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Structural Alterations of the Nucleolus in Mutants of *Saccharomyces cerevisiae* Defective in RNA Polymerase I

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We have previously constructed mutants of *Saccharomyces cerevisiae* in which the gene for the second-largest subunit of RNA polymerase I (Pol I) is deleted. In these mutants, rRNA is synthesized by RNA polymerase II from a hybrid gene consisting of the 35S rRNA coding region fused to the *GAL7* promoter on a plasmid. These strains thus grow in galactose but not glucose media. By immunofluorescence microscopy using antibodies against the known nucleolar proteins SSB1 and fibrillarin, we found that the intact crescent-shaped nucleolar structure is absent in these mutants; instead, several granules (called mininucleolar bodies [MNBs]) that stained with these antibodies were seen in the nucleus. Conversion of the intact nucleolar structure to MNBs was also observed in Pol I temperature-sensitive mutants at nonpermissive temperatures. These MNBs may structurally resemble prenucleolar bodies observed in higher eukaryotic cells and may represent a constituent of the normal nucleolus. Furthermore, cells under certain conditions that inhibit rRNA synthesis did not cause conversion of the nucleolus to MNBs. Thus, the role of Pol I in the maintenance of the intact nucleolar structure might include a role as a structural element in addition to (or instead of) a functional role to produce rRNA transcripts. Our study also shows that the intact nucleolar structure is not absolutely required for rRNA processing, ribosome assembly, or cell growth and that MNBs are possibly functional in rRNA processing in the Pol I deletion mutants.

The nucleolus is the site of rRNA synthesis, rRNA processing, and ribosome assembly (for reviews, see references 16, 35, 37, 44, and 52). Its fine structure has been studied extensively by cytology, using a variety of higher eukaryotic cell systems. In general, three morphologically distinct regions have been recognized by electron microscopy: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). Recent studies using antibodies against known nucleolar proteins have started to reveal some of the molecular components in these regions. For example, RNA polymerase I (Pol I), the enzyme responsible for rRNA gene transcription, is localized mostly in the FC (38), which also contains most of the rRNA genes, as judged by in situ hybridization (2, 49); fibrillarin, the protein component of several ribonucleoprotein particles implicated in rRNA processing, such as U3 small nucleolar ribonucleoprotein particle (snoRNP) (19, 24), is localized in the DFC (29, 36); and ribosomal protein S1 is localized in the GC (20). It is likely that transcription of the rRNA genes takes place in the FC or at the boundary between the FC and the DFC, that the DFC is the major site of pre-rRNA processing, and that the GC, which is outside the DFC, contains ribosomal particles undergoing late assembly steps before the export to cytoplasm. However, details of the molecular architecture of the nucleolus and its functional significance are largely unknown, and even a crucial question regarding the site of rRNA transcription is still under serious debate (23).

In the yeast *Saccharomyces cerevisiae*, the nucleolus appears crescent shaped, occupying a substantial fraction of the nucleus and having close contact with the nuclear envelope (41, 42). Although electron micrographs of thin sections of yeast cells have failed to reveal clear structural details such as the three regions observed with higher eukaryotic cells, the presence of several protein components in the yeast nucleolus has been demonstrated by immunofluorescence microscopy; these components are the largest subunit (A190) of Pol I (10), SSB1 (10), the yeast homolog of fibrillarin (also called NOP1 [17, 39]), and GAR1 (15). Proteins SSB1, fibrillarin, and GAR1 are components of snoRNPs and appear to be involved in rRNA processing (10, 15, 50). As expected from the known association of fibrillarin with U3 RNA in both human cells and *S. cerevisiae* (34, 39), the presence of yeast U3 RNA and also large rRNAs in the nucleolus of isolated yeast nuclei has recently been demonstrated by electron microscopic visualization of DNA-RNA hybrids by using suitable DNA probes (12).

We have constructed several yeast strains which are defective in Pol I. Some strains are temperature-sensitive mutants of the gene (*RPA190*) for the A190 subunit of Pol I (53). Other strains are deletion mutants in which the gene (*RPA135*) for the second-largest subunit (A135) of Pol I is mostly deleted (called Pol I deletion in this report) and rRNA is synthesized by RNA polymerase II from a hybrid gene ("p*GAL7*-35S rDNA") consisting of the 35S rRNA coding region fused to the *GAL7* promoter on a plasmid (27). By immunofluorescence microscopy using antibodies against the known nucleolar proteins SSB1 and fibrillarin, we found that the intact crescent-shaped structure is absent in the Pol I temperature-sensitive strains at nonpermissive temperatures and in the Pol I deletion strains growing in the presence of galactose. Instead, we observed that several granules or dots stained with these antibodies are present in the nucleus. These dots, which we call mininucleolar bodies (MNBs), may represent a constituent of the normal nucleolus and, as discussed in this report, may structurally resemble the prenucleolar bodies (PNBs) observed in higher eukaryotic cells during late mitosis as precursors of the mature inter-

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TABLE 1. Yeast strains and plasmids used

Strain or plasmid	Genotype or characteristics	Reference or source
Strains		
NOY408-1a	*MATα rpa135::LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* pNOY102	27
NOY408-1b	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* pNOY102	27
NOY260	*MATα RPA190 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can^r*	53
NOY259	*MATα rpa190 trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can^r*	53
NOY486	NOY260 carrying YCP50 and therefore Ura$^+$	
NOY488	NOY259 carrying YCP50 and therefore Ura$^+$	
NOY460	*MATα ade2-1 his3-11 trp1-1 leu2-3,112 can1-100* pAA7	Constructed by transforming w303-1a (6) with a DNA fragment carrying *HIS3*, selecting His$^+$, and introducing pAA7 by transformation
prp20/6A	*MATα prp20-1 ade2-101 his3Δ200 ura3-52 tyr1*	Derived from *prp20*/2A described in reference 1
Plasmids		
pAA7	Galactose-inducible expression vector; 2μm, *URA3*	27, 58
pNOY102	pAA7 carrying *GAL7*-35S rDNA	27
YCP50	Yeast-*Escherichia coli* shuttle vector; *URA3 CEN4*	33

phase nucleolus (46; for a review, see reference 11). Our study demonstrates that intact Pol I is important for the maintenance of the intact nucleolar structure. In addition, our study shows that the crescent-shaped structure defined as the *S. cerevisiae* nucleolus is not absolutely required for rRNA processing, ribosome assembly, or cellular growth. Components which have been known to be essential for rRNA processing are present in MNBs and are possibly functional in rRNA processing in the Pol I deletion mutants.

