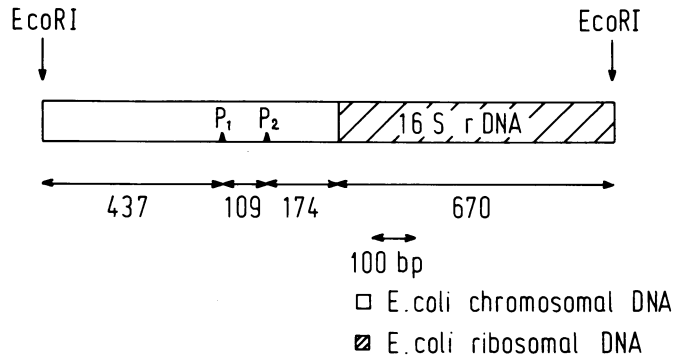

Figure 1. Map of the 1.4 kb EcoRI fragment from λmetA20 DNA, containing the rrnE promoters.
The distances, given in base pairs, are obtained from Refs. 4 and 25, and our own work (16).

the two promoters of the rrnE operon and 670 bp of the m16S sequence (24,25). The start sites, designated P_1 and P_2, are at bp 283 and 174, respectively, upstream from the beginning of the m16S sequence (4,8). After transcription for a short period of time, the newly synthesized RNA chains were hybridized back to the DNA template under R-loop conditions. The resulting R-loop molecules were analysed by electron microscopy.

Fig. 2 gives a schematic drawing of R-loop structures expected when the RNA chains are initiated on either P_1 or P_2. If the transcription period is chosen to be sufficiently short, the distances a_1 or a_2 representing the DNA stretch upstream from the respective initiation sites will be smaller than distance b, which is ahead of the moving RNA polymerase molecule. This will allow us to determine the direction of transcription and to distinguish the transcription initiation sites of nearly all observed molecules. In our experiments we could not assign P_1 or P_2 starts to some molecules which were not sufficiently asymmetrical.

Examples of R-loops with RNA chains initiated on P_1 or P_2 are depicted in Fig. 3. No complicated structures were observed which might have been formed if RNA chains were initiated simultaneously on the P_1 as well as on the P_2 site of the same

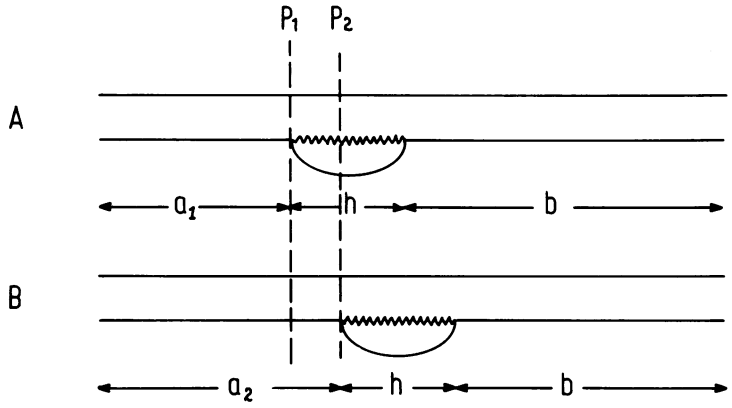

Figure 2. Schematic drawing of transcription R-loop molecules. R-loop molecules expected after initiation on P_1(A) or P_2(B) are depicted. The zigzag line represents the hybridized RNA strand.

