MECHANISMS OF TRANSCRIPTION IN NUCLEOLI OF AMPHIBIAN OOCYTES AS VISUALIZED BY HIGH-RESOLUTION AUTORADIOGRAPHY

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ABSTRACT

In oocytes of *Pleurodeles waltlii*, the method of Miller and Beatty has been combined with a method of high-resolution autoradiography especially suitable for the study of isolated molecules. *In vitro* labeling of RNA by tritiated precursors was carried out with increasing incubation times (1, 4, 15, 24, 48, and 72 h). Silver grains were present over ribonucleoprotein fibrils in amounts sufficient for quantitative analysis of nucleolar DNA transcription.

Statistical analysis of the data revealed that: (*a*) The units of any one nucleolus exhibited a large degree of heterogeneity in their number of grains. (*b*) There was a parallelism between the increasing grain number and the ribonucleoprotein-fibril lengthening as observed along the transcription unit.

KEY WORDS nucleic acids · transcription · nucleolus · oocyte · autoradiography · electron microscopy

The nucleolar genes that code for the precursor molecules of ribosomal RNA (pre-RNA) can be observed in various systems by electron microscopy with the Miller and Beatty method (14, 21, 23, 26, 32, 33). The fundamental organization seems to be independent of species. Nucleolar genes are observed as repeating units; each unit consists of a polarized fibrillar sequence (the transcribed part of the unit) which alternates with portions devoid of ribonucleoprotein (RNP) fibrils that are apparently untranscribed spacers. The main intra- and interspecific variations recorded are caused by fluctuations in the size of the unit (31).

The studies which employ Miller and Beatty's method have helped to elucidate the mechanisms of transcription—in terms of structure—at the molecular level, and the data obtained by means of biological investigations have been largely clarified. The present study is an ultrastructural-autoradiographic analysis of the process of transcription. This was made possible by recent advances in high-resolution autoradiography that use silver grains which are suitable in size and shape for the investigation of spread molecules (2). The study was performed on the oocyte of the urodele amphibian *Pleurodeles waltlii*, the transcription of which has been previously analyzed morphologically (4). Preribosomal-RNA synthesis was investigated on three levels: (*a*) the nucleolus, (*b*) the nucleolar genetic unit, and (*c*) the RNP fibril. Part of this investigation has been presented in a preliminary note (3).

MATERIALS AND METHODS

RNA Labeling

The labeling of nucleolar RNA by tritiated precursors was carried out in vitro. The oocytes were isolated from an ovary fragment which had been obtained from an animal anaesthetized with 1‰ MS 222 Sandoz (Sandoz Ltd., Basel, Switzerland). After immersion of the oocytes in Steinberg-EDTA solution, pH 7.1 (20), for about 30 min, the follicular cells were removed manually with forceps. The only oocytes retained were those in middle or late vitellogenesis. They were incubated at 18°C in Wolf and Quimby's medium (35) (Gibco Bio. Cult., Glasgow, Scotland) for amphibians, which contained 500 μCi/ml of [$^{3-5}$H]uridine (aqueous solution[AS]: 23 Ci/mM) and 500 μCi/ml of [$^{3-5}$H]cytidine (AS: 23 Ci/mM, Commissariat a l'Energie Atomique, Saclay, France). Incubation time ranged from 1 to 72 h.

After each incubation, the level of synthesis activity of the oocyte was thoroughly controlled by examining lampbrush chromosomes in a light microscope by the technique of Gall (11) as modified by Lacroix (15). The oocytes showed lampbrush chromosomes with well-developed lateral loops, thus demonstrating transcription activity.

Electron Microscopy

Nucleolar spreads were produced from oocytes which were incubated in tritiated medium by the technique of Miller and Beatty (22, 23, 24), adapted to this material (4). The preparations were stained with 1% phosphotungstic acid (PTA) in 50% ethanol for 1 min, contrasted by platinum rotative shadowing at an angle of ~10°, and covered by a thin layer of carbon. This procedure was found to be indispensable for the subsequent autoradiographic process (Fig. 1).

Ultrastructural Autoradiography

The method employed in the present study has been described elsewhere (5, 6). In brief, the shadowed grids were individually coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) by the loop interference method (12). This coating technique was found to be the most convenient because the spreading and coating processes had to be done on single grids. After 10 wk of exposure at 4°C, the autoradiograms were developed by a combination (5) of gold latensification (27, 34) and Phenidone (Geigy Chemical Corp., Ardsley, N. J.) development (19). Because of their small size, the grains were easy to individualize and locate on the spread molecular complexes without masking the underlying molecules. In previous studies, the efficiency of the method was found to be 17% (7).

Quantification

Several parallel experiments were carried out with increasing incubation times (1, 4, 15, 24, 48, and 72 h) in a tritiated medium. In all experiments, the labeled nucleoli observed on each autoradiogram resulted from the spread of a single germinal vesicle. All nucleolar spreads were produced from equal-sized oocytes. For a given experiment, oocytes originated from the same animal, and for parallel experiments, oocytes were selected from animals of the same age.

For each grid examined, the first two or three nucleoli observed were photographed at ×10,000. For each nucleolus, the silver grains which fell on at least 10 randomly chosen transcription units were counted. Altogether, the silver grains on 1,455 transcription units were counted. At least 10 units per nucleolus and 10–40 nucleoli per incubation time were investigated. Grains were considered to have been emitted from a given unit when they fell within the area of this unit or within 1 HD (the distance from a line source within which 50% of the total silver grains fall [28]) from its outer edge (see results, Fig. 7). With the present method, the HD value is ~135 nm (18). A selection of the nucleoli, on the one hand, and the fibrillar sequence, on the other hand, was made on the basis of the absence of overlapping and the quality of spreading on the RNP fibrils.

The background noise was always <1 grain/100 μm^2, and therefore was considered to be negligible with respect to the scale of the fibrillar sequence (1.05 μm^2).

To determine the location of the silver grains and the length of the fibrils along the transcription units, each unit was divided longitudinally into 10 equal parts (see results, Fig. 7). Therefore, the average length of each part was:

$$\frac{\text{Length of the unit}}{10} = 0.20\ \mu m$$

The grains were then counted for each of the 10 parts. Their position with respect to the extremities was defined by the position of their projection on the axial fiber of the fibrillar sequence considered.

