Cytological evidence on the ability of the nucleolus organizing regions to assemble pre-existing nucleolar material

S. Sato

Department of Biology, Faculty of Science, Ehime University, Bunkyo-cho 2–5, Matsuyama, Ehime 790 (Japan)
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Summary. The treatment of *Vicia faba* root tips with 5-azacytidine caused unequal allotment of the chromosomes; after one cell cycle, a pair of daughter nuclei was produced, one of which carried the nucleolus organizing regions (NORs) and the other of which lacked them. The pre-existing nucleolar material brought into the NOR-carrying daughter nucleus by the chromosomes disappeared concurrently with the appearance of the new nucleoli, whereas in another daughter nucleus without the NOR a number of small nucleolus-like bodies became visible at random positions. These findings suggest that in addition to ribosomal RNA synthesis the NOR has another role in cells; to stimulate the pre-existing nucleolar material to assemble at the NOR itself.
Key words. 5-Azacytidine; nucleous organizing region; prenucleolar material; silver staining; *Vicia faba.*

Regarding nucleologenesis in higher organisms, some authors have proposed that prior to the beginning of ribosomal (r) RNA synthesis the new nucleolus is formed by pre-existing nucleolar (prenucleolar) material present in the telophase nucleus, and that following rRNA synthesis it grows into a typical nucleolus composed mainly of granular and fibrillar components. The prenucleolar material is believed to originate from the nucleolus in the preceding interphase [1–5]. This proposition is supported by the observation that the prenucleolar material incorporated into the daughter nuclei is soon formed into compact structures termed the prenucleolar bodies and then these bodies diminish in number concurrently with the appearance of the new nucleolus [6, 7]. If the prenucleolar bodies are not digested within the nuclei, this observation inevitably leads to the conclusion that they fuse with each other to form the new nucleolus without rRNA synthesis. According to Lafontaine and Chouinard, however, the prenucleolar material accumulates in the spaces between the chromosomes during early to mid-telophase, suggesting de novo synthesis of the prenucleolar material [8]. A few authors also claim that the prenucleolar material or RNA associated with metaphase chromosomes does not take part in nucleolus reconstruction [9, 10].

If the new nucleolus is actually reconstructed first by the prenucleolar material, the NOR could be expected to stimulate the prenucleolar material to assemble at the NOR itself. Useful information to help to distinguish between this possibility and the other, contradictory propositions concerning nucleologenesis, will be provided if the behavior of the prenucleolar material is examined both in the presence and in the absence of the NOR.

The present author found that when root tip meristems of *Vicia faba* were exposed to 5-azacytidine, pairs of daughter nuclei in which only one of the pair had the NOR were produced at high frequency. The aim of this study is to make a comparative study of the behavior of the prenucleolar material in pairs of nuclei with and without the NOR by means of light and electron microscopy. The results suggest that the NOR actually forces the prenucleolar material to reaggregate at the NOR itself.

Material and methods. Seeds of *Vicia faba* were obtained from commercial sources. The seedlings, germinated in moist sawdust, were grown in a constant-temperature incubator at 22 °C. Actively growing lateral roots of the plants were placed in 20 µg/ml 5-azacytidine (Sigma) with aeration. The incidence of abnormal separation of the chromosomes was examined at 2, 4, 8, and 16 h after the administration of the drug. It occurred at the highest incidence in the specimens treated for 8 h, so specimens treated for 8 h with 5-azacytidine were used for the following preparation. The root tips treated with 5-azacytidine were fixed for 2 h at 4 °C in 4% glutaraldehyde in 1/15 M phosphate buffer (pH 7.0). They were treated in an enzyme mixture, squashed to homogeneity, and spread on glass slides by air-drying [11]. Semi-thin sections of the root tips embedded in Spurr's resin were made with an LKB ultrotome operated by hand [12]. Both the specimens prepared by the air-drying method and those prepared by semi-thin sectioning were stained with $AgNO_3$ solution (1 g of $AgNO_3$ + 1 ml of distilled water) according to the method of Hizume et al. [13].

Some root tips were fixed for 2 h at 4 °C in 3 : 1 acetic alcohol after 5-azacytidine treatment. The chromosome specimens were prepared by enzyme maceration and successive air-drying as described above, and stained in 5% Giemsa liquid in 1/15 M phosphate buffer (pH 7.0). Thin sections, made with an LKB ultrotome from the same block as was used for light microscopy, were stained preferentially for ribonucleoprotein [14]. They were examined with a Hitachi HU-12 electron microscope at 100 Kv.

