Formation of circular polyribosomes in wheat germ cell-free protein synthesis system

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Abstract **We report a morphological study of functioning ribosomes in a efficient and robust cell-free protein synthesis system prepared from wheat embryos. Sucrose density gradient analysis of translated mixtures programmed with luciferase mRNAs having different 5′ and 3′ untranslated regions showed formation of large polysomes. Electron microscopic examination of translation mixtures programmed with those of capped and polyadenylated mRNA revealed that ribosomes assemble into a circular-type polysome in vitro. Furthermore, a series of experiments using mRNAs lacking either cap, poly(A) tail or both also resulted in the formation of circular polysomes, which are indistinguishable from those with the original mRNA. The wheat germ cell-free system may provide a good experimental system for understanding functional ribosomes at the molecular level.**

Key words: Wheat germ; Cell-free translation; Electron microscopic observation; Circular polysome; No-cap non-poly(A) tail

1. Introduction

Over the past 50 years, cell-free protein synthesis systems have mainly been used to elucidate the mechanism of translation, and early reports noted the instability of the system. Indeed, *Escherichia coli* cell-free systems are active only for less than 30 min and systems from reticulocytes or wheat embryos work only for 1–2 h in a test tube [1,2]. Subsequent accumulation of experimental evidence led to the idea that the short life times of reaction in these systems are due to both a shortage of substrate supply, such as amino acids and energy, and an increased concentration of byproducts. In fact, Spirin et al. proposed firstly a new reaction method, a continuous flow cell-free translation system, in which a solution containing amino acids and energy sources is supplied to the reaction chamber through a filtration membrane [3]. This design is significantly more efficient than conventional batch systems: the reaction works for tens of hours and produces hundreds of micrograms per ml reaction volume [3–5]. Recently we have found other limiting factors in wheat germ extract. We demonstrated that conventional wheat germ extracts contain the RNA *N*-glycosidase tritin and other inhibitors of translation such as thionin, ribonucleases, deoxyribonucleases, and proteases, and that these inhibitors originate from the endosperm [6]. Extensive washing of wheat embryos, to eliminate endosperm contaminants, resulted in extracts with a high degree of activity and stability; with the continuous system the reaction maintains translation for 14 days [7]. Further we showed for the first time that incubation led to formation of polysomes in vitro analyzed by sucrose density gradient centrifugation [6]. Based on these findings we proposed a new view of the translation apparatus as an inherently stable system that contrasted with the common belief that the in vitro reconstitution of the translation apparatus leads to an inherently unstable system as the system is built up with hundreds of factors [6,7].

Since the first reports on functioning ribosomes in animal cells as 'beads on a string' on membranes, or 'polysomes' in rabbit reticulocyte cytosol [8,9], a number of electron microscopic (EM) studies on the structure of functioning ribosomes have been published. It is well established that in eukaryotes, from protozoan to human cells, polysomes are arranged in circular and helical configurations, called *circular polysomes* [10–15], in contrast to the linear configurations seen during coupled transcription and translation in prokaryotic cells [16]. The reports of so-called circular polysomes are based on the examination of cellular sections prepared from animal cells and tissues as well as from isolated polysomes. So far, no successful in vitro reconstitution of translation apparatus containing circular polysomes has been reported. This might simply be due to the lack of suitable cell-free translation systems that allow the efficient organization of polysomes.

Here we report results of a morphological examination of ribosomes in a wheat germ cell-free system by transmission electron microscopy. The experimental results reveal for the first time that during translation ribosomes assemble into large circular polysomes which are morphologically indistinguishable from those found in eukaryotic cells in vivo, which may support an explanation for the high efficiency of the system at the morphological level. In addition, an interesting result was that neither the 5′ cap nor the 3′ poly(A) is essential for the architecture of circular polysomes, which is inconsistent with the general understanding.

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2. Materials and methods

2.1. Cell-free protein synthesis

Purification of wheat embryos and preparation of the cell-free extract were performed as described [6], with slight modifications. The reaction mixture (25 μl) contained 6 μl of extract ($200A_{260\,nm}$, $A_{260\,nm}/A_{280\,nm} = 1.53–1.55$); final concentrations of the various ingredients in the translation solution were: 24 mM HEPES/KOH pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 10 μg creatine kinase, 20 units of RNasin as ribonuclease inhibitor, 2 mM dithiothreitol, 0.4 mM spermidine, 0.3 mM of each of the 20 amino acids, 2.7 mM magnesium acetate, 100 mM potassium acetate, 5 μg deacylated tRNA prepared from wheat embryos, 0.05% NP-40, and mRNA. We titrated the mRNA concentration in the translation mixture by means of sucrose density gradient centrifugation and found that 100 nM for capped and 250 nM for non-capped mRNAs gave optimum concentrations leading to large polysome formation. Incubations were done at 26°C. Polysome analysis was done as described previously [6].