Statistical Analysis

Analysis of the variations in the number of grains per transcription unit was essentially based on the evolution of the index of dispersion of their distribution. The definition of the index of dispersion and the interpretation of its evolution with respect to incubation time are detailed in the Appendix. Comparison of the labeling of the different nucleoli was carried out by variance-components analysis.

The degree of significance of the increase of the index of dispersion, with regard to incubation time, was established by linear regression.

RESULTS

Influence of Incubation Time on Labeling

In the above-mentioned experimental conditions, the labeling intensity of preribosomal RNA, represented for each nucleolus by the average

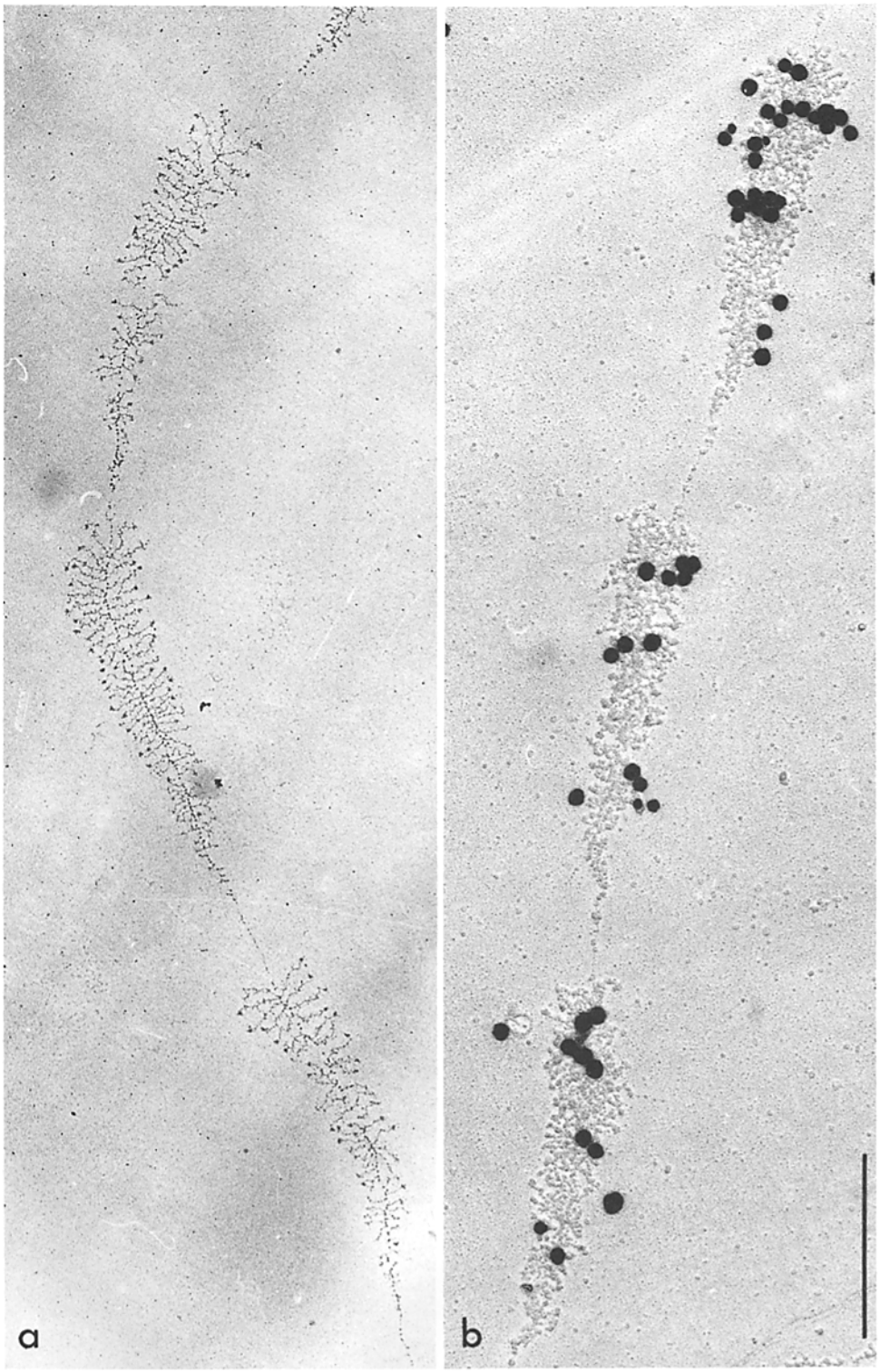

FIGURE 1 Labeling of the nucleolar transcription units in the oocyte of *Pleurodeles waltlii* (incubation in tritiated medium 72 h; uridine + cytidine 1 mCi/ml; AS:23 Ci/mM). (*a*) Spreading by the classical technique and staining with 1% PTA. (*b*) Platinum rotative shadowing and autoradiography with Ilford L4 emulsion, exposure at 4°C for 10 wk, gold latensification, and phenidon development. Bar, 1 μm.

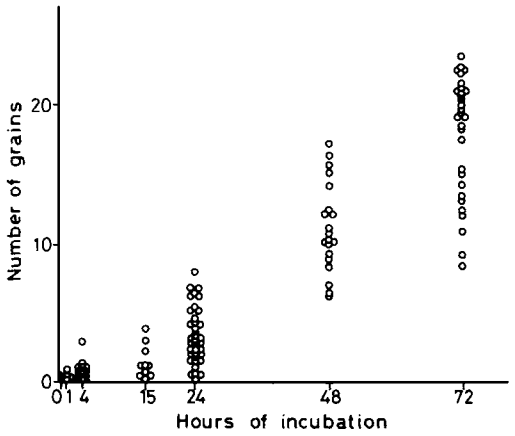

FIGURE 2 Comparison of the labeling of units as incubation times are increased. Each experimental point represents the average number of silver grains per transcription unit in one nucleolus. Approx. 12 units were counted per nucleolus, and 127 nucleoli were examined (see Tables I and II). This diagram clearly shows that the pool was not saturated even after 72 h of incubation.

number of grains per transcription unit, increased as incubation time was lengthened from 0 to 72 h (Fig. 2).