Results and discussion. Figs 1 a–c are representative pictures illustrating the process of unequal allotment of the chromosomes. In early anaphase the daughter chromosomes move toward the poles, but some of them did not separate from the other set of daughter chromosomes migrating towards the opposite pole (fig. 1 a). This abnormal segregation may be due to incomplete condensation of the chromosomes. In advanced anaphase the situation was essentially the same as in early anaphase but the chromosomes began to loosen (fig. 1 b). Breaks occurred in the attenuated chromosome arms during late anaphase to telophase, eventually resulting in larger and smaller daughter nuclei (fig. 1 c). The smaller nucleus, therefore, lacks a part of the daughter chromosome set. In late anaphase a pair of the dark spots indicative of the NORs was seen in the larger nucleus, whereas they were never discerned in the smaller one (fig. 1 d). This means that the NORs were not segregated into the smaller nucleus. Accordingly, in early telophase, new nucleoli became visible only in the larger nucleus but not in the smaller one (fig. 1 e). At the same time, argyrophilic deposits became discernible within the larger nucleus as well as within the smaller nucleus. In advanced telophase, the argyrophilic substance disappeared in the larger nucleus when the nucleoli were fully developed, but a number of small argyrophilic nucleolus-like bodies were seen scattered in the smaller one (fig. 1 f). This was also the case for the whole mount specimen (fig. 1 g). Interestingly, in the abnormal nucleus which failed to segregate, the nucleolus-like bodies were not visible around the large nucleolus, whereas in the region distant from the nucleolus a number of them still remained, which suggests that the prenucleolar material surrounding the NOR was assembled to form the nucleolus (fig. 1 h).

All the thin sections for electron microscopy were stained preferentially for ribonucleoprotein. This technique specifically brings structures containing RNA into relief by bleaching the chromosomes [14]. In metaphase no electron dense material was discernibly associated with the chromosomes.

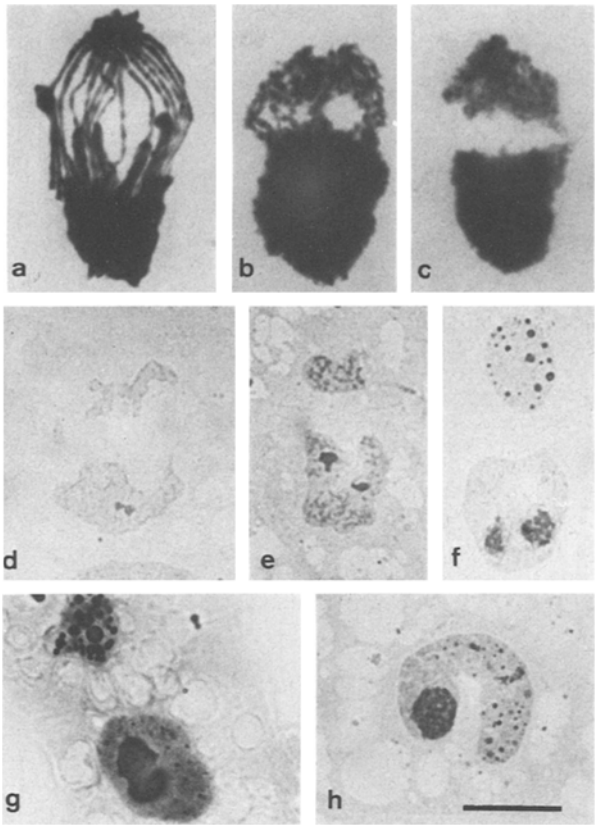

Figure 1. Light micrographs demonstrating the process of unequal allotment of the chromosomes (*a–c*, Giemsa stain) and the behavior of argyrophilic nucleolar material (*d–h*, Silver stain) in the root tip meristems of *Vicia faba*. *a* Anaphase. *b* Early telophase. *c* Advanced telophase. Note that a set of larger and smaller daughter nuclei is produced by unequal allotment of the chromosomes. *d* Anaphase. The nucleolus organizing regions are seen in the larger nucleus but they never occur in the smaller ones. *a* Early telophase. *f* and *g* Advanced telophase. Two large nucleoli are seen in the larger nuclei while many small argyrophilic bodies are seen in the smaller ones. *h* Advanced telophase nucleus which failed to segregate. Note that the argyrophilic bodies are not seen around the nucleolus. *d–f* and *h* Semi-thin sections. *g* Whole mount specimen. Bar 10 μm.