2.2. Preparation of mRNAs

The mRNAs were synthesized by in vitro transcription of the linearized pEU, the cell-free expression vector [7]. This plasmid was derived from pSP65 carrying a luciferase gene under SP6 RNA polymerase promoter control. Capped mRNA encoding luciferase is 2252 nucleotides (nt) long and consists of the sequence m^7GpppGAAUACACGGAAUUCGAGCUCGCCCGGGAAAUCUCAAUG (the underlined sequence is the initiation codon) at its 5′ end, a 1650 nt coding sequence, and a 3′ non-coding region of 549 nt from a type-1 dihydrofolate reductase (*dhfr*) gene of *E. coli* with a poly(A) tail (pA) of 100 adenosines (cap549pA) [7]. A cap549 mRNA was synthesized using a similar DNA construct but lacking the poly(dT) sequence in the plasmid. The other type was mRNA lacking the cap but containing a 71 nt naturally occurring translation enhancer at the 5′ terminus, the Ω sequence from tobacco mosaic virus (GenBank accession number X02144). This mRNA was: Ω with the 549 plus a 1077 nt sequence which is located right after the restriction site in pSP65 lacking 100 nt pA (Ω1626) [7]. The templates for the Ω-containing mRNAs were created by introduction of the sequence into the above luciferase plasmids. After the transcription, the transcription solution was passed through Sephadex G50, and mRNA was purified by repeated ethanol precipitation, and dissolved in water.

2.3. Electron microscopy

The reactions were stopped by addition of 7 volumes of TKM (25 mM Tris–HCl, 50 mM KCl, and 5 mM $MgCl_2$, pH 7.8). Specimens were prepared by the surface spreading method with slight modifications [14,17]. Briefly, fine talc powder was sprinkled over the surface of a hypophase in a plastic Petri dish. The diluted sample was taken in a platinum loop and placed onto the hypophase surface. A clear circular expansion free of talc powder was scooped by the EM grids and was mounted on films of carbon-coated parlodion (Mallinckrodt, St. Louis, MO, USA). The surface was washed with TKM and 0.05% Photo-Flo (Kodak) detergent followed by Pt–Pb metal shadowing on a rotary table (Hitachi VE2020). Observations were made with a Hitachi H7600 EM at 80 kV.

3. Results and discussion

3.1. Polysome formation during protein synthesis in the wheat germ cell-free system programmed with mRNAs lacking cap or poly(A) tail, and both

The 5′ and 3′ untranslated regions (UTRs) of eukaryotic mRNA play a crucial role in the regulation of gene expression: they control mRNA translational efficiency, stability, and localization. To see the effect of UTRs on template activity in the wheat germ cell-free system, luciferase mRNAs with cap and polyadenylated, lacking either cap, poly(A) tail or both were translated under their own optimum concentration in the wheat germ cell-free system and analyzed by means of sucrose density gradient centrifugation. With capped and polyadenylated mRNA (cap549pA), incubation led to formation of polysomes, a shift to heavier polysomes with a concomitant decrease of 80S monosomes (Fig. 1), which is consistent with earlier observations [6]. When experiments were carried out programmed with luciferase mRNA having a 5′ cap and a 3′ UTR of 549 nt, but lacking pA (cap549), or Ω1626 mRNA with a 3′ UTR of 1626 nt lacking both cap and pA (Ω1626), two types of mRNAs gave practically the same polysome profiles. The results are in good agreement with the previous report [7], in which it was shown that these three types of mRNA had similar template activities when measured by [^{14}C]leucine incorporation into protein. Furthermore, mRNA having Ω at its 5′ end, but lacking the pA tail at the 3′ end worked as long as 48 h [7].

The results were not fully explained by the current notion that the cap and pA, found on almost all eukaryotic mRNAs, stimulate translation initiation and stabilize mRNA by their synergistic action. We then searched for a clue by morphological approach.

