Experimental Cell Research 70 (1972) 140–144

ELECTRON MICROSCOPIC AUTORADIOGRAPHIC STUDY OF RNA SYNTHESIS IN YEAST NUCLEUS

W. W. SILLEVIS SMITT,[1] C. A. VERMEULEN,[2] J. M. VLAK,[1]
Th. H. ROZIJN[1] and I. MOLENAAR[3]

[1]*Laboratory for Physiological Chemistry at The State University, Utrecht,* [2]*Laboratory of Genetics at The State University, Groningen and* [3]*Centre for Medical Electron Microscopy, The State University, Groningen, The Netherlands*

SUMMARY

High resolution autoradiography of isolated yeast nuclei has been used to investigate the distribution of newly formed RNA in the nucleus. The nuclei were isolated after incubation of spheroplasts with ^{3}H-uracil for 5 min. Most grains (80%) are located over the dense crescent. In the dense crescent the grains seem to be associated with the nucleolonema. An analysis of the nuclear RNA on polyacrylamide gels shows that the incorporated ^{3}H-uracil is predominantly present in the initial (37S) ribosomal precursor RNA. The EM autoradiographic and biochemical data, taken together, strongly suggest that we have to ascribe a nucleolar function to the dense crescent of the yeast nucleus.

When isolated yeast nuclei are observed by means of the phase-contrast microscope, a crescent-shaped segment of high optical density can be discerned [1]. EM studies have ascertained it to be very likely that this crescent is equivalent to the mammalian nucleolus. A large part of the "dense crescent" is occupied by a structure strongly resembling the nucleolonema of the mammalian nucleolus. Moreover, a correlated biochemical and electron microscopic study has shown that the dense crescent consists mainly of RNA and protein [2].

The exponentially growing yeast cells from which the nuclei are isolated are endowed with a very high rate of synthesis of ribosomal RNA and a high ribosomes content [1]. Thus it is not surprising that yeast nuclei, compared with nuclei of animal cells, contain a relatively large amount of RNA which is mainly ribosomal precursor RNA [3]. Evidence has come from several sources [3–5], that in yeast both species of ribosomal RNA derive from a common large precursor molecule. Hence it is clear that yeast, one of the most primitive eukaryotes, possesses a mechanism for the processing of ribosomal RNA which resembles that of higher eukaryotes. This raises the question whether, in yeast, as in higher eukaryotes, the ribosomal precursors will be confined to the region of the nucleus resembling a nucleolus.

In order to obtain an insight into the intranuclear localization of the ribosomal precursor RNA, we have performed pulse labelling experiments with ^{3}H-uracil followed by electron microscopic autoradiography.

MATERIALS AND METHODS

Organism and isotope

The experiments were performed with spheroplasts [1] of the yeast strain *Saccharomyces carlsbergensis,*

no. 74 from the British National Collection of Yeast Cultures. The spheroplasts were incubated (27°C) at a cell density of 7×10^7 per ml in a medium containing, per ml: 120 mg mannitol, 10 mg glucose, 0.2 μg of resp. thiamine HCl, riboflavin phosphate, nicotinamide, pyridoxal HCl, calcium pantothenate, biotin and inositol, 15 μM$(NH_4)_2SO_4$, 1 μM $MgCl_2$ and 20 μM sodium-potassium phosphate, pH 6.3. After preincubation for 15 min ^{3}H-uracil (spec. act. 20 Ci/mM; Philips Duphar) was added to make a final concentration of 10 μCi/ml. Incubation was then continued for 5 min. After labelling, the spheroplast suspensions were rapidly chilled and the spheroplasts harvested by centrifugation at 3 000 g for 5 min, at 2°C.

Preparation of nuclei

The nuclei were isolated as described elsewhere [1, 6].

Preparation and electrophoresis of RNA

For the extraction of RNA, a suspension of nuclei (2×10^9) was treated with 100 μg DNase (Worthington, RNase free) for 12 min at 27°C [6]. RNA was then extracted essentially as described by Parish & Kirby [6, 7]. Acrylamide gel electrophoresis was performed according to Loening [8] and Bishop et al. [9] at 20–25°C in 2.4% (w/v) acrylamide gels, prepared with 0.12 (v/v) ethylene diacrylate as a cross-linker. After electrophoresis the gels were scanned at 266 nm in a Zeiss spectrophotometer (slit 0.1 × 0.4 cm), that was adapted for this purpose and combined with a Beckman recorder [6]. The gel was then frozen on dry ice and was cut into 0.1 cm slices. These were solubilized in 0.5 ml 1 N NH_4OH in a scintillation vial for 2 h at 20–25°C. For radioactivity measurements 14.5 ml of a 6:23 (v/v) mixture of Triton X-100 and a toluene solution of PPO and POPOP was added. Radioactivity was measured in a Mark I liquid scintillation counter (Nuclear Chicago) [6].