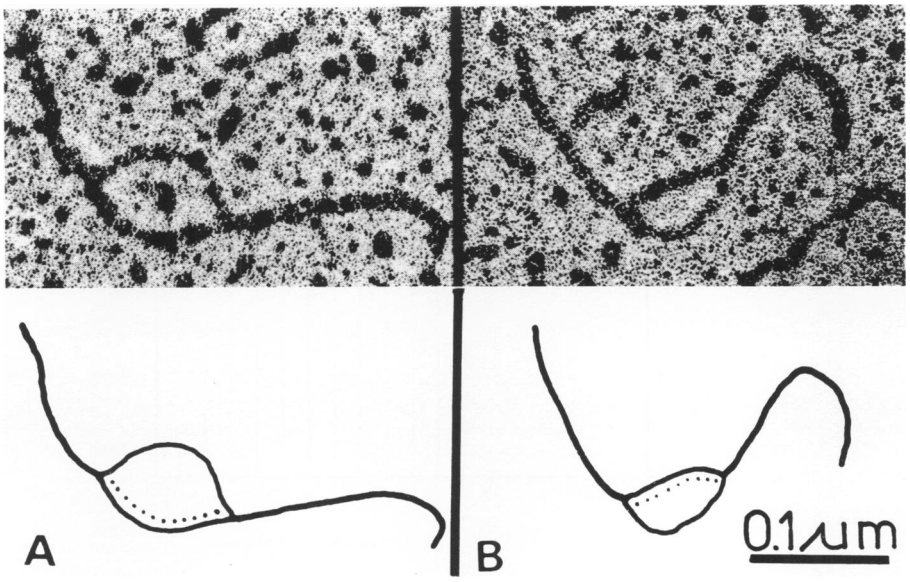

Figure 3. Electron micrographs of transcription R-loop molecules. The 1.4 kb rrnE fragment was transcribed for 45 sec. CTP was present at 5 µM, the other nucleoside triphosphates were at 200 µM. Examples of a P_1 start (A) and a P_2 start (B) are shown.

DNA molecule.

Most R-loops were observed at the KCl concentration optimal for rRNA synthesis (50 mM, see Ref. 26), and only rarely at 100 or 150 mM KCl.

Identification of the transcription initiation sites.

A large number of R-loop molecules, formed after transcription for 30 sec at 50 mM KCl and 200 μM of each of the four nucleotides, was measured (Fig. 4). Defining the fragment terminus lying upstream from the promoters as position zero, and the entire fragment length (1390 bp) as one, we map the transcription initiation sites at positions 0.29-0.31 and 0.35-0.37. These values are in good agreement with the positions 0.31

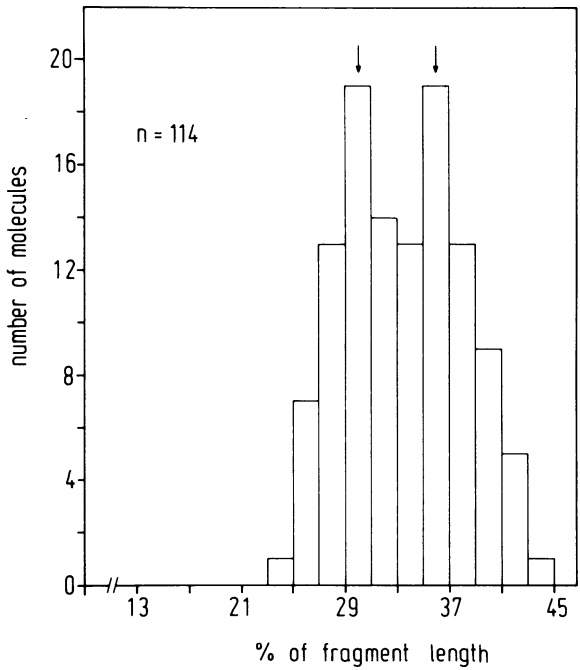

Figure 4. Distribution of the transcription initiation sites.
Transcription was for 30 sec at 200 μM of each of the four NTPs. Assuming that the promoter is located at the shorter arm, we calculated the positions of the initiation sites, and expressed them as percentage of the total length of each individual molecule. The arrows indicate the most probable position of P_1 and P_2.

for P_1 and 0.39 for P_2 which have been derived from nucleotide sequence (4,25) and restriction enzyme analysis (4,16). For the experiments described below, the 0.33-value was chosen as the boundary between P_1 and P_2 starts.

Use of the start sites.

From the molecules in Fig. 4 we estimate that 46% of the RNA chains is initiated on P_1 and 54% on P_2. In doing so we had to omit five molecules which could not be assigned unambiguously. Our data are in good agreement with the results of Gilbert et al. (8) obtained from in vitro transcription experiments. The relative use of P_1 and P_2 is compatible with the ratio of heparin-sensitive versus heparin-insensitive complexes (17, see also the Introduction). However, no firm conclusion can be drawn since the RNA polymerase/promoter ratio in these experiments was quite different (17).