MATERIALS AND METHODS

Materials. The rabbit anti-A190 antibody was prepared in this laboratory (54). Antibodies against SSB1 were provided by J. Broach, Princeton University, Princeton, N.J. Human autoimmune serum containing antifibrillarin was provided by H. Busch, Baylor College of Medicine, Houston, Tex. The anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC), anti-mouse IgG-rhodamine isothiocyanate (TRITC), and anti-human IgG-FITC conjugates were purchased from Sigma Chemical Co., St. Louis, Mo. 3-Aminotriazole, 8-hydroxyquinoline, cycloheximide, zymolyase (100T), β-glucuronidase (type H-2), and 4′,6-diamino-2-phenylindole (DAPI) were also purchased from Sigma. All other chemical reagents were from Fisher Scientific, Fairlawn, N.J., or J. T. Baker Chemical Co., Phillipsburg, N.J. [^{3}H]uridine (50 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. Membrane filters were purchased from Millipore Corp., Bedford, Mass.

Strains and media. YEPD consists of 2% yeast extract, 1% Bacto Peptone (Difco Laboratories, Detroit, Mich.), and 2% glucose. Synthetic galactose medium (SGal) consists of 2% galactose and 0.67% Bacto Yeast Nitrogen Base (Difco), supplemented when indicated with amino acids or bases as described by Sherman et al. (40). The concentration of Casamino Acids used as a supplement was 0.5%. Synthetic glucose (SD) medium is the same as SGal except that 2% glucose is substituted for galactose. Yeast strains and plasmids used in this study are described in Table 1. Cells were grown at 30°C unless otherwise stated.

Preparation of cells for immunofluorescence microscopy. Prefixation of yeast cells and subsequent steps for sample preparation were done essentially as described by Clark and Abelson (9). Exponentially growing cells (the A_{600} of cultures being less than 0.4) were used unless otherwise stated. Formaldehyde (37%) was added directly to the culture such that the final concentration of formaldehyde was 3.7%. After incubation for 30 min at 30°C, cells were removed from formaldehyde solution by filtration through a 0.45-μm-pore-size Millipore filter. They were washed with cold 0.1 M potassium phosphate (pH 6.5) and resuspended in cold 0.1 M potassium phosphate (pH 6.5)–1.2 M sorbitol. The cells were pelleted by centrifugation and resuspended in 100 mM Tris-HCl (pH 8)–25 mM dithiothreitol–5 mM Na_2EDTA–1 M sorbitol and incubated at 30°C for 10 min. Cells were pelleted by centrifugation and resuspended in 0.1 M potassium phosphate (pH 5.8)–1.2 M sorbitol. One-tenth of the volumes of β-glucuronidase (13,000 U/ml) and zymolyase (500 μg/ml) were added. This mixture was incubated at 30°C until the conversion of cells to spheroplasts was completed (15 to 30 min). The spheroplasts were then centrifuged, washed, and resuspended in 0.1 M potassium phosphate (pH 6.5)–1.2 M sorbitol. The cell suspension was then spread over polylysine-coated slides, and the cells were permitted to settle onto the slide for 5 min. The remaining liquid was then removed, and the slides were immersed in −20°C methanol for 6 min and then −20°C acetone for 30 s. The slides were allowed to dry at room temperature and stored at 4°C until further use. In several experiments, methanol and acetone, either each alone or in combination, were also used to fix cells instead of using formaldehyde. The same results were obtained.

Immunofluorescence microscopy. A phosphate-buffered saline (PBS)-bovine serum albumin (BSA) (BSA [0.1 mg/ml] in PBS; PBS is 0.04 M K_2HPO_4, 0.01 M K_2HPO_4, and 0.15 M NaCl) solution was applied to the cells on the slides, and the cells were incubated at room temperature for 5 min. The liquid was removed, and primary antibody was applied. The slides were incubated overnight in a sealed moist chamber at room temperature. They were then washed four times for 5 min each time with PBS-BSA. All subsequent procedures were performed in the dark. Secondary antibody was then applied, and the slides were incubated for 2 h at room temperature in a sealed moist chamber. The slides were washed four times for 5 min each time with PBS-BSA and then two times for 5 min each time with PBS. DAPI (1 μg/ml) was applied to the slides, and the cells were incubated for 5 min. After two more washings with PBS for 5 min each time, the slides were allowed to dry. Mounting medium (90%

glycerol, 10% PBS [pH 9.0], 1 mg of *p*-phenylenediamine per ml) was placed on the slides, and coverslips were applied and sealed with fingernail polish. Slides were stored in the dark at −20°C. Fluorescence microscopy was performed with a Zeiss Axioskop (Carl Zeiss Inc., Oberkochen, Germany), using filters for fluorescein, rhodamine, and UV detection. Pictures were taken with Kodak T-Max ASA 400 black-and-white film.