Preparation for electron microscopy

The nuclear pellets were prepared for electron microscopy as described previously [2] with one modification: 2% solution of glutaraldehyde was used instead of a 5% solution.

Electron microscopic autoradiography

One part of Ilford L-4 Nuclear Research emulsion, diluted with 2 parts of distilled water, was melted and carefully stirred with a glass rod for 14 min at 45°C. The emulsion was then cooled at 0°C for 4 min and kept for 5–10 min at room temperature. Grids containing thin sections were coated with a 50 Å evaporated carbon layer, mounted on alcohol-cleaned objective slides and applied with a monolayer of emulsion according to the wire loop technique described by Caro et al. [10]. Autoradiographs were stored for approx. 4 months at 4°C in light-tight boxes containing silica gel and developed by use of the method of Wisse & Tates [11] which involves gold latensification and elon-ascorbic acid development. This method was modified in such a way that the autoradiographs were developed for 4 min instead of 7.5 min.

Autoradiographs were fixed with hardening fixer for 3 min, washed 3 times in distilled water and carefully removed from the objective slides. Pictures of grains-associated nuclei were taken at ×12 000 with a Philips EM 300 electron microscope. Final magnification for grain counting and surface measurements was ×80 000.

The surface areas of dense crescent and chromatin were estimated by superimposing a regular point lattice (distance between points = 1 cm) on the electron micrographs and counting the points over dense crescent and chromatin area respectively according to Weibel et al. [12]. After normalizing the total number of points to the total number of grains counted, the grain density was expressed as the ratio of number of grains to number of points.

Enzymatic extraction of isolated nuclei

The procedure for the enzymatic extraction of fixed nuclei and the chemical analysis of the nuclei after enzymatic extraction has been described previously

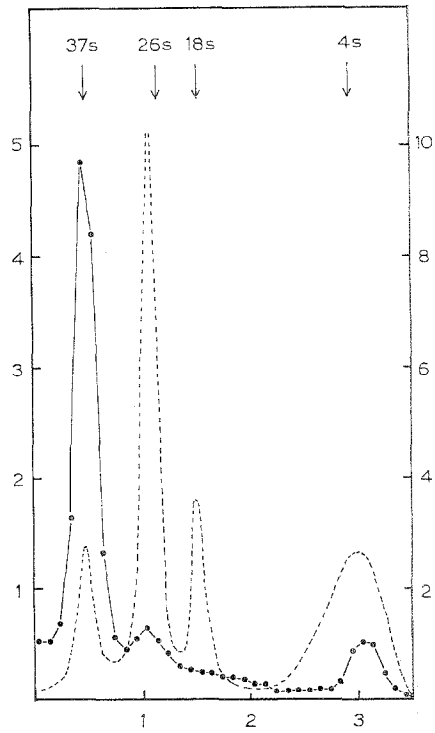

Fig. 1. Abscissa: cm travelled and slice number; *ordinate: (left)* OD_{266}; *(right)* dpm × 10^{-5}.

Acrylamide gel electrophoretic analysis of nuclear RNA (from 1.4×10^9 nuclei). Electrophoresis was for 90 min at 5 mA per gel. ---, optical density; ●—●, ^{3}H-uracil. The optical density in the 4S region is due to degraded DNA.