Silver grains were located over the fibrillar sequences, periodically distributed along the deoxyribonucleoprotein (DNP) fiber. The labeling of the spacers was reduced to the background level regardless of incubation time (Fig. 3). It was also interesting to note that in the long parts of the DNP fiber which did not bear any RNP fibrils, the grain density was also reduced to the background level (Fig. 4). Thus, in the fibrillar part of the nucleolus, autoradiography permitted the direct observation of preribosomal RNA biosynthesis *in situ*.

Heterogeneous Labeling of Transcription Units

A relatively large degree of variability was observed in the labeling of transcription units (Fig. 5). The distribution of the number of the grains per unit was more heterogeneous than a simple random distribution, because the index of dispersion (see Appendix) was much greater than 1 (Table I).

The number of grains per unit also varied with different nucleoli (Fig. 2). For example, after 72 h, the average number of grains, calculated for each nucleolus, ranged from 8.50 to 23.70. Table II specifies that these variations between nucleoli could not be entirely explained by the variations between units within the same nucleolus. More precisely, ~20% of the total variance in the number of grains per unit could be accounted for by variations between nucleoli.

Transcription units within the same nucleolus also exhibited heterogeneous labeling. Fig. 6 and Table III show that the index of dispersion calculated between units of the same nucleolus was significantly greater than 1, and increased significantly ($P < 0.5$) with time.

Finally, the degree of labeling found over any transcription unit was not statistically related to that of the adjacent one. This was clearly shown by the results of a comparative study of adjacent unit labeling on 185 units (Table IV). The median value (m) of the distribution of the number of grains per unit was determined for each nucleolus. Each unit was then classified into one of four categories by comparing (a) the number of its grains (g) to the median: $g \leq m$ or $g > m$, and (b) the number of grains of the subsequent unit to the median: $g \leq m$ or $g > m$. Depending upon whether $g \leq m$ or $g > m$ for the unit, the proportion of subsequent units in which $g \leq m$ was 0.58 or 0.56, respectively. This meant that the proportion was in both cases identical, and any contiguous units displayed very similar, more or less different, or quite different numbers of grains.

Spatial Distribution of Grains within Units

The curves in Fig. 7 show the distribution of grains in 216 units after 24, 48, and 72 h of incubation. At each incubation, the average number of grains was found to differ significantly ($P < 0.01$ at 24 h, $P < 0.001$ at 48 and 72 h) from one tenth of a unit to another. In fact, the average number of grains as computed for each tenth of the unit increased quasi-linearly from the beginning of the unit to the seventh tenth and was constant from the eighth to the last one. Note that the shape of these curves paralleled the curve which represents the average length of the RNP fibrils from the beginning to the end of the units (Fig. 7).

A study was also undertaken to compare the number of grains on two longitudinal halves of the same unit (Fig. 8). The index of dispersion between the number of grains of the longitudinal halves of the same unit was computed on 218

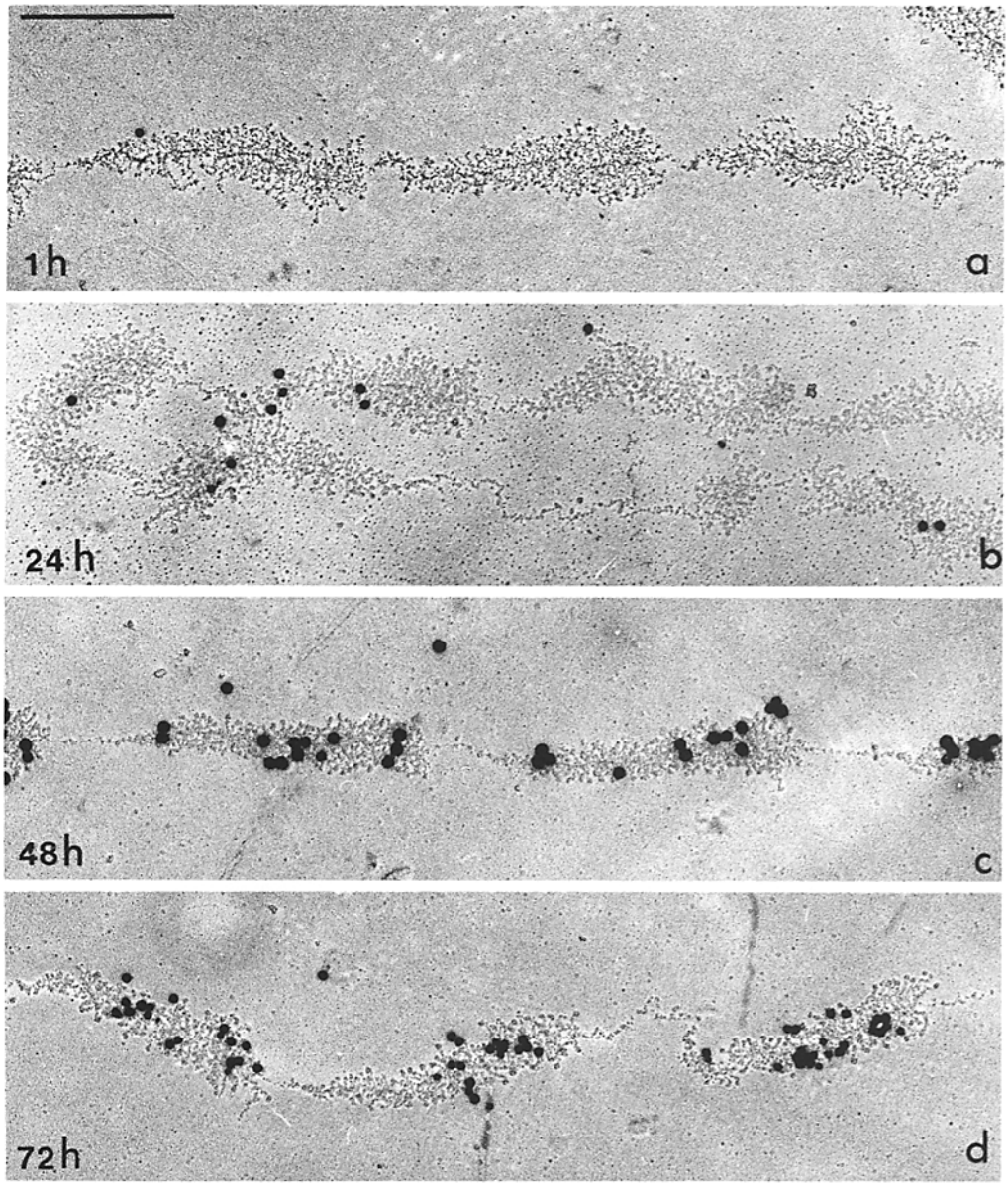

FIGURE 3 Effect upon the units of increasing incubation times in tritiated medium. This resulted in an increase in numbers of grains which fell over the units while the spacers were devoid of silver grains, regardless of the incubation time. Bar, 1 μm.

units after 72 h of incubation, and the average value was 1.07 ± 0.24. This shows that the labeling of the two halves of the same unit was homogeneous.