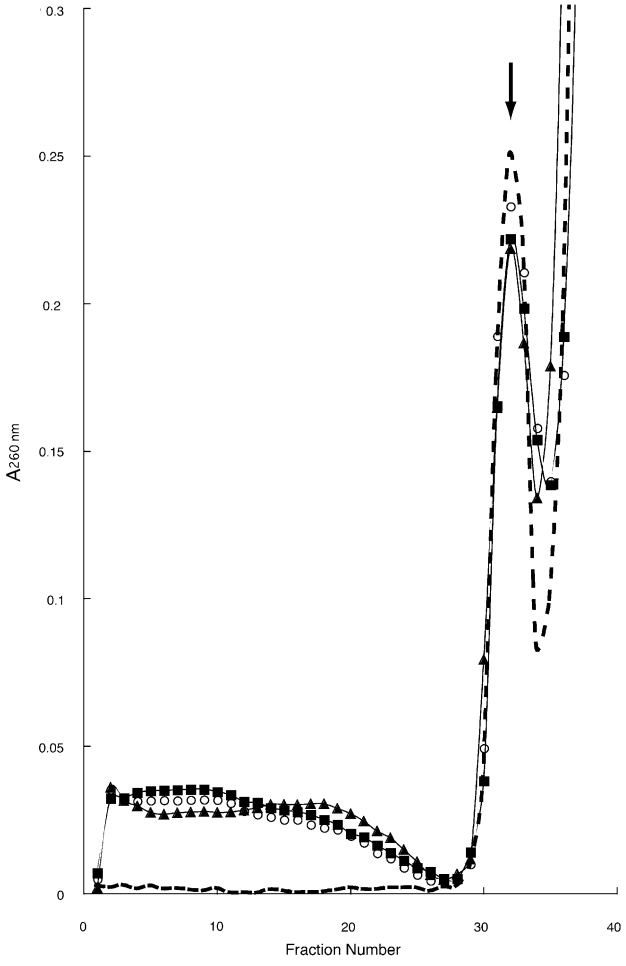

Fig. 1. Polysome profiles formed in translation reaction. Cell-free translation reactions programmed with either cap549pA (■), cap549 (▲), or Ω1626 (○) mRNAs were incubated for 2 h. Each 30 μl reaction mixture aliquot was loaded onto a linear 10–50% sucrose gradient in 25 mM Tris–HCl, pH 7.6, 100 mM KCl, and 5 mM $MgCl_2$ and centrifuged and fractionated from the bottom of the tubes and the $A_{260\,nm}$ measured as described. The dotted line shows the sedimentation profile of 15 μl unincubated reaction mixture (the arrow points to 80S ribosomes).

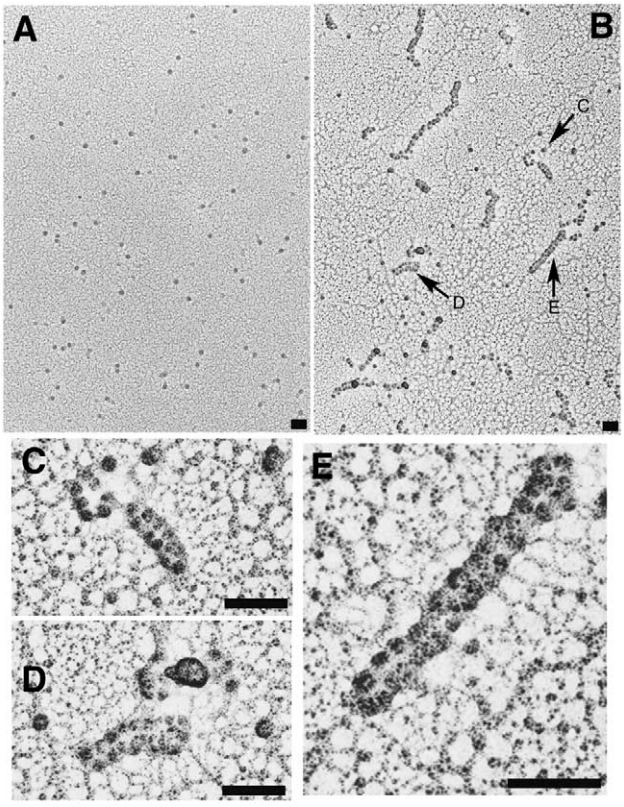

Fig. 2. EM images of polysomes formed during wheat cell-free translation. Protein synthesis was done at 26°C for 1 h with luciferase mRNA having the 5′ cap and pA (cap549pA), and specimens were prepared by surface-spreading and shadowing. A: Without mRNA. B: With capped and polyadenylated luciferase mRNA. C–E: Higher magnification of c, d and e polysomes shown in B. Bars equal 100 nm.