Electron microscopy. Cells were grown under the same growth conditions used for the preparation of the immunofluorescence slides (see above). Fixation, prestaining, and embedding of the cells were done according to a modification of the method described by Clark (8). The cells were collected by filtration, washed with 0.1 M potassium phosphate (pH 7), and then resuspended in the same buffer containing 4% paraformaldehyde–0.5% glutaraldehyde. The cells were allowed to fix for 1 h on ice and were then incubated for 1 h at room temperature. After completion of the primary fixation, the cells were pelleted by centrifugation, washed three times in 0.1 M potassium phosphate (pH 7), and then incubated in a buffer containing 20 mM Tris-HCl (pH 8), 5 mM Na_2EDTA, 25 mM dithiothreitol, and 1 M sorbitol for 10 min at 30°C. These cells were then washed once with 0.1 M potassium phosphate-citrate (pH 5.8)–1.2 M sorbitol. The cell walls were digested by suspending the cells at an A_{600} of 10 to 20 in 0.1 M potassium phosphate-citrate (pH 5.8)–1.2 M sorbitol to which 0.1 volume of β-glucuronidase (13,000 U/ml) and 0.1 volume of zymolyase 100T (5 mg/ml; ICN ImmunoBiologicals) had been added. This mixture was incubated at 30°C for 2 h. After the digestion, the cells were washed once in 0.1 M potassium phosphate (pH 7) and then suspended in 2% OsO_4 in 0.1 M potassium phosphate (pH 7) for 1 h at room temperature. The cells were washed with distilled water and then prestained with 2% aqueous uranyl acetate for 1 h at room temperature. The cells were washed again with distilled water and then dehydrated through an ethanol dehydration series in preparation for embedding in Poly/Bed 812 resin (Polyscience) (see reference 8 for details of the dehydration and embedding procedures). Sections of these cells were stained with 1% aqueous uranyl acetate and counterstained with Reynold's lead acetate stain. The stained sections were then viewed on a Philips 410 electron microscope.

Pulse-labeling of cellular RNA. Yeast strains (which were Ura^+) were grown in appropriate media lacking uridine. At selected times after addition of inhibitor or temperature shift, aliquots of cultures were incubated with [^{3}H]uridine (10 μCi/ml; 0.05 μg/ml). After 15 min, the labeling was stopped by the addition of 2 volumes of 10% trichloroacetic acid containing 5 mg of uridine per ml. The resulting precipitates were collected by filtration onto 0.45-μm-pore-size Millipore filters, air dried, and measured for ^{3}H radioactivity. The differential synthesis rate of rRNA was determined by RNA-DNA hybridization using a suitable rDNA probe as described previously (53).

