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Probing the structure of ribosome assembly intermediates *in vivo* using DMS and hydroxyl radical footprinting

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ABSTRACT

The assembly of the *Escherichia coli* ribosome has been widely studied and characterized *in vitro*. Despite this, ribosome biogenesis in living cells is only partly understood because assembly is coupled with transcription, modification and processing of the pre-ribosomal RNA. We present a method for footprinting and isolating pre-rRNA as it is synthesized in *E. coli* cells. Pre-rRNA synthesis is synchronized by starvation, followed by nutrient upshift. RNA synthesized during outgrowth is metabolically labeled to facilitate isolation of recent transcripts. Combining this technique with two *in vivo* RNA probing methods, hydroxyl radical and DMS footprinting, allows the structure of nascent RNA to be probed over time. Together, these can be used to determine changes in the structures of ribosome assembly intermediates as they fold *in vivo*.

1. Introduction

Ribonucleoprotein (RNP) complexes typically undergo many structural changes during their lifecycles, from transcription of the RNA to maturation of the complex and degradation. Because the full lifecycle of an RNP cannot often be reconstituted in the test tube, footprinting methods for probing RNA structure in the cell have gained increasing attention [1,2]. Here, we describe methods for probing the structure of the 16S ribosomal RNA in *Escherichia coli* cells following metabolic labeling of pre-rRNA. This approach can be adapted to high-throughput investigations of RNP dynamics.

The bacterial ribosome is made up of three rRNAs and over 50 proteins that assemble into the large (50S) and small (30S) subunits. Assembly of the subunits is coupled with pre-rRNA synthesis [3–5] and requires only a few minutes during logarithmic growth [6]. Near the end of the assembly process, an intermediate 17S form of the pre-rRNA is trimmed to its mature (16S) length in several steps by ribonucleases. Several features likely contribute to the rapidity of subunit biogenesis in the cell. First, the 5′-to-3′ polarity of co-transcriptional assembly limits the opportunities of forming non-native RNA secondary structures, because 5′ regions of the rRNA are able to fold before the 3′ regions have been transcribed [5]. Second, more than 15 assembly factors and additional RNA and protein modification enzymes facilitate assembly and carry out the final steps of subunit maturation [7,8]. Third, homeostasis of free ribosomal proteins ensures a constant pool of protein components [9]. In eukaryotes, ribosome assembly is aided by over 200 assembly factors and is also coupled to transport of assembly intermediates from the site of transcription in the nucleolus to the cytoplasm [10].

Because the bacterial ribosomal subunits can be reconstituted *in vitro* using only free rRNA and ribosomal proteins [11,12], they have long served as a model for the assembly of ribonucleoprotein complexes. Despite this, ribosome biogenesis in living cells remains poorly understood. One reason for this is the low abundance of assembly intermediates, which constitute 2–5% of total rRNA in *E. coli* under normal growth conditions [6]. Moreover, the predominant intermediates are difficult to isolate using sucrose gradient sedimentation, because they tend to migrate near the mature subunits.

Previous studies increased the concentration of ribosomal intermediates by using temperature-sensitive strains or conditional mutations that stall assembly under non-permissive conditions such as low temperature [13]. Ideally, one should study the path

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of assembly in real time, under normal conditions. The kinetics of ribosome synthesis or protein binding has been measured by pulse-labeling cells with isotopically-modified nucleosides or amino acids and then analyzing via scintillation counting [6] or mass spectrometry [14]. These approaches reveal the protein composition of assembly intermediates. When complemented by structure probing of the RNA, a more complete picture of the assembly process can be obtained.

We present here a technique for probing the structure of ribosome assembly intermediates in *E. coli* (Fig. 1). To synchronize pre-rRNA synthesis, we take advantage of the role that two intracellular small molecule effectors play in regulating the initiation of bacterial rRNA transcription. In *E. coli*, rRNA transcription is repressed during starvation through both the stringent response (ppGpp) and the concentration of the initiating NTP [15]. During the late stationary phase and extended periods of starvation, the concentration of the initiating NTP becomes the primary repressor of ribosomal RNA operon (*rrn*) promoter activity [15]. When nutrients become available, there is a rapid increase in intracellular NTP concentration and pre-rRNA synthesis is reactivated. For log phase cells subject to a nutrient upshift and cells diluted from stationary phase into fresh media, *rrn* promoter activity increases within 1 min [15].

In order to label nascent transcripts when pre-rRNA transcription resumes, we pulse-labeled starved cells with 4-thiouridine (4sU) just before feeding [16]. After extracting the total RNA from the cells, the nascent RNA labeled with 4sU can be conjugated to a biotinylation reagent via a reversible disulfide bond [17]. The labeled RNA is captured on streptavidin beads and analyzed by direct primer extension or high-throughput sequencing. In this way, it is possible to get snapshots of the ribosome assembly process after transcription begins again.