As mitosis progressed to late anaphase, however, electron dense material began to accumulate on the surface of the chromosomes. At the beginning of telophase, the interchromosomal region was filled with tightly packed electron dense material (fig. 2 a). The appearance of the electron dense material was the same both in the larger and in the smaller nuclei. In advanced telophase, however, one or two large nucleoli began to develop in the larger nucleus, and the electron dense material was loosely associated with them (fig. 2 b). The appearance of the new nucleolus strictly corresponded in timing with the disappearance of the electron dense material distributed in the interchromosomal region, indicating that the new nucleolus is reconstructed by the electron dense material dispersed in the space between the chromosomes. In contrast, no nucleolus developed in the smaller nucleus. Instead, a number of electron dense nucleolus-like bodies, which apparently corresponded to the argyrophilic bodies detected under the light microscope, were seen among the chromosomes (fig. 2 b). It is apparent that the electron dense material aggregated at random positions to form the nucleolus-like bodies in the smaller nucleus. A higher magnification revealed that these bodies were essentially fibrous but some ribosome-like particles sometimes seemed to be present in them (fig. 2 c).

The behavior of the prenucleolar material examined in the present work can be summarized as follows: (1) the previously dispersed nucleolar material began to accumulate on the chromosomes during anaphase and was consequently brought into the daughter nuclei, (2) in early telophase the prenucleolar material universally occupied the interchromosomal spaces of both the larger and the smaller nuclei, and (3) with the progress of telophase, the new nucleolus became visible concurrently with the disappearance of the prenucleolar material in the larger nucleus, whereas a number of the nucleolus-like bodies formed in the smaller nucleus. The results (1) and (2) are completely parallel with those obtained in plants gown under normal conditions [11]. 5-Azacytidine, therefore, seems not to result in any abnormality in the behavior of the prenucleolar material although it is known to cause variable biological effects on cells [15]. It is also impossible that 5-azacytidine differentiates the fate of the prenucleolar material between the larger and the smaller nuclei because 5-azacytidine can be supposed to be present at the same concentration in both the nuclei. It is, therefore, concluded that the NOR is responsible for the fate of the prenucleolar material incorporated into the daughter nuclei. More specifically, the NOR stimulates the prenucleolar material to accumulate around the NOR itself and form a primitive nucleolus.

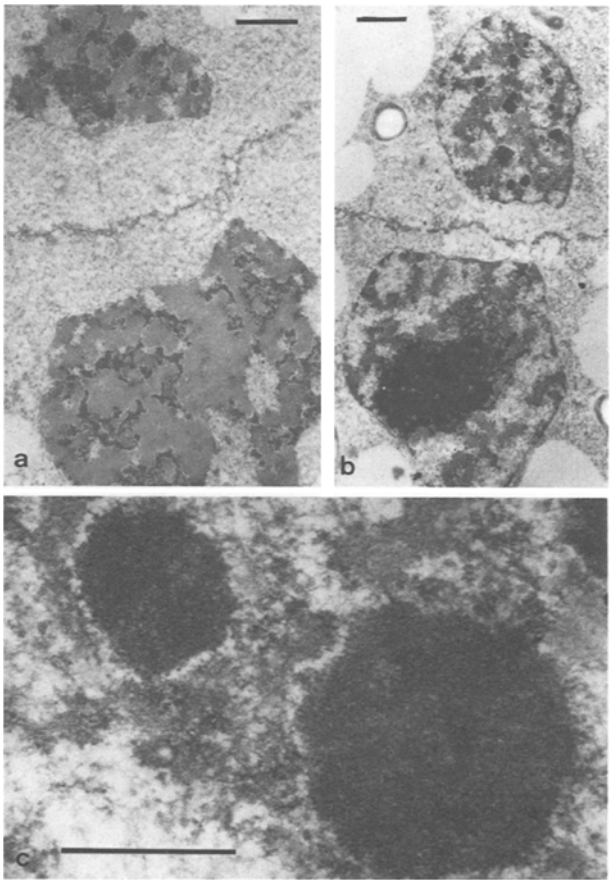

Figure 2. Electron micrographs showing electron-dense prenucleolar material visualized by ribonucleoprotein preferential staining. *a* Early telophase. The prenucleolar material fills the spaces between the chromosomes. Bar 1 μm. *b* Advanced telophase. Bar 1 μm. A large nucleolus in the larger nucleus while many nucleolus-like bodies are seen in the smaller one. *c* Magnified view of the nucleolus-like bodies. They are essentially fibrous. Bar 0.5 μm.