3.2. Morphological examination of functioning ribosomes

EM studies have established that in eukaryotes, from protozoan to human cells, polysomes in specimens prepared from cells or tissues are arranged in circular and helical configurations called circular polysomes [9–15]. These circular polysomes appear to reflect the configuration of the polysomes in eukaryotic cells producing proteins at high rates. To see the morphological architecture of the polysomes in the cell-free translation system, we next carried out morphological examination of ribosomes by transmission electron microscopy (Fig. 2A–E). In the absence of template only monoribosomes were visible (see dispersed dots in Fig. 2A). When the mixture was incubated in the presence of luciferase mRNA having the 5′ cap and pA (cap549pA), elliptic polysomes became visible and long bar-shaped structures that resemble beads on a string and have the appearance of circular polysomes (arrows marking items c and d for smaller, and e for large polysomes in Fig. 2B). High magnification electron micrographs of the elliptic polysomes (Fig. 2C,D) and of one of the large polysomes (Fig. 2E) confirm that the configurations we observed are in fact indistinguishable from those polysomes in cells in which active protein synthesis takes place [9–15]. Although the EM method does not allow direct visualization of mRNA strands, it was possible to estimate their length by measuring polysome sizes. The length of one nucleotide in mRNA on polysomes has been estimated to be 0.34 nm by EM [18]; the length from phosphorus to phosphorus in a fully extended mRNA strand was determined to be 0.59 nm by physicochemical methods [19]. The average length of the larger polysomes in our images is 246 nm, which is less than half of the calculated total length of the coding region, 561 nm (561 = 1650 nt encoding luciferase × 0.34 nm), thus supporting the idea that ribosomes populate along with the open reading frame (ORF), not in a linear or fully extended arrangement. Based on the size of the luciferase ORF and the estimated average number of 21 ribosomes within larger polysomes, one can calculate that a single ribosome contains about 66 nucleotides. The calculated number is in reasonable agreement with that obtained by cryo-EM, where it was estimated that a single wheat ribosome could cover 57–91 nucleotides [20]. Our observations indicate a high and also constant rate of initiation and elongation, and in conjunction with the observed high efficiency of translation [6,7], they imply that the translation machinery of the cell-free system retains the characteristics of intact cells in both function and structure. We believe that this is the first report of successful reconstitution of so-called circular polysomes in vitro and that a high efficiency of the system could be a result of the formation of this eukaryote-type polysome during the translation reaction. The term ‘circular polysome’ was used to indicate the similarity in shape with those reported in the literature [9–15], and that does not necessarily mean a real organization of polysomes.

It is believed that the circular polysomes are formed by an interaction between cap and pA and several protein factors such as initiation factors (eIFs) and poly(A) binding protein [21–23]. As a first step to insight into the molecular architecture of the circular polysomes, we undertook morphological analyses in a similar way as above, but with mRNA lacking pA. Luciferase mRNA having the 5′ cap and a 3′ UTR of 549 nt but no pA (cap549) was synthesized; this message had a template activity of up to 67% of the control mRNA (cap549-pA) [7]. It was surprising to see that mRNA lacking pA led to the formation of double-row and twisted-bar circular polysomes (Fig. 3A,B and C,D) with shapes indistinguishable from the control template (Fig. 2B,E), although somewhat smaller in size. The results are in agreement with functional in vivo studies on yeast, where mRNAs with or without pA had comparable template activity [24]. They are, however, inconsistent with the current understanding of the organization of eukaryotic polysomes that implies an interaction of the 5′ cap and the 3′ pA. EM observation of the incubation mixture programmed with Ω1626 mRNA lacking both cap and pA revealed formation of circular polysomes that are very similar to those obtained with 5′ capped luciferase mRNA containing a 549 nt 3′ UTR and a pA (compare B and E in Fig. 2 and C and D in Fig. 3), but appeared to be somewhat smaller in size. The latter observation can be explained if the mRNAs in our system are primarily degraded from their 3′ end by exonucleases such as the exosome complex as pointed out previously [7,25,26]. The fact that this mRNA attaching a longer 3′ tail, by 1077 nt, originated from pEU did not cause any gaps, but simply gave elongated or linear configurations in the picture provides additional evidence that pA and other special terminal sequences in the 3′ UTR of mRNA are not directly involved in construction of the circular polysomes. The results are consistent with our previous report [7] that translation does not depend on a specific sequence or pA but depends only on the length of