DISCUSSION

This study revealed transcription activity of nucleolar chromatin through the use of electron microscope autoradiography applied to isolated and spread molecules. The data obtained were suitable for quantitative analysis of the variations in transcription-unit-synthesis activity with respect to incubation time. Such quantitation was possible because of two factors. First, the numbers of grains obtained with the autoradiographic method used were ample for statistical analysis, and sec-

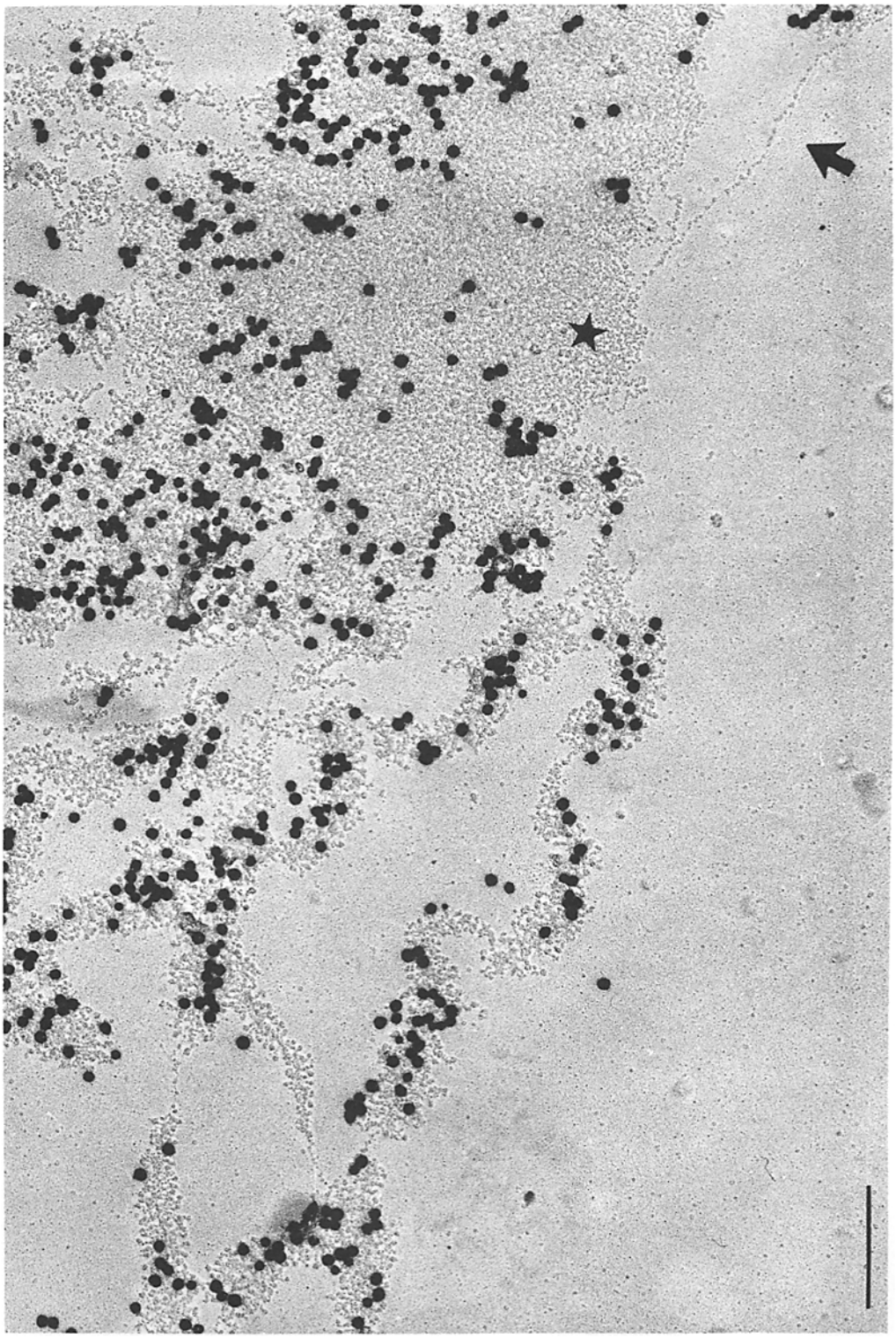

FIGURE 4 Nucleolar spread labeled by autoradiography (incubation for 48 h). High concentration of silver grains over easily distinguishable fibrillar sequences. Arrow: unlabled bare DNP fiber; star: this part of the nucleolus apparently consists of nontranscribing packed DNP fiber. Bar, 1 μm.

