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The Primitive† Ribosome Model

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A new model is proposed on the origin of crystallizable ribosomes and the kinetics of ribosome crystallization. The model assumes that ribosome crystallizability is a property the ribosomes have only in a well-defined period of their life cycle and implies a close relationship between cellular differentiation and structural rearrangements at the ribosome level. The model is used to interpret a variety of cases in which ribosome crystallization occurs, such as chick embryo tissues during development, tissue cultures treated with different antibiotics, chick adult tissues infected by viruses, lizard oocytes, degenerating cells and dedifferentiated cellular systems.

1. Introduction

In 1964 Crain, Benitez & Vatter reported the occurrence of intracellular regular arrays of ribosomes in chick embryo dorsal root ganglia (Crain, Benitez & Vatter, 1964).

In 1966 and 1967 Byers reported that cooling induces in different tissues of 1–2 day chick embryos the formation of regular aggregates of ribosomes apparently identical with those of Crain, Benitez & Vatter. In addition, Byers has recognized, for the first time, that these aggregates are true crystals by showing the crystallographic group to which they belong (Byers, 1966, 1967). The basic unit of these crystals is a tetramer of ribosomes, and crystallization can occur either in two-dimensional sheets of ribosomes belonging to the P4 symmetry group or in three-dimensional stacks of P4 sheets belonging to the P422 symmetry group.

In 1966 Ghiara & Taddei reported the presence in lizard oocytes of regular aggregates of ribosomes which later have been recognized as identical with the P4 and P422 crystals (Ghiara & Taddei, 1966; Ghiara, Taddei & Filosa, 1966; Taddei, 1968, 1972).

† The term "primitive" is not used here in an evolutionary sense but as defined in section 4.

A precursor work on ribosome crystallization can be considered the finding of Bellairs (1961) that chick blastoderm degenerating cells contain "parallel bands" of "aggregated cytoplasmic granules" not seen in healthy cells, since it was later recognized that these aggregates are P4 ribosome crystals (Webster & Gross, 1970; Birks & Weldon, 1971).

The first report on the occurrence of ribosome tetramers in precooled chick embryo tissues is by Humphreys, Penman & Bell (1964), and detailed investigation on tetramers has been reported by Bell, Humphreys, Slater & Hall (1965), Humphreys & Bell (1967), Carey (1970, 1971), Carey & Read (1971), Byers (1971) and Carey, Hobbs & Cook (1972).

Morimoto, Blobel & Sabatini (1972*a*,*b*) reported a series of experiments on ribosome crystallization kinetics and derived a general conclusion which can be considered the first theoretical model on the ribosome crystallization process.

In this paper it will be shown that the experimental evidence of Morimoto, Blobel & Sabatini, as well as many other experimental results accumulated since 1964, are well interpreted by an alternative model.

2. The Morimoto, Blobel & Sabatini Model

The expression "Morimoto, Blobel & Sabitini model" (MBS model) refers to the set of conclusions about ribosome crystallization derived by Morimoto *et al.* (1972*a*,*b*).

These conclusions are obtained from four main groups of experimental results on chick embryos submitted to cooling.

(1) During the first period of cooling there is an extensive disaggregation of polysomes with the formation of a monomer peak which reaches the maximum after $\simeq 30$ min. Later, after 3 hr of cooling, the ribosome crystallization process starts, and its evolution is characterized by the decrease of the monomer peak and a corresponding increase of the tetramer one. The interpretation of this behaviour is that the crystallizable ribosomes derive from polysomes through the chain:

$$\text{normal polysomes} \rightarrow \text{monomers} \rightarrow \text{tetramers}.$$

(2) A cycloheximide treatment prevents the formation of tetramers and also prevents polysome disaggregation. Morimoto *et al.* connect these two effects and say that cycloheximide prevents the formation of tetramers exactly because it limits the availability of polysome-derived-monomers, i.e. of crystallizable ribosomes.

(3) A puromycin treatment releases monomers from polysomes by the artificial termination of the nascent polypeptide chain and does not prevent

the formation of tetramers. Puromycin, far from preventing polysome disaggregation, favours monomerization. Therefore its effect is considered a further confirmation that the availability of crystallizable ribosomes depends strictly on the possibility of obtaining free monomers from the normal cell polysomes.

(4) The ribosome tetramers do not contain messenger RNA but they are active in a cell-free system with artificial messengers. For this reason Morimoto *et al.* say that crystallizable ribosomes are "inactive but potentially active monomers".