A number of nucleolus-like bodies have been found in the microspore nucleus of *Zea mays* in which the NOR is lost[16,17]. When the cultured cells of *Xenopus laevis* and of mammals are exposed to colcemid, a number of micronuclei result and many nucleolus-like bodies are formed in some of them[18-21]. No fully developed nucleoli have been found in the micronuclei with many nucleolus-like bodies. These findings seem to be well compatible with those of the present study. Inhibitors of rRNA synthesis such as ethidium bromide and cordycepin can also induce the appearance of numerous nucleolus-like bodies, but they do not result from exposure to inhibitors of the synthesis of protein or extranucleolar RNA[6,22,23]. Recently, actinomycin D has also been shown to prevent the assembly of the prenucleolar material at the NORs[24]. This means that rRNA synthesis may be involved in the mechanism which draws the prenucleolar material to the NORs. 5-Azacytidine does not inhibit RNA synthesis[15]. Therefore, reconstruction of the nucleoli in the larger nuclei suggests that the NORs still have the ability to synthesize rRNA even after they have been abnormally segregated. To clarify this, a further study is required, focusing on whether or not NORs which have been abnormally segregated can incorporate rRNA precursors.

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Confirmation of the structure of nisin and its major degradation product by FAB-MS and FAB-MS/MS

M. Barber, G. J. Elliot, R. S. Bordoli[a], B. N. Green[a] and B. W. Bycroft[b]

Department of Chemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD (UK), [a] VG Analytical Ltd, Wythenshawe M23 9LE (UK), and [b] Department of Pharmacy, University of Nottingham, Nottingham NG7 2RD (UK),
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Summary. FAB-MS has been applied to the analysis of a nisin complex and FAB-MS and FAB-MS/MS data from the major component used to provide confirmation of the amino sequence and positions of the sulphur-bridged rings in these highly modified peptides.
Key words. Nisin, modified bacterial peptide, FAB-MS, FAB-MS/MS.

The nisins are a group of several closely related modified peptides with antimicrobial activity which are produced by various strains of *Streptococcus lactis*. They have an inhibitory effect on the growth of gram-positive organisms, and also limit sporulation of a range of *Bacilli* and *Clostridia*. This latter property has been exploited by the food industry and nisin is now very widely employed as a preservative for processed foods, particularly milk products[1].

It has been known for more than 30 years that the nisin complex is a mixture of peptides and over this period various groups have investigated their structures. The most comprehensive investigation on the principal active component was carried out by Gross and Morrell who proposed[2], on the basis of extensive chemical studies, the structure shown in figure 1. This structure is based on a 34 amino acid residue peptide, incorporating a number of unusual modified residues. These consist of two dehydroalanines (Dha) and one dehydrobutyrine (Dhb), together with a lanthionine (Ala–S–Ala) residue and four β-methyl-lanthionines (Abu–S–Ala). The α-centers of the α-aminobutyric acid (Abu) moieties in the β-methyllanthionines are of the D-configuration. The remaining amino acids are all assumed to be of the L-configuration. The lanthionine and β-methyl-lanthionine residues introduce sulphur bridges at various points in the molecule, giving rise to 4.5 and 7-residue cyclic units and one very striking 4.4-residue bicyclic system.

Peptides sharing the unusual structural features of nisin, notably the presence of several sulphur-linked cyclic units, have also been isolated from a number of other micro-organisms.

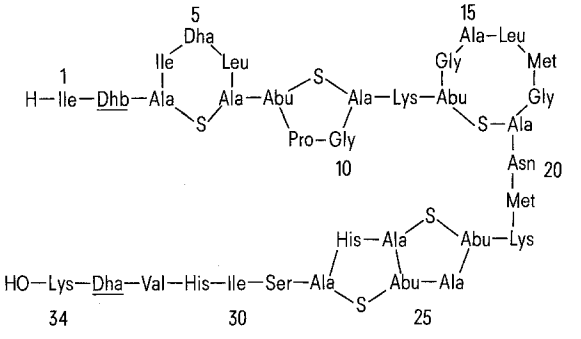

Figure 1. The structure of nisin.