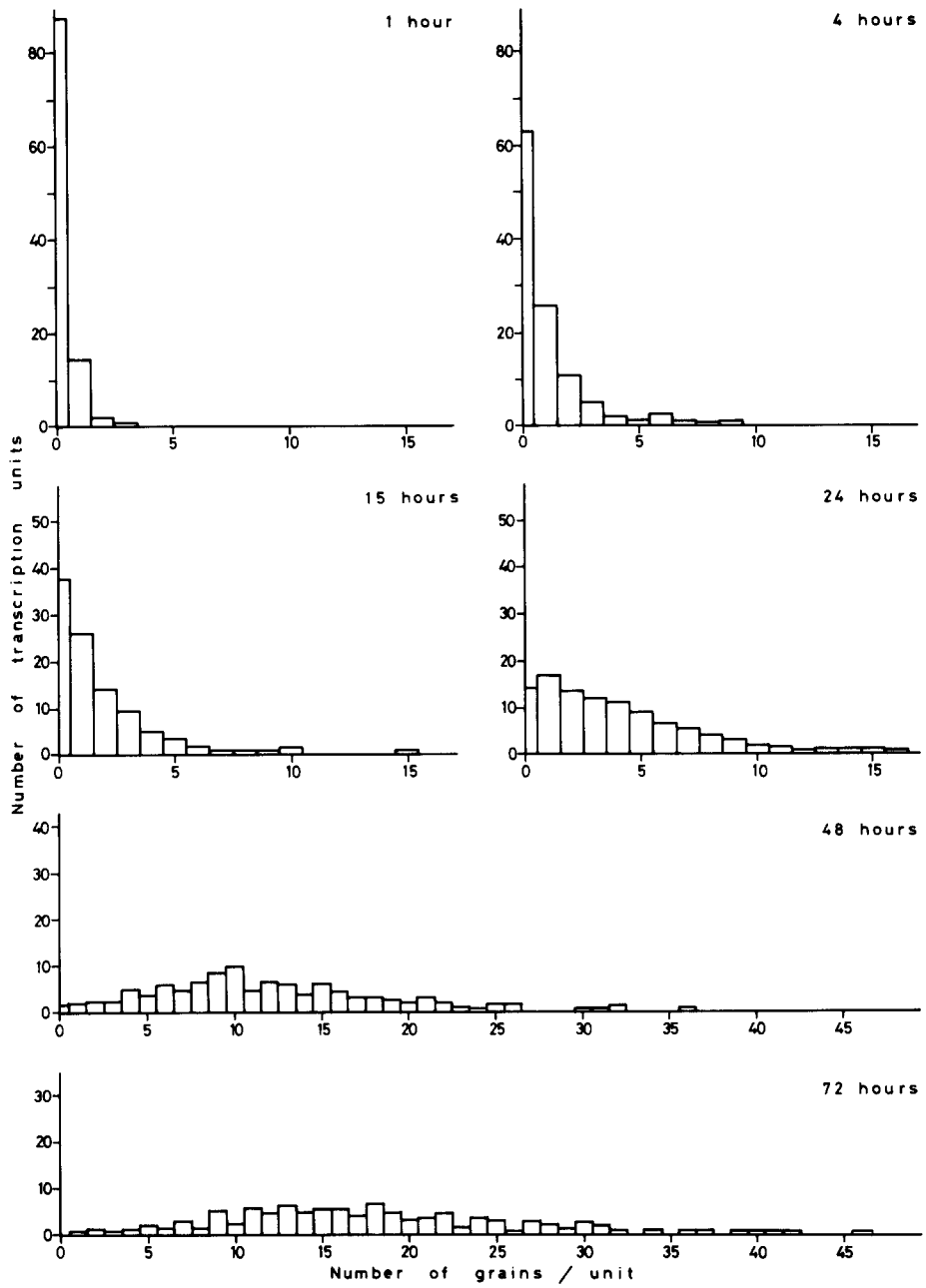

FIGURE 5 Comparison of the histograms which shows the distribution of the number of silver grains per unit for increasing incubation times (1, 4, 15, 24, 48, and 72 h). Note that the labeling heterogeneity increases with time. See text for interpretation.

ond, the size and the shape of the silver grains permitted a precise localization of the labeling of each unit. In view of the results, the following points can be stressed: (*a*) The endogenous nucleotide pool of the oocyte was not saturated by exogenous precursors, even after long periods of incubation in tritiated medium. (*b*) The units of any one nucleolus exhibited a large degree of heterogeneity in their number of grains. (*c*) There was a parallelism between increasing grain number and RNP lengthening as observed along the transcription unit.

TABLE I

Distribution of the Number of Grains per Unit

Incubation time	Number of units	Average number of grains per unit	Variance of the number of grains per units	Index of dispersion
h		*mean ± SE*	s^2	$d = s^2/mean$
1	110	0.23 ± 0.06	0.31	1.35
4	256	0.96 ± 0.10	2.55	2.55
15	152	1.41 ± 0.13	4.47	3.17
24	415	3.46 ± 0.16	9.94	2.87
48	215	11.13 ± 0.40	33.06	2.97
72	307	16.90 ± 0.46	62.25	3.69

TABLE II

Distribution of the Average Number of Grains per Unit of Nucleolus

Incubation time	Number of nucleoli	Average number of units per nucleolus	Variance between nucleoli	Variance between units of a nucleolus	Percent of the total variance caused by variations between nucleoli	P^*
h			s^2_N	s^2_U	$s^2_N/(s^2_N + s^2_U)$	
1	12	9.5	0.002	0.30	0.01	0.05
4	19	13.5	0.35	2.22	0.14	0.001
15	10	15.2	0.97	3.61	0.21	0.001
24	38	10.9	2.89	7.15	0.29	0.001
48	19	11.3	5.26	28.21	0.16	0.001
72	29	10.6	15.34	47.60	0.24	0.001

* Significance level of the variance between nucleoli.

Large Size of the Oocyte Pool

In oocytes during vitellogenesis, the labeling of nucleolar RNA is made particularly difficult by the large size of endogenous nucleotide pools (17). Recent biochemical evidence shows that these pools are not saturated by exogenous precursors even after very long periods of incubation, 120 h for *Xenopus laevis* (8) and 50 h for pleurodele (9), for example. It is known that more prolonged in vitro incubation results in changes of RNA synthesis as shown by the retraction of the loops in the lampbrush chromosomes (9). In the conditions used in this study, the saturation of nucleotide pools was not found to occur at 72 h (Fig. 2). In the absence of pool saturation, we found it was useful to compare the results as incubation times were increased. This led to our development of a procedure for quantitation, as described in the Appendix, and allowed us, in our opinion, to provide a significant analysis of the distribution of grains.

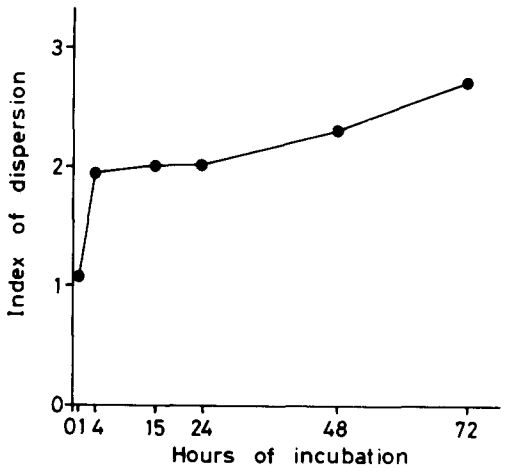

FIGURE 6 Index of dispersion variations for the distribution of the number of grains per unit with respect to incubation time. ●——●——●: Average dispersion calculated for each nucleolus. The index is always >1, thus precluding a random distribution.