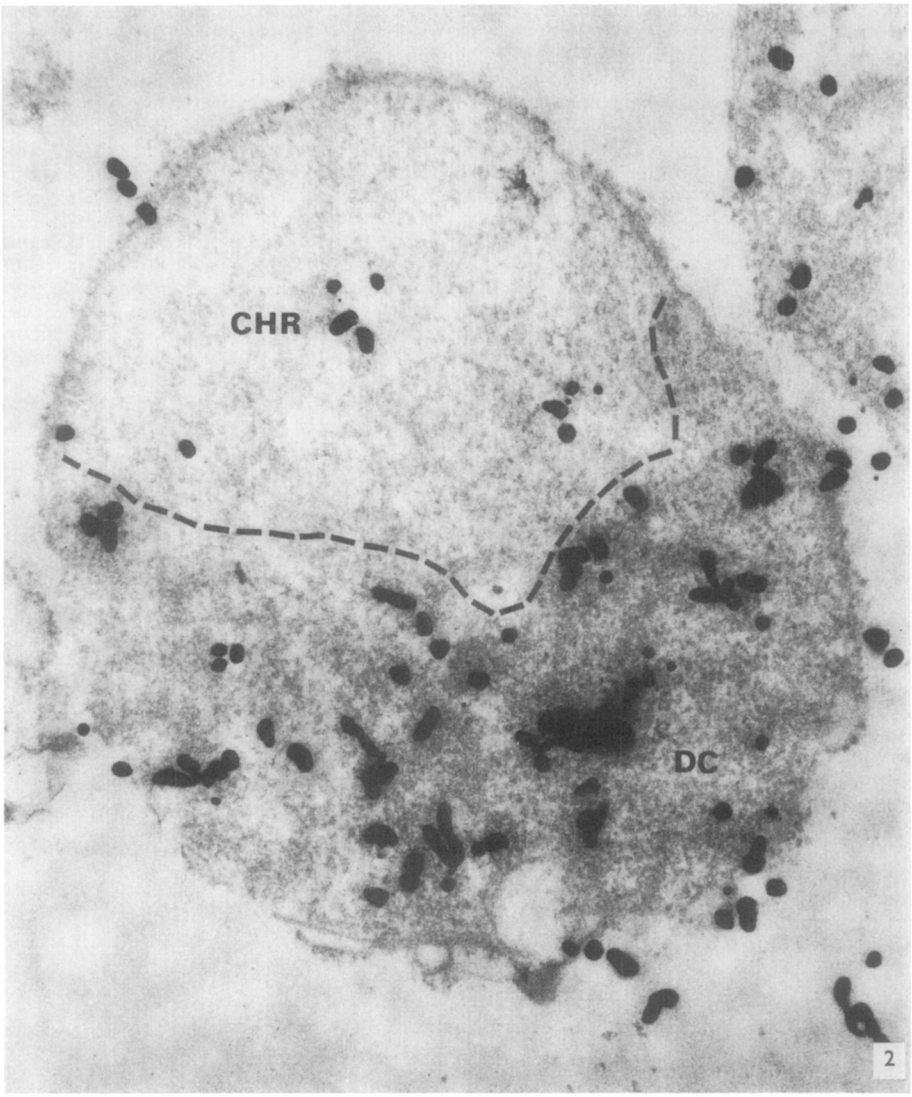

Fig. 2. Yeast nucleus (× 66 000). The grains are mainly located on the dense crescent and preferentially on its nucleolonema. *dc*, dense crescent; *chr*, chromatin. For further explanation see text.

[2]. The fixed nuclei (2×10^9) were resuspended in 2 ml 0.5 mM $MgCl_2$-0.01 M Tris-HCl, pH 7.4, containing either RNase (30 μg/ml) or RNase (same conc.) + DNase (30 μg/ml).

RESULTS

An analysis of the nuclear RNA after a 5 min pulse with ^{3}H-uracil is presented in fig. 1. The radioactivity profile shows a predominant 37S peak and some radioactivity in the 28S region. Some rapidly labelled polydisperse RNA and 4–7S RNA is present. It is clear from the radioactivity pattern of the nuclear RNA that the radioactivity is predominantly present in the 37S and 28S ribosomal precursors [3, 6].

Table 1. *Grains counted in 20 EM autoradiographs of yeast nuclei*

	Dense crescent	Chromatin	Total
Grains (A)	903	205	1 108
Points[a]	1 741	1 397	3 138
Points, normalized to total grains (B)	615	493	1 108
Ratio (A/B)	1.5	0.4	1.0

[a] Details on surface measurements are given under Materials and Methods

In fig. 2 an EM autoradiograph of a yeast nucleus is shown. The grains are not distributed equally over the nucleus, but concentrated in the region occupied by the dense crescent. In order to gain more insight into the distribution of grains over the dense crescent and the chromatin respectively, the grain density was determined in 20 nuclei. The results are shown in table 1: the grain density in the dense crescent is at least thrice as high as in the chromatin.

In table 2 the results of controls are presented. We have shown previously [2] that the chemical composition of fixed nuclei which have been incubated in buffer without enzyme is the same as the composition of nuclei that are not incubated ("cold control"). Incubation of the glutaraldehyde-fixed nuclei with RNase or RNase and DNase for 0.5 h releases some 80 % of the RNA and 90 % of the radioactivity: this shows that the radioactivity was incorporated only in RNA. On EM autoradiographs of the RNase-treated nuclei it appeared that only a few grains per nucleus were present (not shown).