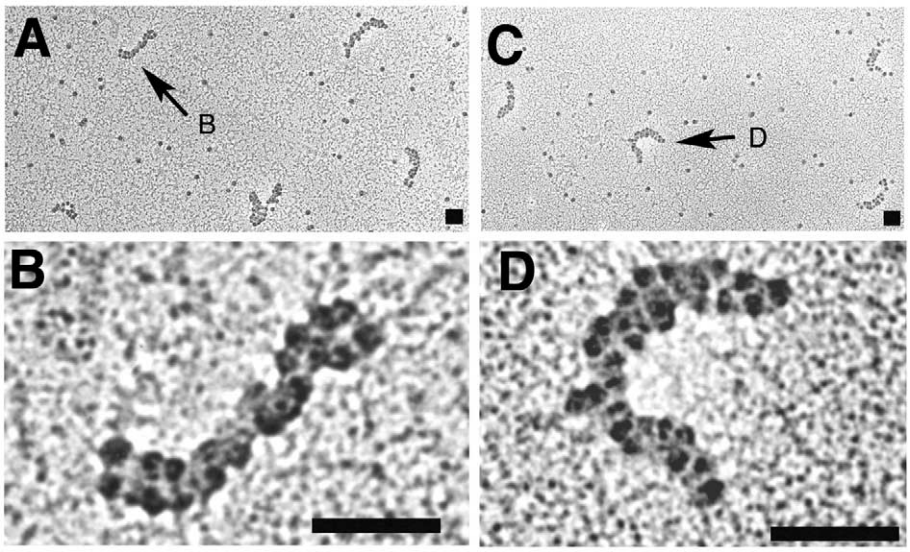

Fig. 3. Formation of circular polysomes programmed with mRNAs lacking both cap and poly(A) tail. Translation reactions were programmed with the cap549 mRNA for A and the Ω1626 mRNA for B and incubated for 1 h. Specimens were prepared and analyzed as in Fig. 1. Bars equal 100 nm.

the 3′ UTR. Similar structures with elliptic shapes were observed, when the experiments were done using smaller mRNAs such as that for *dhfr* and green fluorescent protein (data not shown), attaching the same 5′ and 3′ UTRs as for the above luciferase mRNA.

Our biochemical and morphological examinations indicate that neither the 5′ cap nor the 3′ pA is essential for the architecture of circular polysomes; this is inconsistent with the general understanding [21–23]. Obviously there are two explanations for the apparent discrepancy: (1) wheat embryos or plants, different from an animal system, developed a unique mechanism for the organization of the circular polysomes with Ω beside the cap but in the absence of a poly(A) tail, or (2) it is a general mechanism that neither 5′ cap nor 3′ pA is directly involved in the architecture of circular polysomes. Although the widely accepted understanding is that the circular polysomes are formed by an interaction between cap and pA with protein factors, it should be noted that the idea is based on a conclusion derived from two separate approaches, morphological observations and biochemical binding experiments with mRNA and proteins, as well as from protein–protein interaction studies. All of those biochemical assays were all carried out under conditions that do not reflect the functioning translation apparatus. We do not have enough data to explain the apparent discrepancy at this moment, however, we see a possible cause for this in our morphological and biochemical results which were obtained through examination of a highly efficient cell-free system, which contained few, if any, translation inhibitors such as nucleases. Our results, in fact, appear consistent with those of early studies using purified polysomes devoid of protein factors. Pfuderer et al. first proposed a helical model of polysomes based on a hydrodynamic analysis [27], and Shelton et al. suggested from their EM observations the possibility that polysomal mRNA has a natural tendency to bend [10]. Recently Christensen et al. provided EM results further supporting this notion by showing the direction of the bend relative to ribosomal orientation on the mRNA [12]. This point may be in part supported by more recent studies on the fine structure of prokaryotic ribosomes. Agrawal et al. showed a curved path of mRNA within ribosomes by cryo-EM [28], and Yusupov et al. demonstrated a kink in the mRNA backbone of about 45° between the adjacent A and P site codons in the crystal structure of complex 70S [29]. Considered together, it can be speculated that the formation of circular polysomes may be the result of the intrinsic tendency of mRNA to bend rather than a consequence of initiation reaction-coupled events. In any case, we believe that our system is ideal for further study of the architecture of circular polysomes and of the functional structure of ribosomes at the molecular level.

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