Heterogeneity of Labeling in Transcription Units

The origin of this variability could be one or more of four factors: (*a*) A heterogeneous distribution of labeled precursors related to the nonsaturation of the pool in the neighborhood of the different units. (*b*) A morphological variability of the transcription units. (*c*) A heterogeneous functioning of the transcription units. (*d*) A heteroge-

TABLE III

Variations of the Number of Grains between Units of the Same Nucleolus

Incubation time	Number of nucleoli	Average number of units per nucleolus	Average intra-nucleolus index of dispersion ± SE
1	12	9.2	1.08 ± 0.09
4	19	13.5	1.95 ± 0.31
15	10	15.2	2.01 ± 0.41
24	38	10.9	2.02 ± 0.25
48	19	11.3	2.33 ± 0.22
72	29	10.6	2.73 ± 0.28

TABLE IV

Relationship between the Number of Grains of Two Adjacent Units along a DNP Fiber

	Next unit		
Unit	$g > m$	$g > m$	Total
$g \leq m$	58	42	100
$g > m$	48	37	85

See the text for definition of m.

neity caused by the autoradiographic procedure.

An irregular distribution of labeled precursors in the culture medium or even within the oocyte could be at the origin of heterogeneous labeling. Heterogeneity between units of the same nucleolus would mean that this irregularity was located within the nucleolus itself. If this were true, some definite parts of the nucleolar DNP fiber would bear transcription units with more intense labeling than others, which is not consistent with the statistical evidence which reveals that two contiguous transcription units could, in fact, bear very different numbers of silver grains. Therefore, one must consider an irregularity in the distribution of labeled precursors at a more elemental scale than the nucleolus—the transcription unit. However, if this phenomenon entirely explained labeling heterogeneity, this heterogeneity should decrease as incubation time is increased, which was not the case. In fact, the index of dispersion of the grain/unit distribution increased with the duration of incubation. Therefore, factors of heterogeneity, other than the irregular circulation of labeled molecules, must be taken into consideration.

On the basis of the morphological evidence alone, a functional variability of the nucleolar transcription units would not be expected. The high degree of similarity of the nucleolar transcription units of the pleurodele is well known (4). In our material, the average length of the transcription units was 2.02 μm, with a standard deviation (s) of 0.16 μm and an index of dispersion (d) of 0.01. It was also possible to obtain a precise analysis of the potential heterogeneity of the

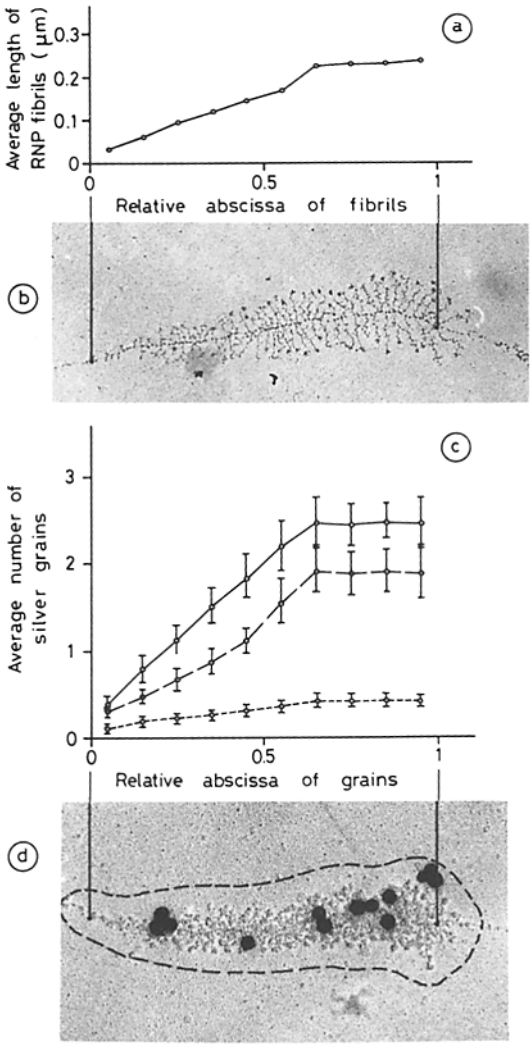

FIGURE 7 Comparison between the length of the RNP fibrils and the number of grains from the first to the last tenth of the units. (*a*) Average length of the RNP fibrils. (SE is from 0.001 to 0.019). (*b*) Illustration of the unit, which indicates the position of the two extremities—the beginning and the end. (*c* and *d*) Average number of grains falling on each tenth after 24, 48, and 72 h of incubation (O-----O 24 h, O— —O 48 h, O——O 72 h); the bars indicate SEs. See text (Materials and Methods, and Results) for interpretation. Because of the scattering of grains in the vicinity of the first tenth, it was not possible to locate more precisely the initiation point: from −1HD to the apparent origin, the labeling lies between 0.01 ± 0.01 for 24 h of incubation and 0.04 ± 0.01 for 72 h of incubation.

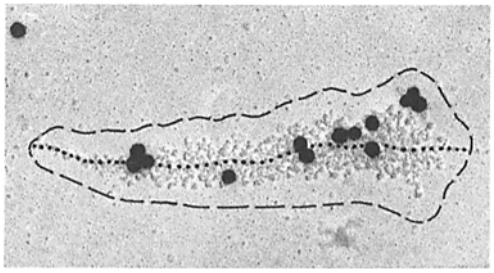

FIGURE 8 Illustration of the method used to control the homogeneity of the labeling of the two halves of the same unit, taking the HD into account. The index of dispersion between the number of grains of the two longitudinal halves of 218 units was 1.07, which indicates that the autoradiographic method itself cannot account for the heterogeneous labeling of the units (see Results and Discussion).

density of RNP fibrils in 49 control units not subjected to autoradiography. The mean value was 98.8 fibrils per unit with $s = 5.5$ and $d = 0.31$. It is clear that the values of the index of dispersion in these measurements are too small to explain the heterogeneity of labeling. Therefore the magnitude of labeling heterogeneity observed must be explained on some basis other than morphological variability.

