Electron microscopic analysis of the transcription of the *Bacillus subtilis* riboflavin operon inserted into the hybrid plasmid pLP102

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We used electron microscopy and computer analysis to study the *Bacillus subtilis* riboflavin operon transcription. The size of the riboflavin operon has been shown to be 2.40 ± 0.15 MDa. Transcription maps of the riboflavin operon in vitro and in vivo have been obtained. In the riboflavin operon region four transcripts are sythesized in vivo having the following approximate boundary positions in the map of pLP102: 46–52, 46–72, 62–72 and 55–58 map units. The riboflavin operon is transcribed from three promoters, at 46, 58 and 72 map positions. A scheme of the riboflavin operon transcription in *B. subtilis* is suggested.

Riboflavin operon (Bacillus subtilis) *Transcription* *Electron microscopy*

1. INTRODUCTION

Riboflavin is a biologically active substance, vitamin B_2. There are 6 structural genes of riboflavin biosynthesis, organized in an operon, in *Bacillus subtilis* cells [1]. The basic structural organization and riboflavin biosynthesis regulation have been elucidated by a series of biochemical and genetic experiments [2–5]. In particular, the existence of at least two operator regions in the riboflavin operon [5] has been demonstrated. One operator controls the early stages of riboflavin biosynthesis, the other controls the later ones. At the same time, the riboflavin operon transcription scheme has not yet been revealed. Here, we examined the location of the riboflavin operon promoter and terminator regions by electron microscopy.

2. MATERIALS AND METHODS

The *Eco*RI and *Bam*HI endonucleases were from Boehringer Mannheim. *E. coli* RNA polymerase was kindly provided by B. Kucheryavy (Institute of Biological Physics, USSR Academy of Sciences, Pushchino). DNA was isolated as in [6]. RNA was isolated as described [7]. The pLP102 DNA-RNA hybridization was performed for 30 h at 56°C (for reference see [8]). The DNA-RNA polymerase binding method has been described in [9]. Transcription with *E. coli* RNA polymerase in vitro was carried out for 15 and 1 min [10]. In the 1 min synthesis the transcription mixture was heated for 5 min at 70°C to cleave short transcripts from the DNA-RNA polymerase-RNA ternary complex, then RNA was isolated from DNA with 2 M NaCl, concentrated by alcohol precipitation and used in hybridization experiments. Electron microscopy was done according to Davis et al. [11]. The hyperphase contained 50 mM Tris-HCl, 5 mM Na-EDTA, 50% formamide (pH 8.5), and 50 μg/ml of cytochrome *c*. Deionized water was used as a hypophase. All samples were platinum-shadowed at an angle of 7°. Photographs were taken with a Philips EM 400T electron microscope with a magnification of about $10000\times$. DNA length measurements were made with the help of an HP9864 digitizer connected to an HP9825 desktop computer (Hewlett-Packard, USA). A set of programs for data manipulation was kindly pro-

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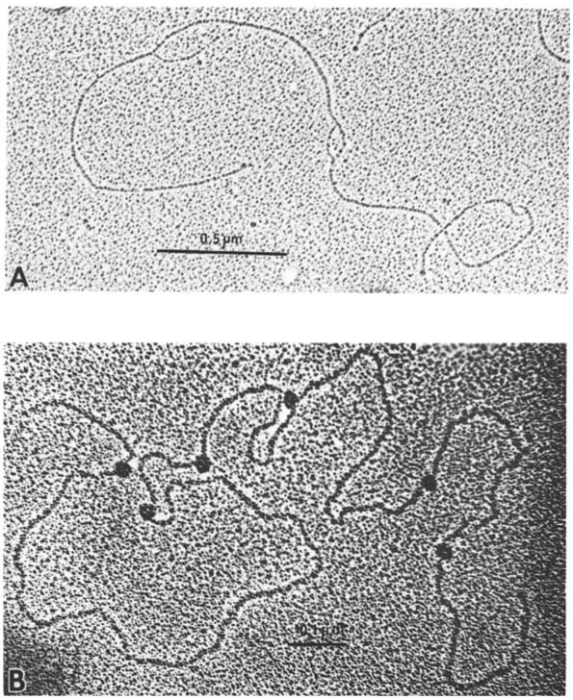

Fig.1. Electron micrographs of R-loops on pLP102-*Bam*HI DNA (A) and of *E. coli* RNA polymerase-DNA complexes (B).

vided by Dr Eu.I. Golovanov. At least 100 DNA molecules were used in each experiment to obtain the histogram.

3. RESULTS AND DISCUSSION

The recombinant plasmid pLP102 was the subject of our studies [12]. pLP102 consists of the pBR322 vector molecule with a *B. subtilis* DNA fragment (6.3 MDa) inserted at the *Eco*RI restriction site and containing the riboflavin operon, *spoO* genes responsible for sporulation, and probably a small DNA region participating in lysine biosynthesis. Two basic methods were used here, the method of R-loop formation in the DNA/complement RNA hybridization region and the method of RNA polymerase-DNA binding.