Table 2. *DNA and RNA content and radioactivity of fixed nuclei, following incubation with nucleases*

Conditions	DNA (%)	RNA (%)	Radioactivity (%)
Control (cold)	100	100	100
RNase	97	23	12
RNase + DNase	20	22	13

The results are expressed as percentages of the DNA and RNA content and amount of radioactivity of control nuclei

We did not perform a quantitative study of the grain distribution within the dense crescent. However, the grains seem to be associated with the (electron-dense) nucleolonema.

DISCUSSION

An analysis of the pulse-labelled nuclear RNA on acrylamide gel has shown that the main body of nuclear radioactivity is present in the 37S ribosomal precursor (fig. 1). These results have prompted us to investigate the distribution of radioactivity within the nucleus by means of electron microscopic autoradiography. From the grain densities, given in table 1, it can be concluded that per unit of nuclear volume about three times more radioactive RNA is present in the dense crescent than in the chromatin. As we know from phase contrast- and electron microscopy that nearly half of the nuclear content is taken up by the dense crescent, this means that the greater part of the nuclear radioactive RNA is located in the dense crescent. As, according to the electrophoresis pattern, most of the nuclear radioactivity is present in the 37S ribosomal precursor RNA, it is tempting to assume that most, and possibly all of the 37S precursor is located in the dense crescent. In this connection it is worthwhile to mention our observation that the grains are preferentially located above the nucleolonema of the dense crescent. Our results from electron microscopic autoradiography support our view from previously

published data [2, 3] that the dense crescent of the yeast nucleus may be functioning as a nucleolus.

The loose and sponge-like structure of the dense crescent may be related to the high rate of synthesis of ribosomal RNA in exponentially growing yeast. The nucleus of the yeast cell is very actively engaged in the synthesis of ribosomal RNA. This is illustrated not only by a very high content of ribosomal precursor RNA [3, 6] but also by the data of Retèl & Planta [13] and Halvorson et al. [14, 15]. These authors have estimated that a strikingly large part of the genome (about 2%) is occupied by rRNA cistrons. Thus it is not surprising that in exponentially growing yeast cells a nucleolus comprises a large part of the nucleus.

In order to answer definitively the question whether the dense crescent has a nucleolar function, we have performed experiments that lead to the isolation of dense crescents relatively free of chromatin. A report of these experiments will be published in a forthcoming paper [16].

The authors are grateful to Mr J. A. Oosterbaan, Mr H. Meiborg, Mr M. Veenhuis and Mr R. Schiphof for their skilful technical assistance.

The present investigations were supported, in part, by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the advancement of Pure Research (Z.W.O.).

REFERENCES

1. Rozijn, T H & Tonino, G J M, Biochim biophys acta 91 (1964) 105.
2. Molenaar, I, Sillevis Smitt, W W, Rozijn, T H & Tonino, G J M, Exptl cell res 60 (1970) 148
3. Sillevis Smitt, W W, Nanni, G, Rozijn, T H & Tonino, G J M, Exptl cell res 59 (1970) 440.
4. Retèl, J & Planta, R J, Europ j biochem 3 (1967) 248.
5. Taber, R L & Vincent, W S, Biochim biophys acta 186 (1969) 317.
6. Sillevis Smitt, W W, Vlak, J M, Schiphof, R & Rozijn, T H, Exptl cell res 71 (1972) 140.
7. Parish, J H & Kirby, K S, Biochim biophys acta 129 (1966) 554.
8. Loening, U E, Biochem j 102 (1967) 251.
9. Bishop, D H L, Claybrook, J R & Spiegelman, S, J mol biol 26 (1967) 373.
10. Caro, L G, Tubergen, R P van & Kolb, J A, J cell biol 15 (1962) 173.
11. Wisse, E & Tates, A D, Proc fourth Europ reg conf electron microscopy (ed D S Bocciarelli) p. 465. Tipografia Poliglotta Vaticana, Roma (1968).
12. Weibel, E R, Kistler, G S & Scherle, W F, J cell biol 30 (1966) 23.
13. Retèl, J & Planta, R J, Biochim biophys acta 169 (1968) 416.
14. Schweizer, E, McKechnie, C & Halvorson, H O, J mol biol 40 (1969) 261.
15. Schweizer, E & Halvorson, H O, Exptl cell res 56 (1969) 239.
16. Sillevis Smitt, W W, Vlak, J M, Molenaar, I & Rozijn, T H. In preparation.

Received July 20, 1971