The start sequence of the first rrnE promoter is pppApUpCp- while that of the second promoter is pppCpUpCp- (8). This difference should provide a tool to suppress initiation on the second promoter by reducing the CTP concentration of the incubation mixture. In the presence of 5 μM CTP, still 50% of the RNA chains is initiated on P_2 (Fig. 5A). However, at 3 μM CTP the percentage of P_2 starts is strongly reduced to 19% (Fig. 5B). The trend towards P_1 preference at lower CTP concentration is statistical highly significant ($P < 0.0003$; see Materials and Methods). Further reduction of the CTP concentration to 2 μM or lower strongly decreased the number of R-loop structures observed. Apparently, transcription is now inhibited irrespective of the promoter used. Halted elongation or abortive initiation are possible explanations. The latter phenomenon has been reported for the A_1 and A_2 promoters of T_7 (27).

It is apparent from our experiments that initiation on P_2 hardly occurs in the presence of 3 μM CTP. It was recently reported that also the extent of RNA initiation on the A_1 and A_2 promoters of T_7 dropped precipitously at substrate concentrations of about 3 μM (27). Earlier, much higher concentrations of the start nucleotide had been reported to be required for RNA initiation (28,29). Gilbert et al. (8) reported that at CTP

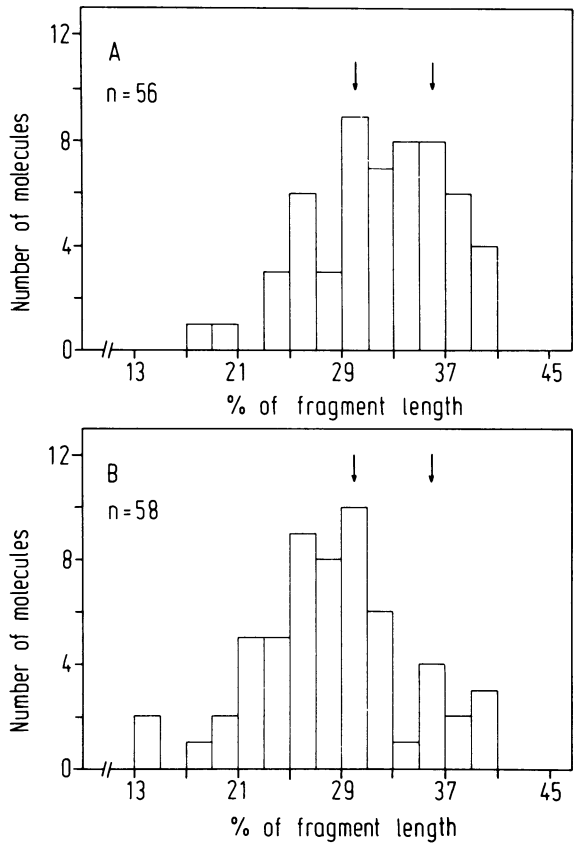

Figure 5. The effect of CTP concentration on initiation site use. Transcription was for 45 sec at 200 μM ATP, UTP, GTP and 5 μM (A) or 3 μM (B) CTP. See further the legend to Fig. 4.

concentrations below 50 μM, transcription from P_2 was more strongly affected than that from P_1; at 5 μM CTP transcription from P_2 was almost negligible whereas we observed hardly any effect in the presence of 5 μM CTP. A possible explanation might be sought in the assay conditions which are somewhat different; the UTP concentration is lower and the RNA polymerase/DNA ratio is higher in the experiments of Gilbert et al.

Initiation events on P_1 and P_2; possible differences.

The promoters P_1 and P_2 of rrnE are about equally sensitive

to ppGpp (data not shown), but differ in the stability of the polymerase-promoter complex towards heparin (17, see also above). Possible differences in initiation features may also be reflected in the length distribution of transcripts after a fixed short time. We therefore analysed the length distribution of the RNA-DNA hybrids from the R-loop experiments of Figs. 4 and 5 and found a highly significant difference between P_1 and P_2 (Fig. 6), the transcripts from P_2 being shorter.