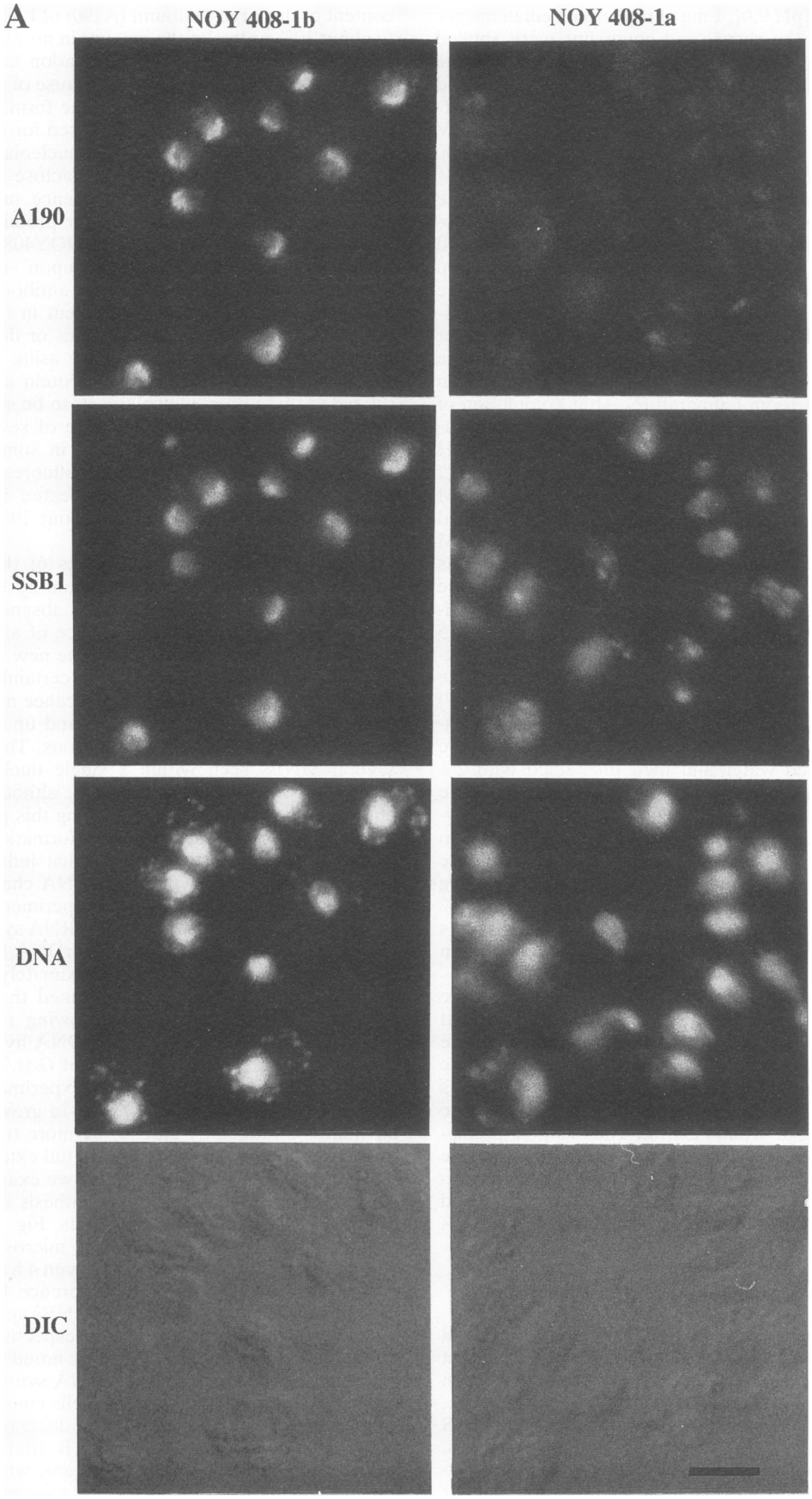
RESULTS

The nucleolar structure in Pol I deletion mutants growing in the absence of Pol I. Strain NOY408-1a does not have intact Pol I because the gene for the A135 subunit of Pol I has been deleted. This strain can grow in media containing galactose, since rRNA is synthesized by Pol II from the p*GAL7*-35S rDNA fusion gene. By using Western immunoblot analysis, it was previously shown that the A135 subunit is not detectable in crude extracts from this strain and that the cellular content of the largest subunit (A190) of Pol I is also reduced, to about 10% or less of the amount in normal control strains, presumably because of its degradation caused by the absence of the A135 subunit (27). Because of the importance of Pol I and/or rRNA synthesis for the formation and maintenance of nucleolar structure proposed for higher eukaryotic cells (3, 4), we were curious about nucleolar structure in this Pol I deletion strain growing in galactose media and examined the cells by immunofluorescence microscopy, using antibodies against known nucleolar proteins. As shown in Fig. 1, the control cells (strain NOY408-1b) showed the normal crescent-shaped structure upon staining with anti-A190, anti-SSB1, and antifibrillarin antibodies, while such a crescent-shaped structure was absent in the Pol I deletion cells. Instead, several small granules or dots (MNBs) were recognized by immunofluorescence using the anti-SSB1 or antifibrillarin antibody. The two protein antigens are colocalized in these dots, which appear to be scattered throughout the nucleoplasm. The presence of several MNBs in a single nucleus was observed, and in some cells up to 10 MNBs were present. The immunofluorescence seen with anti-A190 was very slight, as expected from the cellular content of A190, which is only about 10% or less of that present in the control cells.

Comparison of nuclear structures of the Pol I deletion strain with those of the control strain by thin-section electron microscopy also revealed the absence of the normal nucleolar structure and the presence of a new structure in the Pol I deletion strain (Fig. 2). The new structure (Fig. 2; NOY408-1a, arrows), which almost certainly corresponds to MNBs observed by immunofluorescence microscopy, has a diameter of about 0.2 to 0.5 μm, and up to three of them were recognized in some thin sections. The possibility that several MNBs seen within a single nucleus are actually contiguous has not been excluded, although we have not obtained any micrographs supporting this possibility.

One possible explanation for the formation of MNBs with such a large size is to postulate that individual snoRNPs interact with the many growing rRNA chains on an rRNA gene, as recently demonstrated experimentally for the U3 snoRNP (24). In NOY408-1a cells, rRNA synthesis is carried out by Pol II from the p*GAL7*-35S rDNA hybrid gene on a plasmid, which is present in approximately 20 to 50 copies per cell (27). Therefore, we examined the possibility that MNBs are present only when growing rRNA chains are transcribed from the p*GAL7*-35S rDNA hybrid genes.

It is known that the expression of *GAL7* is repressed by glucose (22, 48). In fact, previous experiments showed that the synthesis of rRNA in NOY408-1a growing in galactose medium is inhibited by glucose by more than 90% (27) and that the inhibition takes place to its full extent within 30 min after the addition of glucose. Thus, we examined the effects of glucose repression of rRNA synthesis on the pattern of MNBs in this strain. As shown in Fig. 3, MNBs were observed by immunofluorescence microscopy with anti-SSB1 and antifibrillarin antibodies even 4 h after the addition of glucose, and no significant difference from the control pattern was observed. Therefore, MNBs appear to represent a structural unit which can exist irrespective of the process of rRNA transcription. (It should be noted that even though a nearly complete inhibition of rRNA synthesis takes place quickly upon addition of glucose, cells continue to grow and divide for a considerable time. The decrease in growth rate becomes evident only at about 6 h after the addition of glucose. At 4 h after glucose addition, when cell numbers increased about twofold [both direct microscopic counts and