Our initial aim was to determine the size of the riboflavin operon and position within the hybrid plasmid pLP102. With this end in view we hybridized the pLP102 DNA with the total RNA isolated strictly under the following conditions. First, RNA was isolated from *B. subtilis* strain CHP335, which constitutively synthesizes riboflavin, so that we obtained a higher mRNA content of the riboflavin operon in total RNA. Second, RNA isolation was performed at an early stage of exponential growth (16 h), i.e. long before the sporulation process, and, consequently, the operation of the *spoO* genes located next to the riboflavin operon. These conditions made it possible to achieve preferential hybridization between complementary RNA and the riboflavin operon. This allowed us to determine the location and size of the riboflavin operon. As shown in fig.2, the riboflavin operon constitutes 26% of the pLP102 DNA total length (46–72 map units), i.e. 2.4 ± 0.15 MDa.

The first step in determining the riboflavin operon promoters and terminators consisted in mapping the R-loop boundary distribution (contour line in fig.3). The histogram peaks are potential regions of transcript initiation and/or termination. Still, the map does not allow us to determine the R-loop positions, if their ends are concentrated in the 69–73 map unit region. To solve the problem of the transcript end location we used differential analysis of the R-loop boundaries, which allowed us to take into account only the transcripts with an end corresponding to a potential region of the transcription start or termination, i.e. points 1, 1A, 2, 2A, 3, 3A in fig.3. Fig.3 shows that the right

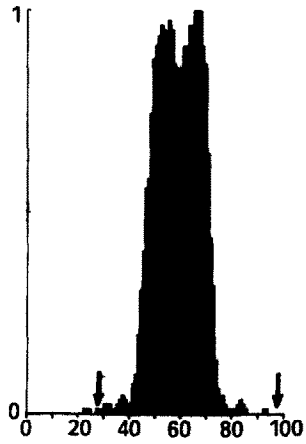

Fig.2. Histogram of R-loops on pLP102-*Bam*HI DNA. Abscissa, calibrated in % of pLP102 DNA length (map units); ordinate, in relative amount of R-loops. The arrows at the *Eco*RI restriction site limit the *B. subtilis* DNA inserted region.

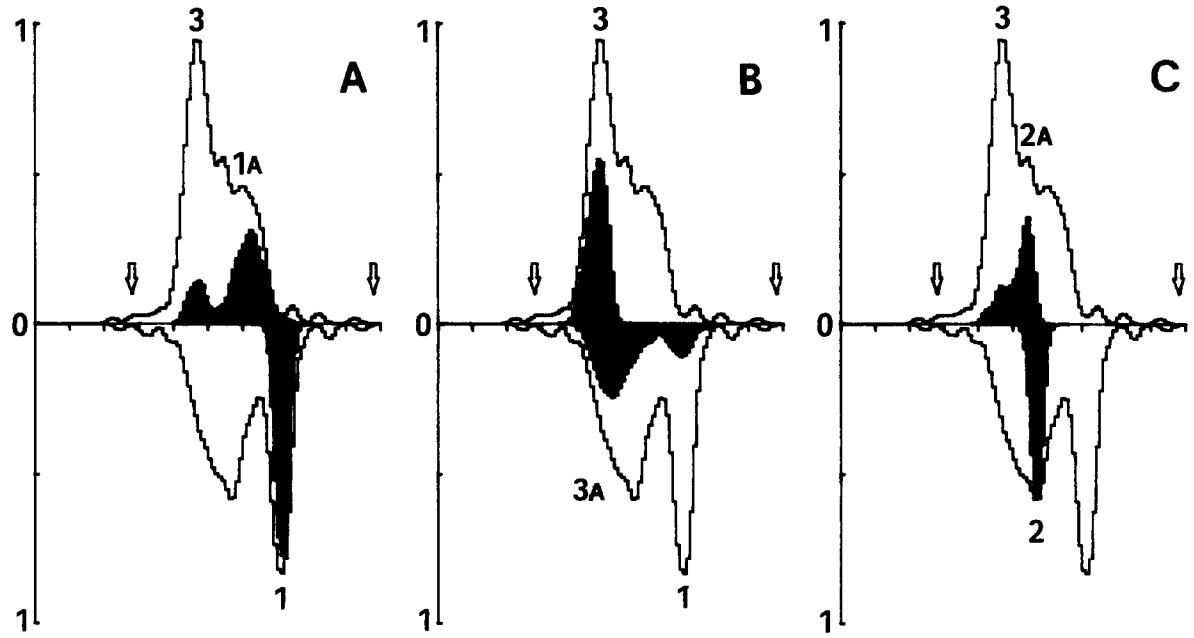

Fig.3. Histograms of R-loop boundaries on pLP102-*Bam*HI DNA (contour line). Abscissa as in fig.2; ordinate represents an amount of left ends (upwards) and right ends (downwards) at a given point. The hatched areas are submaps of the boundary distribution of R-loops, with one end in regions 69–73 map units (A), 40–49 map units (B), 55–58 map units (C).

ends of two transcripts differing in size are located within the 69–73 map unit region. One has the size of the riboflavin operon (46–72 map units), indicating the existence of polycistron mRNA; the other is shorter, and its left end corresponds to peak 1a with a maximum at map position 62. Similarly, R-loops with left ends within the 44–49 unit region (fig.3B) have right end maxima at 52 and 72 map units. Thus, the analysis of the R-loops with the ends located in regions 1 and 3 suggests the existence of 3 transcripts of different sizes, with the following average boundary map positions: 46–52, 46–72, 62–72. Mapping of the middle region of the riboflavin operon (fig.3C) shows that the transcripts corresponding to peak 2 are those mainly located at point 2A, although their left ends may reach peak 3 (46 map units).