4sU is photoactive and has been widely used to study RNA-protein complexes by in-cell photocrosslinking [16,18]. More recently, metabolic labeling with 4sU has been used to isolate RNA from yeast [17] and metazoan cells [19] for transcriptome studies. To our knowledge, this represents the first use of 4sU to isolate newly synthesized RNA in bacteria.

A variety of reagents, such as dimethylsulfate (DMS), ribonucleases, N-methylisatoic anhydride (SHAPE) and hydroxyl radicals, have been used to probe RNA structures *in vitro* [20,21]. DMS and hydroxyl radical (X-ray) footprinting are particularly useful for probing RNA structures in the cell. DMS is lipophilic and readily passes the cell membrane [22,23]. Hydroxyl radicals can be generated *in situ* by synchrotron or gamma radiation [24–26]. We have successfully used hydroxyl radicals and DMS to probe rRNA *in vivo* with a time resolution of 30 s. Both techniques will be presented here.

Time-resolved X-ray-dependent hydroxyl radical footprinting has been used to study the kinetics of *in vitro* RNA folding [27] because sufficient hydroxyl radicals to probe RNA can be generated in a few milliseconds using a synchrotron X-ray source with high flux density [28]. X-ray footprinting has also been used on frozen *E. coli* cells to characterize the structure of the small subunit of the ribosome and examine the effect of ribosome assembly factor deletion [29]. Because the hydroxyl radical is produced *in situ* by photolysis of the water, no harmful permeabilization of the cell is needed. Another advantage of X-ray footprinting is that the extent of cleavage can be controlled by varying the X-ray dose [30].

The hydroxyl radicals break the RNA strand by abstracting a proton from the ribose C4′, C5′ or C1′ [31]. The probability of cleavage depends on the solvent accessibility of the ribose, and thus reflects the RNA tertiary structure or protein interactions. Because hydroxyl radicals cleave the RNA backbone in a base-independent manner, hydroxyl radical footprinting provides structural data on the RNA at single-nucleotide resolution, unlike many other chemical probing techniques.

In contrast to hydroxyl radical, DMS methylates adenine N1, cytosine N3 and guanine N7, and therefore the extent of modification reports on the RNA secondary structure and other interactions with the RNA bases. Methylation of A N1 and C N3 is detected by pausing of reverse transcriptase during primer extension. Methylation of G N7 is usually only detected by primer extension if it subsequently causes depurination. DMS has been extensively used to probe RNA *in vitro* [32–34], and because it is highly reactive, can also be used to monitor RNA assembly in real time [35]. It has also been used *in vivo* for transcriptomics studies [36]. High-throughput DMS probing of rRNA structure has been performed in bacteria, yeast, and mammalian cells [36–38]. While DMS only modifies certain bases, it does not require a synchrotron, is reproducible, and easy to scale up.

The protocols below describe X-ray and DMS footprinting of RNA in actively dividing cells in order to map changes in the small ribosomal subunit that take place during assembly. The reader can choose to use either technique or both depending on the resources of the lab and the requirements of the experiment.

2. Materials and methods

2.1. Overview of starvation recovery

During normal cell growth, the ribosomes being synthesized in the cell are in various states of assembly, giving a time-average of the different pre-rRNA intermediates. To synchronize transcription of the rRNA, we exploited the fact that rRNA transcription in *E. coli* is repressed during starvation by the stringent response and then activated when nutrients become available [15]. As illustrated in Fig. 2, little 17S pre-rRNA is present in *E. coli* MRE600 cells suspended in a minimal medium lacking phosphate. Upon addition of a rich medium containing phosphate, 17S pre-rRNA is detected within 1–2 min, consistent with the kinetics of pre-rRNA synthesis during recovery from stationary phase [15]. When 4sU is added to

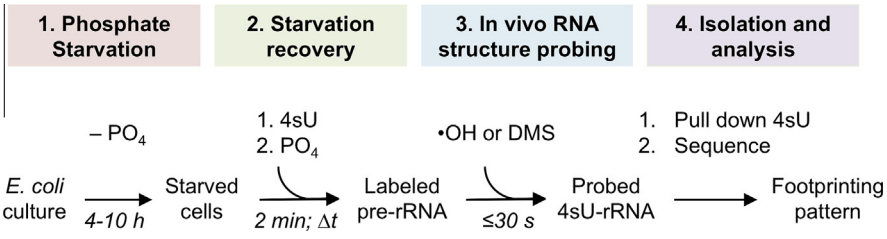

Fig. 1. Overview of time-resolved *in vivo* footprinting protocol. The method consists of three major parts: (1) starvation recovery, (2) *in vivo* RNA footprinting, and (3) isolation and analysis of the RNA. In the first phase, pre-rRNA transcription is synchronized by starving cells of phosphate, then adding nutrients and phosphate to trigger *de novo* ribosome biogenesis. The modified nucleotide 4-thiouridine (4sU) is added to the media prior to food. During the recovery from starvation, 4sU-labeled nascent rRNA is probed with hydroxyl radical or DMS in the second phase. In the third phase, the 4sU-labeled RNA is isolated from harvested cells and sequenced.