From the results listed above the conclusion is obtained that "crystallizability is an intrinsic property of the inactive monomers derived from the normal cell polysomes after the discharge of the polypeptide chain".

3. Discussion of the MBS Model

(A) FIRST OBJECTION

An outstanding characteristic of the ribosome crystallization process in chick embryos is the fact that it occurs massively in early embryonic tissues and then it decreases during development until it disappears almost completely in adult organs (Maraldi & Barbieri, 1969; Morimoto *et al.*, 1972*a*,*b*).

The MBS model however does not offer a direct explanation of this important characteristic. Indeed, if we accept that ribosome crystallizability is an intrinsic property of normal inactive monomers, knowing that cooling commonly induces an extensive monomerization of polysomes, we should find ribosome crystallization much more frequently in adult and differentiated cells too.

Morimoto, Blobel & Sabatini are aware of this logical implication of their model and try to overcome it by invoking extra factors and by saying that "the relative abundance of ribosomes...the sensitivity to cooling...the intracellular environment" are all factors which vary with the type of tissue and the stage of development, and all may affect the availability of ribosomes susceptible to crystallization by cooling. But there are many objections to these arguments.

(1) "The relative abundance of ribosomes" is not a critical factor in ribosome crystallization since this can occur in a wide range of cytoplasmic ribosomal densities. Maraldi, Biagini, Simoni, Bersani & Barbieri (1972) observed, for example, that in the first stages of segmentation the ribosome crystallization can still occur even with an extremely low density of ribosomes. On the other hand, in well differentiated embryonic and adult cells, ribosome crystallization normally does not occur even when there is an extensive monomerization, however high the ribosome density is, and however long

the hypothermic treatment (Simoni, Biagini, Maraldi, Barbieri & Bersani, 1973).

(2) "The sensitivity to cooling" is also a factor which is relatively far from being of critical importance. Apart from the fact that ribosome crystallization can occur at different temperatures of cooling (Goessens, 1972), and with different periods of hypothermic treatment (Byers, 1966), it must be noted that cases exist in which ribosome crystallization occurs without any cooling at all (Birks & Weldon, 1971; Moretti, Zitelli & Baroni, 1972; Mottet & Hammar, 1972).

(3) "The conditions in the intracellular environment" do not seem a better argument to invoke because ribosome crystallization can occur in a highly heterogeneous class of environments, in cells of very different tissues, in proliferating and in degenerating systems, in nuclei and in cytoplasms, during mitosis and in interphasic cells (Barbieri, Simonelli, Simoni & Maraldi, 1970).

In addition, a drastic change in the cytoplasmic conditions—such as the treatment with Vinblastine sulphate which produces sequestration of large protein aggregates and induces the formation of long helices of ribosomes with a spectacular change in the cytoplasmic morphology—does not repress ribosome crystallization at all (Maraldi, Simonelli, Pettazzoni & Barbieri, 1970).

Therefore it is concluded that the decrease and disappearance of ribosome crystallization during development cannot be attributed to "secondary" effects but has to be coherently foreseen by the models describing ribosome crystallization.

(B) SECOND OBJECTION

The decrease of the monomer peak and the corresponding increase in the tetramer peak during cooling seems to leave little doubt about the conclusion that tetramers derive from normal polysomes. However this effect is only a qualitative one and may well receive an opposite interpretation. On examining the kinetics table of Morimoto *et al.* (1972*b*, p. 358) it is possible to see that the maximum amount of monomers, found between the first 30 and 60 min cooling, accounts for $\simeq 40\%$ of the total ribosomes, and, after 22 hr of cooling, the monomers still represent a little less than 30%. This decrease of $\simeq 10\%$, while the amount of polysomes remains practically constant, obviously cannot explain the increase in tetramer percentage from the initial 0% to the final $\simeq 30\%$. This quantitative disagreement may be explained by other considerations.

Ribosome microcrystals can be recovered in many subcellular fractions whose sedimentation properties range between those of the nuclear and the

microsomal fractions (Barbieri, Pettazzoni, Bersani & Maraldi, 1970; Barbieri, Bersani, Simoni & Maraldi, 1973).

We refer to all these pellets as the "macrosomal" fraction, and we distinguish it from the classical mitochondrial fraction first because it is much "broader" and second because the use of hypotonic media and (occasionally) of detergents confers quite a different morphological aspect to it.