A series of experiments has been performed to study the in vitro transcription of the riboflavin operon using *E. coli* RNA polymerase. As shown in fig.4A, *E. coli* RNA polymerase actively recognizes and transcribes the region of the *B. subtilis* riboflavin operon. A correspondence is observed between points 1, 2 and 3, for both in vitro and in vivo systems. The nucleotide sequence of the average *E. coli* promoter is known to be homologous to that of the promoters recognized by the major form of the *B. subtilis* RNA polymerase [13]. In the light of this homology it is clear why the *E. coli* enzyme recognizes the riboflavin operon promoters.

To determine the direction of the transcription hybridization of short RNAs synthesized in vitro on pLP102 DNA for 1 min was performed (not shown). Differential analysis of the R-loop boundaries showed that one of the ends of the short transcripts corresponds to points 1, 2 and 3 in the transcription boundary map, whereas the opposite ends of these R-loops in the case of one transcript shifted to the left, closer to peak 3, and in the other cases to the right towards peaks 1 and 2, unambiguously indicating that at point 3 the transcription is directed from left to right, and at points 1 and 2 from right to left.

One more proof of the promoter's localization at points 1, 2 and 3 resulted from the study of sites of *E. coli* RNA polymerase tight binding to pLP102 DNA (fig.4B). It is known that RNA

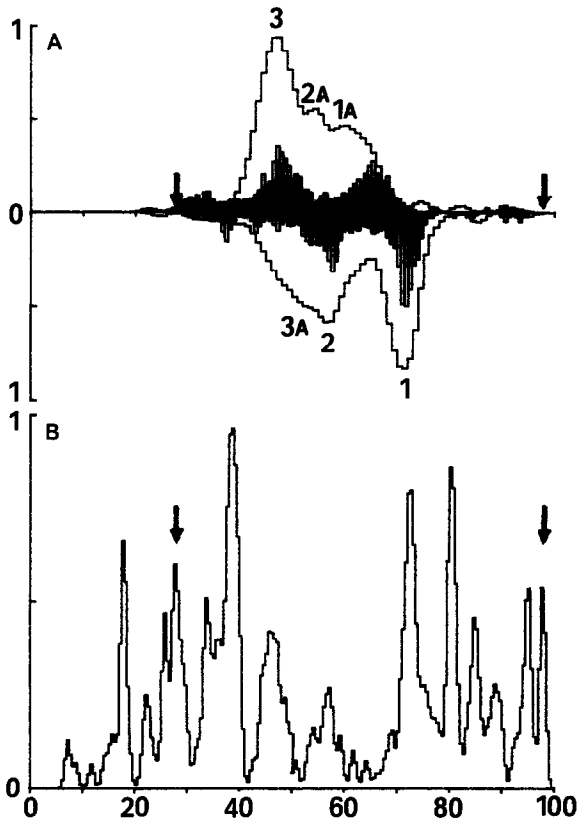

Fig.4. (A) Histograms of R-loop boundaries on pLP102 DNA (RNA synthesized in vivo – contour line; RNA synthesized in vitro – hatched areas). (B) Map of *E. coli* RNA polymerase binding sites on pLP102-*Bam*HI DNA. Arrows indicate *Eco*RI sites.

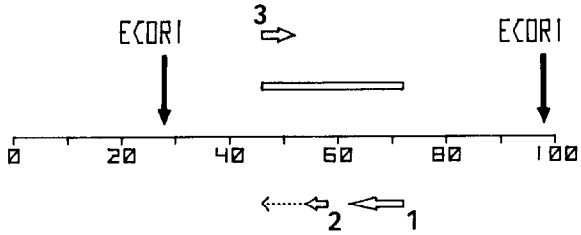

Fig.5. Schematic representation of *B. subtilis* riboflavin operon transcription in vivo. The figure indicates promoter positions; the arrows show the direction of transcription and the positions of terminators. The rectangle corresponds to polycistron mRNA, with the direction of synthesis as yet undetermined.

polymerase binds preferentially to promoters [14–17]. In the region of the riboflavin operon the tight binding sites of *E. coli* RNA polymerase are mapped; they correspond to peaks 1, 2 and 3 in R-loop boundary maps in vivo and in vitro.

The results obtained allowed us to suggest a diagram of the riboflavin operon transcription in *B. subtilis* cells (fig.5).

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