This suggests the possibility of a difference in the functioning of the units. A possible explanation of the labeling variations observed between transcription units of the same nucleolus could be the existence of asynchronous variations in the transcription activity of the units. This hypothesis is in agreement with recent studies by Hackett and Sauerbier (13), Scheer et al. (30), and McKnight and Miller (21). These reports have suggested that each gene possesses its own "control element" so that activation and inhibition of transcription units occur at different times.

The autoradiographic process itself could be at the origin of this heterogeneity, however. The number of grains generated by a radioactive source might alternatively give zero, one, or several grains. Several sources might generate a single grain. The further dispersion that results from this variability factor should not, however, lead to an increase of the index of dispersion over incubation time (see Appendix) if the emulsion is perfectly homogeneous at the scale of the unit. In the present case, if the emulsion had been heterogeneous at the unit's scale, the two longitudinal halves of the same unit should have been statistically heterogeneous in terms of their number of grains, which was not the case.

Significance of the Labeling Distribution within the Transcription Unit

According to current concepts (23), lateral fibrils materialize the 40S preribosomal RNA in the course of biosynthesis. A given RNA molecule is initiated well before the elongation of the preceeding molecule is completed. The completion of the synthesis of the 40S RNA molecule is followed by the immediate release of the corresponding RNP fibril. This successive initiation process permits the observation of the transcribed part of the nucleolar genic unit, and so this hypothesis is essentially confirmed by autoradiography (1, 3, 25).

The present analysis of grain distribution along the unit revealed that at the end of the unit, the number of grains ceased to increase and the RNP fibrils stopped lengthening. The concordance between autoradiographic and morphological evidences precludes a mere contraction of these fibrils as the cause of cessation of RNP-fibril lengthening. Such a phenomenon could result from various causes: (*a*) a "quenching" caused by a difference of density of the fibrils along the unit or by a dose dependence in electron microscope autoradiography (29); (*b*) a contraction of the longer fibrils; (*c*) influence of the nonsaturation of the oocyte pool upon the kinetics of the transcripts along the DNA matrix; or (*d*) a biological difference between the two parts of the unit.

A larger number of fibrils in the widest part of the unit could result in a quenching because of a more compact source of decays. This is not, however, in agreement with the fact that the density of the fibrils did not vary significantly along the units (Fig. 9). Besides, the plateau was maintained at each length of incubation period, starting from the seventh tenth, regardless of the level of labeling (Fig. 7), which precludes a saturation of the emulsion. As for dose dependence, it was negligible because the average density in the first six tenths of the units was 2.37 grains/μm^2 and 2.84 in the remaining four tenths. If one assumes a 17% efficiency (7), this falls in the portion of the curves published by Salpeter and Szabo (29) in which the differences in anticipated efficiency do not exceed a few percent. Finally, self adsorption is not possible in a thin (a few nanometers thick) bidimensional source.

The plateau in lengthening has been interpreted as reflecting an increase in secondary structure (16). This could not entirely explain the data in this case; one would expect an increasing number

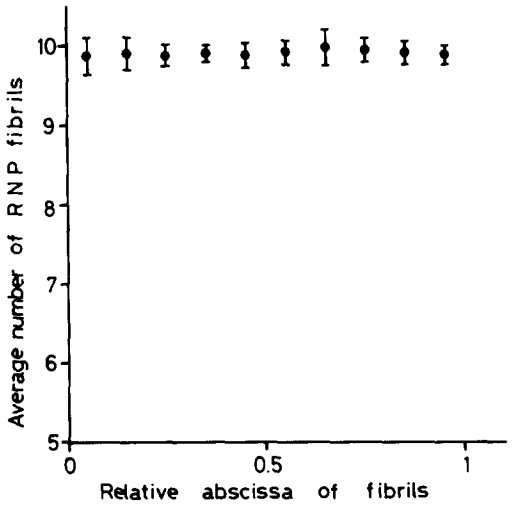

FIGURE 9 Average number of fibrils counted in each tenth of 49 transcription units. The density of the fibrils did not vary significantly along the transcription unit. Bars indicate SEs.

of grains and therefore the absence of a plateau in the labeling.

Nonsaturation of the endogenous nucleotide pools by exogenous percursors cannot be entirely rejected as a cause of differences in labeling between the shorter and longer transcripts. It is unlikely as far as the plateau is concerned, because this plateau was still observed for long periods of incubation (48 and 72 h) in conditions closer to saturation.

Therefore, one is left with the fourth hypothesis, namely, that there are differences between those fibrils which belong to the upward slope of this curve and those which belong to the plateau. The results might suggest the following interpretations, among others: (*a*) Preribosomal 40S RNA synthesis is completed as soon as the RNP fibril reaches its maximal length, and there is no liberation of the corresponding RNP fibrils. This results in a storage of these fibrils, for some period of time, along the DNP fiber. The DNA segment responsible for transcription is shorter than the fibrillar sequence. (*b*) Cessation of RNA synthesis at the end of the unit is only an appearance and results from the superposition of the RNA synthesis and the processing that takes place simultaneously or almost simultaneously. This interpretation is in partial agreement with that of Franke et al. (10), who suggested, on the basis of morphological criteria, that such RNA molecular cleavage arises from the initiation of synthesis which would begin before the apparent initiation point of the transcription unit in the spacer segment. At the present time, there are no biochemical data to explain this surprising result. However, it is strongly suggestive of a dichotomy between morphological and biochemical evidences.

These results were presented at the Fifth Nucleolar Workshop of the European Cell Biology Organization (ECBO), in Salamanca, Spain, in June 1977.

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APPENDIX

ANALYSIS OF THE HETEROGENEITY OF THE NUMBER OF GRAINS PER TRANSCRIPTION UNIT

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Analysis of the heterogeneity of the number of grains counted in different transcription units is easiest when the endogenous pool of precursors is saturated—the distribution of the tritiated precursors in the endogenous pool may be considered to be homogeneous and thus is not itself a factor in heterogeneity. In the present case, however, the endogenous pool was not saturated even after long incubation periods (72 h), and thus the distribution of the tritiated precursors within the endogenous pool cannot be considered homogeneous. However, as incubation time increases, the distribution of the tritiated precursors tends to become more and more homogeneous; it is therefore of interest to analyze the variations in the heterogeneity of grain counts as a function of incubation time. As a matter of fact, as incubation time increases the variance of the number of grains increases, too. This, however, would be the case even if the number of grains followed a simple Poisson distribution, because the average number of grains per transcription unit also increases with incubation time. Thus, the variance of the number of grains provides no information about the origin of the observed heterogeneity. It is therefore of interest to find a measurement which can provide such information. The measurement proposed, the ratio of the variance of the number of grains to the mean value of this number, is the so-called "Index of Dispersion" (1).