A
NOY 408-1b
NOY 408-1a
A190
SSB1
DNA
DIC

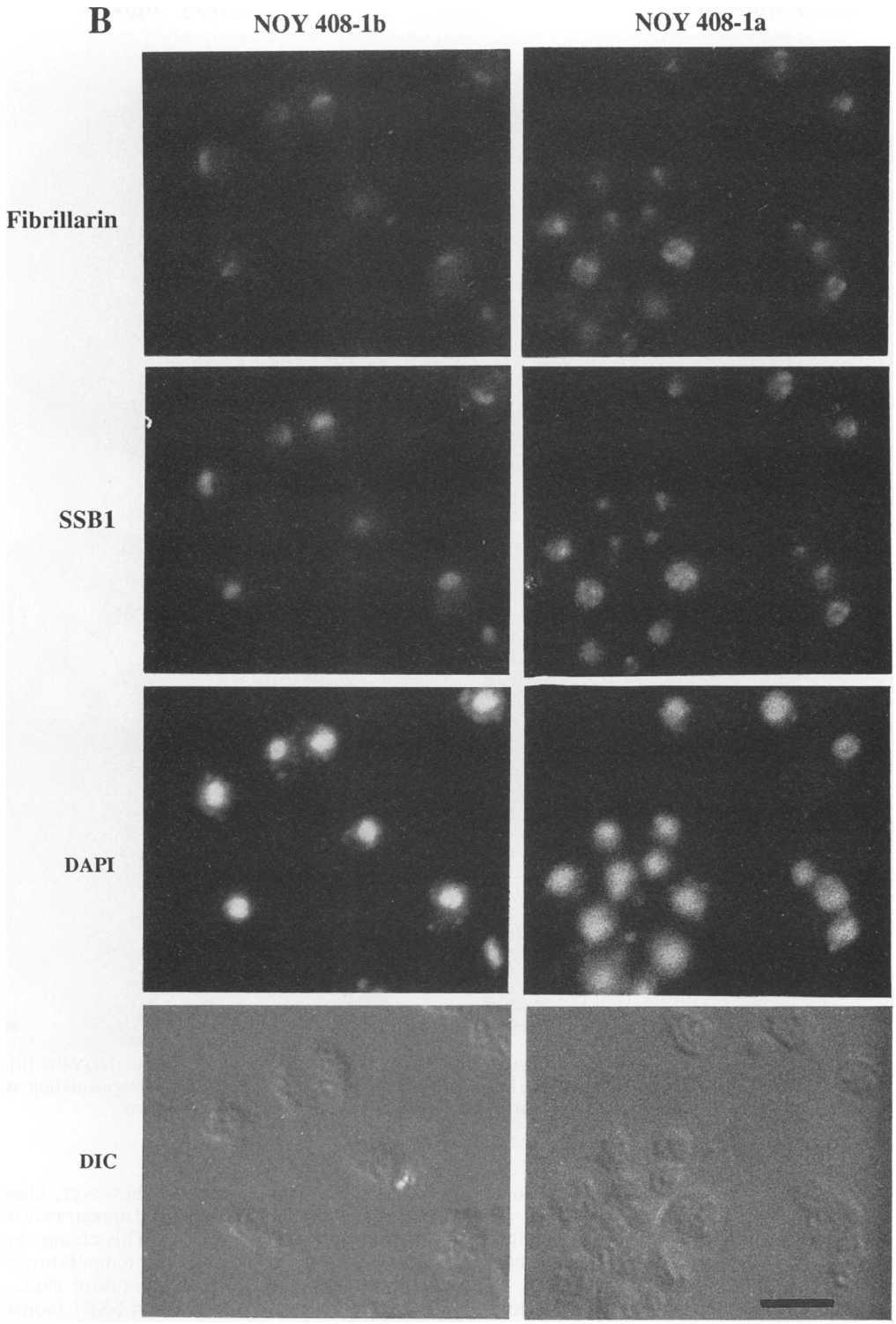


FIG. 1. (A) Double-label immunofluorescence of A190 and SSB1 comparing the Pol I deletion mutant (NOY408-1a) growing in galactose with the control strain (NOY408-1b). Yeast strains NOY408-1a and NOY408-1b were grown in SGal-Casamino Acids-Trp-Ade at 30°C to an A_{600} of between 0.1 and 0.3. Cells were prepared for immunofluorescence microscopy as described in Materials and Methods. They were stained with a 1:500 dilution of rabbit anti-yeast Pol I A190 subunit and a 1:1,000 dilution of mouse YN2Cl serum containing anti-SSB1. The anti-A190 staining was revealed by a 1:2,000 dilution of goat anti-rabbit IgG-FITC conjugate. The anti-SSB1 staining was revealed by a 1:200 dilution of goat anti-mouse-TRITC conjugate. DNA was stained with DAPI. The yeast cells were photographed with Nomarski differential interference contrast (DIC). (B) Double-label immunofluorescence of fibrillarin and SSB1. Growth conditions, preparation of cells for immunofluorescence microscopy, and SSB1 staining were as for panel A. Yeast cells were additionally stained with a 1:500 dilution of human scleroderma serum IgG containing antifibrillarin. Detection of human IgG was with a goat anti-human IgG-FITC conjugate. Bar, 5 μm.