Checking the distribution of RNA between the different subcellular fractions of five-day old chick embryo homogenates before and after a standard cooling revealed the results shown in Table 1. These data indicate that there is a very little shift of RNA between the different subcellular fractions, before and after cooling, provided that microcrystals are not extensively disaggregated.

TABLE 1

Distribution of RNA between different subcellular fractions of five-day old chick embryo homogenates before and after standard cooling

Fraction	Before cooling %	After cooling %
Nuclear (not purified)	25	25
Macrosomal	25	30
Microsomal	40	35
Supernatant	10	10

But Morimoto, Blobel & Sabatini state explicitly that in their experiments "crystalline sheets of ribosomes are broken down into tetramers during homogenization" and obviously this disaggregation shifts the tetramer pool from macrosomes to microsomes. In this way the decrease of the monomer peak reflects only the fact that the continuous addition of tetramers to the microsomal fraction "dilutes" the monomers reducing their percentage with respect to the total amount of ribosomal particles.

(c) THIRD OBJECTION

The cycloheximide effect supports the MBS model, but it may also receive a different explanation as will be seen. The puromycin effect, on the contrary, does not seem in good agreement with the MBS model. If we accept the argument that puromycin leads "by artificial termination of protein synthesis to the formation of a *large* pool of monomers prone to crystallization", this artificial monomerization would be expected to make an "extra" number of crystallizable ribosomes available. Thus an increase of the tetramer peak

"over" the value found in untreated embryos would be expected, but the evidence of Morimoto *et al.* shows the contrary, i.e. that the tetramer peak of puromycin treated embryos is always lower than the tetramer peak of the controls.

(D) FOURTH OBJECTION

If ribosome crystallizability is an "inherent property of normal non-programmed monomers", it would be found in any inactivity period of the ribosome life cycle, despite the fact that the monomers with which we are dealing are newly or previously synthetized ribosomes. This expectation is not sustained by the experiments. It has been reported that a study of the incorporation rate of labelled uridine into the microcrystal fractions reveals that a large majority of crystallizable ribosomes are neo-synthetized particles (Biagini, Simoni, Maraldi, Bersani & Barbieri, 1972).

4. The Primitive Ribosome Model

The primitive ribosome model is based on the assumption that crystallizability in the P4 symmetry group is a property the ribosomes have only in one period of their life cycle, a period which begins with the completion of their biogenesis and ends irreversibly with their engagement in protein synthesis. In this period, we say that the ribosomes are in a "primitive state" or that they are "primitive" ribosomes. We will discuss this model in three different sections corresponding to the three parts or hypotheses which form the model itself.

(A) THE FIRST HYPOTHESIS

The first hypothesis of the primitive ribosome model—that ribosomes can crystallize immediately after the completion of their biogenesis—is in great part linked to the finding of intranuclear ribosome microcrystals (Barbieri, Simonelli, Simoni & Maraldi, 1970). Up to now enough evidence has been collected to exclude the possibility that nuclear ribosome microcrystals are cytoplasmic contamination, (Barbieri *et al.*, 1970), and this conclusion is strongly supported by the finding (Zitelli, Baroni & Moretti, 1970) that chorioallontoic cells infected by *Herpesvirus hominis* type 2 show a high increase in intranuclear crystallization.

In addition we conclude that nuclear crystallizable ribosomes are destined for cytoplasmic exportation from two considerations.

(1) The intranuclear ribosome microcrystals are associated with the granular compartment of the nucleolus devoted to cytoplasmic ribosome biogenesis.

(2) The extremely limited occurrence of these microcrystals is compatible with a very short permanence of crystallizable ribosomes inside the nucleus.

This does not imply that the processing of ribosomal precursors to 80 s mature ribosomes is always completed inside the nucleus, or that ribosomes are normally exported from nucleus to cytoplasm as 80 s particles. In fact the possibility exists that cooling is responsible for the intranuclear aggregation of ready-to-export subunits into 80 s particles.

(B) THE SECOND HYPOTHESIS

The second hypothesis—i.e. that protein synthesis activity requires a basic irreversible conformational change after which the ribosomes are no longer crystallizable and cannot therefore derive from "normal" polysomes—is based on two considerations.

The starting point can be considered the experimental fact that the crystallizable ribosomes are inactive and, at least in a great majority of cases, freshly synthetized ribosomes. This obviously means that ribosomes crystallize "preferentially" in the first inactivity period of their life cycle.