This difference cannot be due to an experimental artifact since the P_1 and P_2 R-loops compared were taken from the same electron micrographs, and only after the length measurement assigned to P_1 or P_2. Since the distribution range is in agree-

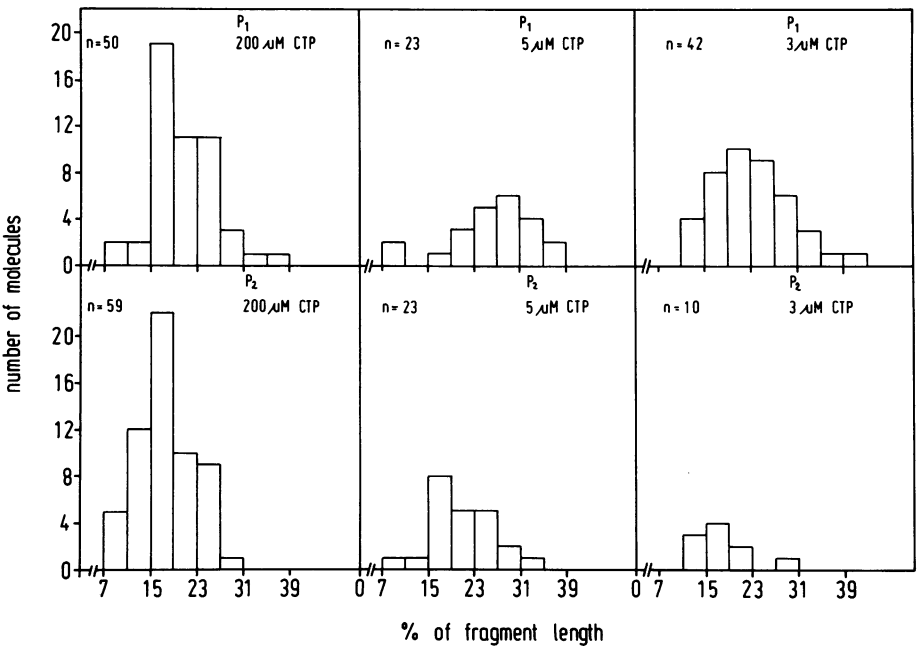

Figure 6. Length distribution of transcription R-loops.

The lengths of the RNA-DNA hybrids formed in the experiments of Figs. 4 and 5 were measured and expressed as percentage of the total length of each individual molecule. Some molecules to which the orientation could not be assigned unambiguously were not included. The statistical values for the differences between P_1 and P_2 were $P < 0.0116$ (200 µM CTP); $P < 0.0003$ (5 µM CTP); $P < 0.0035$ (3 µM CTP).

ment with continuing initiation during the experiment the difference must be either due to a different rate of elongation or a different average "waiting time" before the RNA polymerase starts elongating. Because transcription of all molecules was stopped at the same instant a shift in lengths measured would result. A difference in elongation rate is highly improbable since the transcribed trajects are largely identical, and the 100-nucleotide stretch between P_1 and P_2 has a base composition similar to the succeeding part. Since the minimal elongation rate calculated from our data -12 to 18 nucleotides per sec- is in the range generally found transcription must have proceeded normally. We have to conclude that some difference in an initiation event, most probably in the formation of a productive DNA-RNA polymerase complex, exists between P_1 and P_2. While we cannot at this stage describe this difference in molecular terms it is another feature distinguishing P_1 from P_2, and may be involved in the function of tandem promoters *in vivo*.

CONCLUDING REMARKS

We could distinguish the first and second initiation site of the rrnE operon by electron microscopy and found an equal use of P_1 and P_2 at high concentrations of all nucleoside triphosphates. Our results agree with our previous binding studies *in vitro* (16,17). Recently, the second initiation site of another rrn operon, rrnB, was found to possess an even higher affinity for RNA polymerase than the first (30). We found a clear difference between both initiation sites: P_2 has a somewhat longer average "waiting time" which we cannot yet identify in kinetic terms.

While demonstrating the complexity and subtlety of initiation on the rrn promoters our data do not yet allow an appraisal of the intriguing tandem promoter system whose importance is clear from its conservation in all rrn operons examined.

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