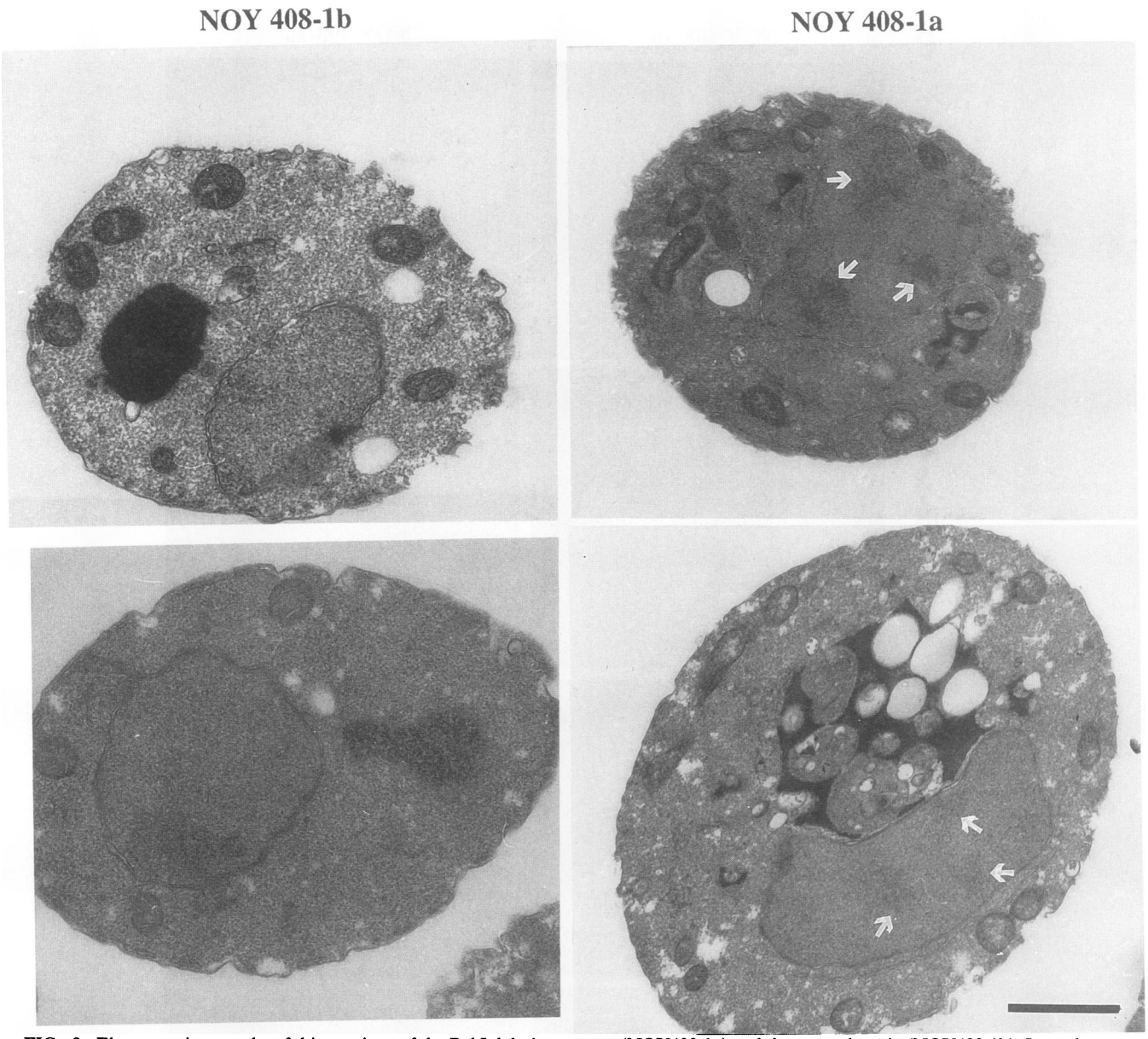


FIG. 2. Electron micrographs of thin sections of the Pol I deletion mutant (NOY408-1a) and the control strain (NOY408-1b). It can be seen that in the control strain, the electron-dense nucleolar regions are localized along the nuclear envelope without fragmentation, whereas several electron-dense bodies, presumable MNBs (indicated by white arrows), exist in the mutant nucleus. Bar, 1 μm.

viable cells counts; our unpublished experiments], we do not see any significant changes in the pattern of MNBs as mentioned above. Thus, it appears that neither the maintenance nor the formation of MNBs is affected by the absence of rRNA synthesis.)

Alterations of nucleolar structure in temperature-sensitive mutants with altered Pol I subunits. We previously isolated temperature-sensitive mutants that have alterations in the largest subunit (A190) of Pol I and display defects in the synthesis of rRNA at nonpermissive temperatures (53). We examined possible changes in the nucleolar structure at restrictive temperatures in one of these mutants, NOY259. As shown in Fig. 4, most of the mutant cells growing at a permissive temperature (23°C) had the crescent-shaped nucleolar structure similar to that of the wild-type cells. Upon a temperature shift-up to 37°C, however, disappearance of the crescent-shaped structure and appearance of a MNB-like pattern were observed (Fig. 4). This change took place in a majority of cells at 2 h after the temperature shift and was complete by 4 h after the temperature shift. Double-label immunofluorescence of A190 and SSB1 indicated the presence of both proteins in the same subnuclear structure, and we also call them MNBs.

In experiments similar to that shown in Fig. 4 (2-h sample shown), [^{3}H]uridine incorporation into total RNA was examined. The incorporation in the mutant was about 25% of that in the control strain at 4 h after the temperature shift (Table 2). The synthesis of rRNA in the mutant at 6.5 h after the shift was previously estimated to be about 10% of that of control cells, using RNA-DNA hybridization analysis of