On the other hand if we accept the alternative that ribosomes can crystallize in *any* inactivity period of their life cycle we cannot explain "directly" many experimental results, above all the decrease of ribosome crystallization during development. In this way the validity of the second hypothesis is partly based on the possibility of offering a simple explanation of the many cases in which ribosome crystallization occurs, as will be discussed.

(C) THE THIRD HYPOTHESIS

The third hypothesis concerns the role that cooling has in the ribosome crystallization process. It states that the crystallizable ribosomes before cooling were still at the stage of ribosomal precursors which are then slowly processed, during cooling, into mature ribosomes.

The validity of this assumption could be tested, in principle, by checking if, during cooling, a continuous decrease of 45 s RNA and a corresponding increase of the 28 s+18 s RNA actually takes place in respect to the total RNA of the cells. Unfortunately this experiment has not been done, and at present can only be indicated as a key test for the third hypothesis of our model. In this section two considerations concerning this hypothesis are discussed.

It is well established, even if no interpretation has been advanced so far, that in a standard hypothermic treatment of chick embryos, tetramers or ribosome microcrystals begin to appear only after 3 hr cooling (Byers, 1966), whereas the pool of monomers released from polysomes is fully available after the first 30 min (Morimoto *et al.*, 1972*a*,*b*). According to our model at

least a part of this 3 hr interval is needed to permit the slow maturation, at low temperatures, of ribosome precursors into mature primitive ribosomes.

The second consideration concerns the interpretation of the cycloheximide effect of Morimoto *et al.* in the context of our model. One effect of the cycloheximide is to inhibit the processing of the 45 s RNA into mature ribosomal RNA (Higashi, Matsuhisa, Kitao & Sakamoto, 1968; Craig & Perry, 1970). The primitive ribosome model requires that this processing be undisturbed to permit ribosome precursor maturation into primitive crystallizable ribosomes, and therefore it is not surprising that a cycloheximide treatment suppresses ribosome crystallization.

5. Ribosome Crystallization During Development

According to the primitive ribosome model the possibility of inducing ribosome crystallization (provided the ribosomes have the right set of components to realize a space group) depends essentially on two conditions:

(1) The cell has to be in an active state of ribosome production with a convenient reservoir of ribosome precursors.

(2) After the completion of their biogenesis the ribosomes have to remain a significant amount of time in the primitive state.

This last condition requires some consideration about the possible "duration" of the primitive state.

During mitosis there is a selective inhibition of protein synthesis initiation (Fan & Penman, 1970) so that the ribosome precursors that mature during mitosis are blocked in the primitive state for a much longer period of time than the precursors which mature during interphase. [Incidentally, we think that this is the reason for the well-established report that ribosome crystallization is greater in mitotic cells compared with interphasic ones (Byers, 1967)]. If this description is correct it is clear that during the cellular cycle the duration of the primitive state varies from a maximum during mitosis to a minimum during interphase, and the average of these times can be called "mean life" of the primitive state.

The decrease of ribosome crystallization during development can now be interpreted by two combined effects. Differentiation brings about a decrease in the proliferative power of the cells which progressively decreases the percentage of freshly synthesized ribosomes. Simultaneously, differentiation brings increasing efficiency in the production of ready-to-function ribosomes, and the primitive state becomes a progressively shorter period of the ribosome life cycle.

In principle it is possible to invoke at least three different mechanisms to explain this last effect.

(i) In early embryonic cells the high rate of cell division makes it possible that the rate of ribosome exportation is greater than the rate of ribosome utilization in protein synthesis. According to this idea differentiation is simply accompanied by a progressively better balanced proportion between the (cytoplasmic) demand and the (nuclear) production of ribosomes.

(ii) The attachment of inductive factors to the ribosomes ends the primitive state. The concentration of these factors increases during differentiation and consequently the percentage of ribosomes which can remain for relatively long periods of time in the primitive state decreases.

(iii) The primitive ribosomes contain an inactivation factor which has to be selectively removed in order to permit their activity, and this removal becomes increasingly more rapid during cell differentiation.

These considerations may appear pure speculation, but if the general outline of the interpretation is right then two important experimental consequences must follow:

(a) Ribosome crystallization must be observed not only in embryonic cells but also in adult tissues which are in a convenient proliferative state. This expectation is completely confirmed by experience. Ribosome crystallization never takes place in well-differentiated organs like liver, myocardium, etc., but adult proliferating tissues show an acceptable quantity of crystalline sheets of ribosomes (Maraldi, Marini & Barbieri, 1972; Simoni *et al.*, 1973).