When the variability of g, the number of grains per transcription unit, is solely determined by the random disintegration of the radioactive atoms incorporated in the unit, g has a Poisson distribution (1). In this case, the variance of g, $v(g)$, is equal to its mean value, $m(g)$, and the index of dispersion is equal to one. When there are additional causes of variability, the distribution of g is more dispersed than a Poisson distribution (2) and the index of dispersion is greater than one.

As a matter of fact, three types of factors determine the magnitude of the variability of g: (a) the number of molecules of precursor incorporated in a unit (I) may vary; this will be referred to as the "incorporation" variability. (b) For a given I value, the number of molecules which are labeled (I^*) may also vary; this will be referred to as the "labeling" variability. (c) Finally, for a given I^* value, the number of grains counted over this unit (g) can also vary; this will be referred to as the "revelation" variability. Among these three possible sources of variability, the first one indicates some variability in the functioning of the units themselves, whereas the last two are introduced by the experimental method used to draw inferences about this functioning. It will be shown in the discussion that follows the way in which the

variations of the index of dispersion of g with incubation time can provide information about the possible existence of an incorporation variability.

Revelation Variability

The mean and variance of the total number of grains counted over a transcription unit which incorporated a given number of labeled molecules of precursor (I^*) are expressed respectively as:

$$m(g; I^*) = I^* \cdot \mu$$

and

$$v(g; I^*) = I^* \cdot \sigma^2 \qquad (1)$$

where μ and σ^2 are the mean and the variance of the number of grains generated by a single molecule of tritiated precursor. For Eq. 1 to hold, however, it is necessary that the different numbers of grains generated by each of the I^*-labeled molecules in the unit be statistically independent; that is, neither positively correlated, as would be the case if the emulsion was heterogeneous in thickness or crystal density, nor negatively correlated, as would be the case if the emulsion was saturated by a very large number of disintegrations per unit area.

Labeling Variability

The number of labeled molecules of precursor incorporated in a unit (I^*) can also vary; if one takes into consideration these variations, the mean and the variance of the number of grains given in Eq. 1 become:

$$\begin{aligned} m(g) &= m(I^*) \cdot \mu \\ v(g) &= m(I^*) \cdot \sigma^2 + \mu^2 \cdot v(I^*) \end{aligned} \qquad (2)$$

where $m(I^*)$ and $v(I^*)$ are the mean and the variance of the number of labeled molecules of precursor incorporated by a unit.

The number of labeled molecules of precursor incorporated in a unit is determined by both the total number of molecules of precursor which are incorporated (I) and the proportion of these which are actually labeled. The mean and the variance of the number of labeled molecules within a transcription unit which incorporated a given number of molecules of precursor (I) are expressed respectively by:

$$\begin{aligned} m(I^*; I, P^*) &= I \cdot P^* \\ v(I^*; I, P^*) &= I \cdot P^* \cdot (1 - P^*) \end{aligned} \qquad (3)$$

where P^* is the proportion of labeled precursor in the neighborhood of the transcription unit. If the endogenous pool is not saturated, P^* itself can vary; if one takes into consideration these variations, the mean and the variance of I^* given in Eq. 3 become:

$$\begin{aligned} m(I^*; I) &= I \cdot m(P^*) \\ v(I^*; I) &= I \cdot m(P^*)\,[1 - m(P^*)] \\ &\quad + I(I - 1)v(P^*) \end{aligned} \qquad (4)$$

where $m(P^*)$ and $v(P^*)$ are the mean and the variance of the distribution of P^*.

Incorporation Variability

The total number of molecules of precursors which are incorporated in a unit (I) can also vary; if one takes into consideration these variations, the mean and the variance of I^* given in Eq. 4 become:

$$\begin{aligned} m(I^*) &= m(I) \cdot m(P^*) \\ v(I^*) &= m(I) \cdot m(P^*)\,[1 - m(P^*)] \\ &\quad + m(I)\,[m(I) - 1]v(P^*) \\ &\quad + v(I) \cdot [m(P^*)^2 + v(P^*)] \end{aligned} \qquad (5)$$

where $m(I)$ and $v(I)$ are the mean and the variance of the total number of molecules of precursors incorporated in a unit.

Variations of the Dispersion Index of the Number of Grains with Incubation Time

By including the three possible sources of variability considered, the mean and the variance of the number of grains counted over a transcription unit can be obtained from Eqs. 2 and 5:

$$\begin{aligned} m(g) &= \mu \cdot m(I) \cdot m(P^*) \\ v(g) &= \sigma^2 \cdot m(I) \cdot m(P^*) \\ &\quad + \mu^2 \cdot \{m(I) \cdot m(P^*)\,[1 - m(P^*)] \\ &\quad + m(I)\,[m(I) - 1]\,v(P^*) \\ &\quad + v(I)\,[m(P^*)^2 + v(P^*)]\} \end{aligned} \qquad (6)$$

From (6) the index of dispersion of g is therefore given by:

$$d(g) = \frac{\sigma^2}{\mu} + \mu \left\{ \left[1 - m(P^*) \right] + [m(I) - 1] \cdot \frac{v(P^*)}{m(P^*)} + \frac{v(I)}{m(I)} \cdot \left[m(P^*) + \frac{v(P^*)}{m(P^*)} \right] \right\}$$

To interpret the variations of the index of dispersion with incubation time, it is necessary to assume that the biological process under study is in a stationary state, i. e., that the distribution of I [and particularly $m(I)$ and $v(I)$] does not vary with incubation time. As incubation time increases, the proportion of labeled precursors increases and the distribution of the labeled molecules within the endogenous pool becomes more and more homogeneous; that is, $m(P^*)$ increases whereas $v(P^*)$ decreases. If the number of molecules of precursor incorporated in the different transcription units is the same [i. e., if the variance $v(I)$ is null], the $d(g)$ is then expected to decrease. Reciprocally, if the $d(g)$ increases with incubation time, as is the case for the experimental results presented here, one is led to the conclusion that the number of molecules of precursor incorporated in the different units is not the same; thus, the functioning of the transcription units itself is heterogeneous.

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