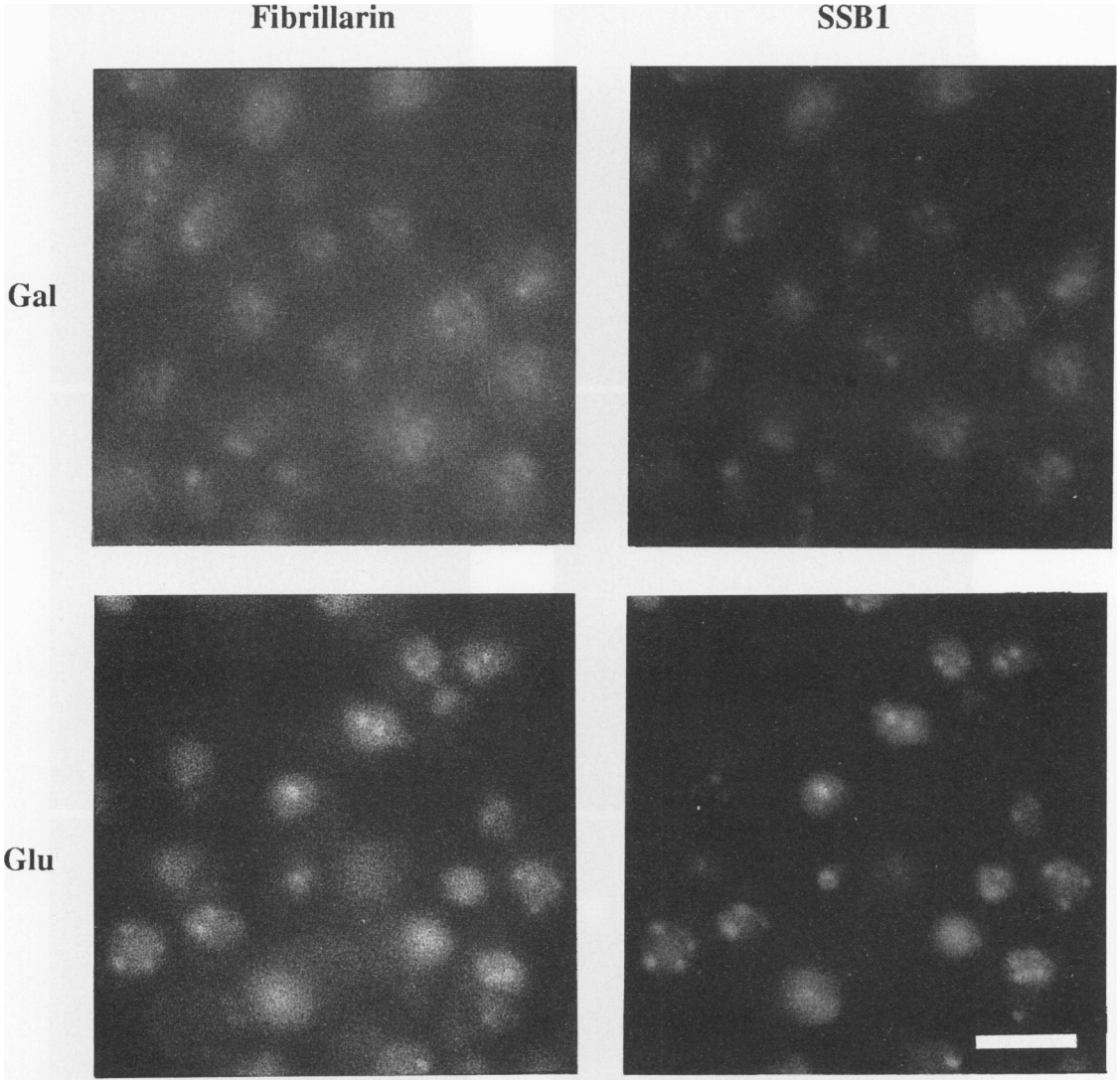


FIG. 3. Double-label immunofluorescence of fibrillarin and SSB1 comparing NOY408-1a cells grown in YEP-Gal with cells treated with excess glucose. NOY408-1a was grown at 30°C in YEP-Gal. At an A_{600} of 0.2, glucose was added to a final concentration of 2%. After 4 h, cells were prepared and stained for immunofluorescence microscopy as for Fig. 1B (Glu). Control cells (Gal) were those taken just before the addition of glucose. Bar, 5 μm.

pulse-labeled RNA (53) (another estimation method yielded the value of 3.6%, as shown in Table 2). Our interpretation of the results is that as a result of a large reduction of rRNA synthesis or a structural alteration of Pol I or both, the intact crescent structure disintegrates into substructures (MNBs), but inactive Pol I is associated with MNBs in this case.

Alterations of nucleolar structure under other conditions affecting rRNA synthesis. We carried out several experiments with normal yeast strains (with intact Pol I enzyme) to determine whether the conversion of the crescent structure to MNBs in the above-described Pol I mutants is due simply to the absence of transcription of chromosomal rRNA genes or to the absence of intact Pol I. First, we examined effects of amino acid starvation and a protein synthesis inhibitor, cycloheximide, both of which are known to inhibit rRNA synthesis (reviewed in reference 51; see also reference 25). For the former, we used 3-aminotriazole, which causes histidine starvation (55). We examined cells after treatment with 3-aminotriazole (10 mM) for 1 h and cells after treatment with cycloheximide (10 μg/ml) for 1 h. In parallel cultures, we confirmed that incorporation of [^{3}H]uridine into total RNA during a 15-min pulse-labeling period was inhibited greatly by these inhibitors (Table 2). The results of double-label immunofluorescence staining using anti-A190 and anti-SSB1 antibodies are shown in Fig. 5. As can be seen, these treatments did not cause fragmentation of the structure, as seen by staining with anti-SSB1; that is, no MNB-like pattern was observed under these conditions. (In Fig. 5A, it can be seen that the A190-stained region is clearly separated in a significant fraction of cells treated with cycloheximide. In other experiments, a similar pattern was observed in cells treated with 3-aminotriazole. This phenomenon is reminiscent of segration of nucleolar components observed for mammalian cells after treatment with actinomycin D [see reviews in references 5 and 43] and is discussed later.) 8-Hydroxyquinoline (20 μg/ml, 1 h), which was reported to inhibit RNA synthesis (21), caused a partial inhibition of [^{3}H]uridine incorporation into total RNA in the