(b) If we induce the dedifferentiation of differentiated tissues we have to reassume ribosome crystallization. This expectation has been confirmed in at least two cases. Liver, kidney and spleen from adult chickens affected by Mareck's disease virus—which induces a high proliferation of dedifferentiated cells in these organs—show, after a standard hypothermic treatment, a high degree of ribosome crystallization totally absent in the controls (Simoni *et al.*, 1973).

Zitelli, Baroni & Moretti (1970) have reported that the chorioallantoic membrane of 16-day old eggs infected with *Herpesvirus hominis* type 2 show, after cooling, a high number of intracellular ribosome microcrystals, totally absent in the controls. The conclusion of Zitelli *et al.* (1970) that: "the presence of ribosome crystals in infected cells is a consequence of the viral infection that induces a large ectomesodermic proliferation of dedifferentiated cellular elements" is completely consistent with the primitive ribosome model because of the close connection it presumes between cellular differentiation and ribosome crystallizability.

6. Interpretation of Other Experiments

Our model can also easily be used to explain the appearance of ribosome crystallization in many heterogeneous cases.

(1) In a degenerating cellular system it is possible that the switching-off of protein synthesis is more rapid than the switching-off of ribosome biogenesis, which can also be temporarily stimulated as a compensation mechanism, bringing about the accumulation into the cytoplasm of primitive ribosomes prone to crystallization. This seems to be the explanation for the "abnormal" ribosome crystallization found in chick embryo cells which show signs of cellular suffering and degeneration (Webster & Gross, 1970; Birks & Weldon, 1971; Mottet & Hammar, 1972).

(2) In other systems the need may arise for ribosome storage so that the cell produces ribosomes without using them, a fact which automatically brings about the accumulation and storage of groups of primitive ribosomes like the crystalline bodies observed in lizard oocytes by Ghiara, Taddei & Filosa (1966). It should be noted that in this system crystallizable ribosomes obviously no longer need to be "freshly synthesized ribosomes" as in rapidly growing embryos.

(3) Antimetabolic treatments which have the effect of inhibiting or lowering protein synthesis without interfering at least with the last stages of ribosome biogenesis have an effect favourable to ribosome crystallization. They permit the maturation of the ribosomes, but not their engagement in protein synthesis which results in an increased duration of the primitive state. For example, tissue cultures of fibroblasts derived from 11-day old chick embryos normally show a low degree of ribosome crystallization (less than $\simeq 1\%$ of cells contain microcrystals). But the same cells treated with puromycin or with actinomycin-D or with Vinblastine sulphate for some hours before cooling show a much higher number of cells containing microcrystals, as well as a higher percentage, in each cell, of crystallized ribosomes (Maraldi *et al.*, 1970, 1973).

A similar effect of stimulating ribosome crystallization has been reported by Moretti, Zitelli & Baroni (1972) in tissue cultures treated with rifampycin, with the additional fact that this antibiotic can induce ribosome crystallization without any cooling at all.

It must be noted that the time factor is crucial in these treatments and a noticeable increase in crystallization occurs only if they last for an adequate period of time. On the other hand the puromycin treatment by Morimoto *et al.* lasted for only a few minutes at 37° because the release of the nascent polypeptide chain is a quick event and has an almost immediate effect on normal polysomes.

We conclude therefore that the degree of ribosome crystallization of a chosen cellular system can be changed only by a profound change in its metabolism, and it is largely independent of the manipulations which can be induced on its normal polysomes.

(4) Byers (1971) has reported that free ribosomes derived from chick embryo polysomes can form tetramers *in vitro*, and this report seems to support the MBS conclusion that crystallizable ribosomes derive from normal polysomes. However, we have already seen in section 3 that ribosome microcrystals derive from the heterogeneous fraction of the macrosomes which, during homogenization, are easily disrupted, at least in part, in light fragments. In this situation it is impossible to discriminate between normal polysomes and aggregates of primitive ribosomes and in our opinion it is exactly the presence of this last kind of aggregate which is responsible for the tetramerization *in vitro* described by Byers. On the other hand the assumption can be proved or disproved in a direct way by checking if ribosome tetramerization *in vitro* can be obtained from the polysomes of an adult well differentiated organ.

At present the tetramerization *in vitro* described by Byers requires ribosomes derived from embryonic tissues, and this fact legitimizes the conclusion that only primitive ribosomes can crystallize.

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