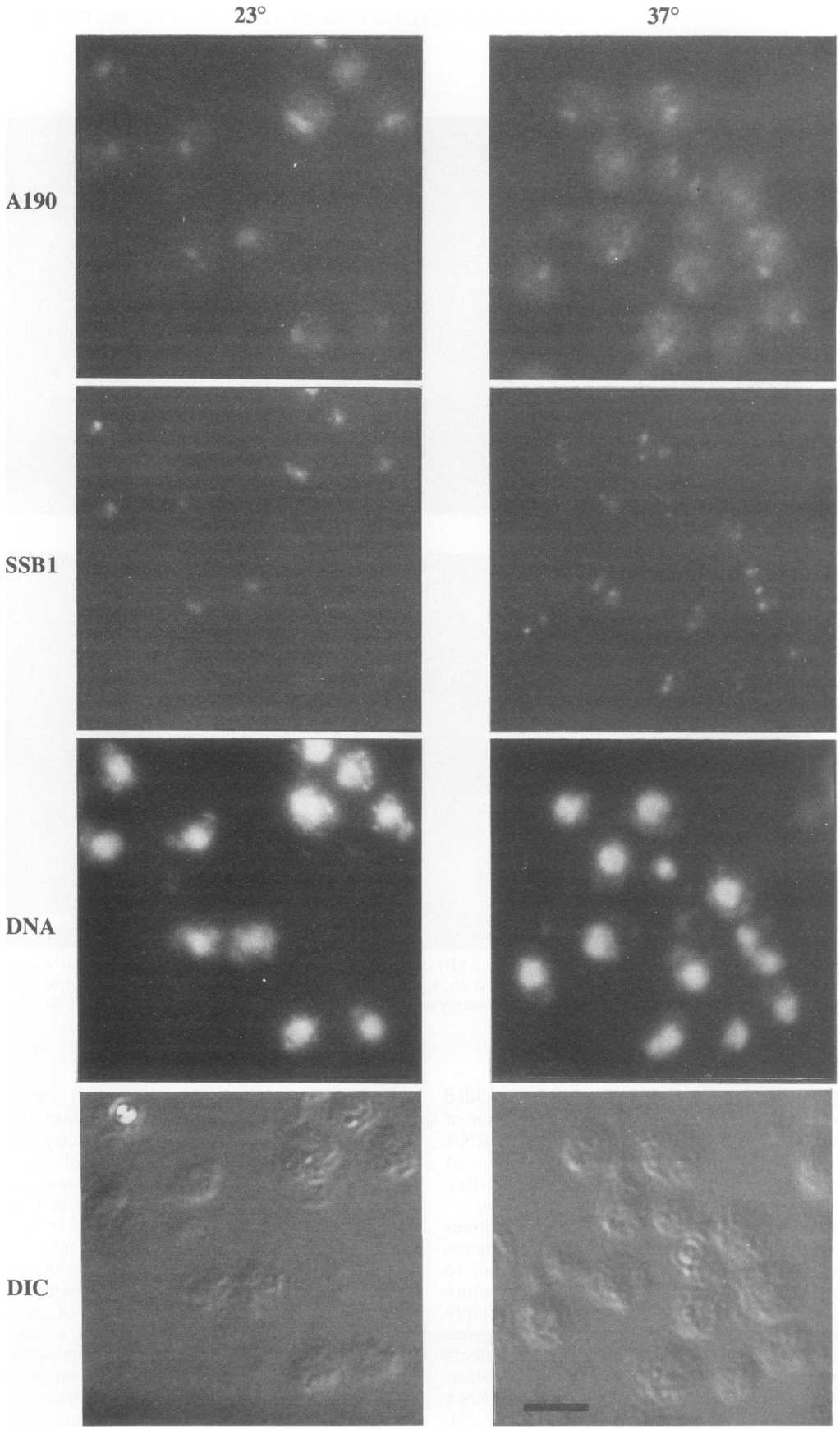


FIG. 4. Double-label immunofluorescence of A190 and SSB1 in a temperature-sensitive mutant of A190. NOY259 (*rpa190-1*) was grown in SD at 23°C to an A_{600} of 0.2. Half of the culture was shifted to 37°C, and the remaining half was kept at 23°C. At 2 h after the shift, cells from cultures at both temperatures were prepared and stained for immunofluorescence microscopy as for Fig. 1A. DIC, differential interference contrast. Bar, 5 μm.

TABLE 2. Rates of [^{3}H]uridine incorporation into RNA under conditions used to examine nucleolar structures

Inhibitor and conditions	[^{3}H]uridine incorporation (% of control) into[a]:		Nucleolus fragmentation (MNB formation)
	Total RNA	rRNA	
3-Aminotriazole (10 mM, 1 h)	5.6	4.5	−
Cycloheximide (10 μg/ml, 1 h)	20	4.2	−
8-Hydroxyquinoline (20 μg/ml, 1 h)	46	ND	−
rpa190-1 mutation			
37°C, 4 h	25	ND	+
37°C, 6.5 h[b]	19	3.6	+
prp20-1 mutation (37°C, 1 h)	9.1	ND	+
SRP1 depletion in NOY481[c] (12 h after shift to glucose)	17	9	+

[a] Cells were pulse-labeled with [^{3}H]uridine for 15 min, and aliquots were taken to determine the incorporation into total RNA. In some experiments, other aliquots were used to determine differential incorporation into rRNA (^{3}H in rRNA/^{3}H in total RNA), using RNA-DNA hybridization. For example, for 3-aminotriazole experiments, total incorporation was 5.6% of the control value, and differential incorporation into rRNA was 81% of the control value. Thus, relative incorporation into rRNA in 3-aminotriazole-treated cells was 4.5% (5.6% × 0.81) of the control value. In other experiments, measurements of differential incorporation into rRNA were not done (ND).

[b] The data were obtained previously (partly published in reference 53). In this case, the [^{3}H]uridine pulse was 10 min, and the incorporation into total RNA and that into rRNA (calculated from the hybridization data as explained above) were 19 and 3.6%, respectively, of the control value. In the present study, we measured [^{3}H]uridine incorporation at 4 h after the temperature shift-up.

[c] The data were taken from the experiments to be published elsewhere (57); see also reference 58 and Discussion. Using a strain in which the expression of SRP1 is under galactose control, incorporation of [^{3}H]uridine into RNA was measured by 10-min pulse-labeling at 12 h after the shift from galactose to glucose. The incorporation into total RNA and that into rRNA (calculated from the hybridization data) were 17 and 9%, respectively, of the value for the control strain (NOY477) in the glucose medium. It should be noted that the corresponding values were 66 and 57%, respectively, at 10 h after the shift to glucose, when about half of the cells showed the unfolded nucleolar structure and about half of the cells showed fragmented nucleolar structures. Conversion to MNB-like structures was complete by 12 h after the shift.

strain that we used but did not cause any significant alteration of the nucleolar structure, as judged by immunofluorescence staining with anti-A190 and anti-SSB1 (Fig. 5).

We also examined cells in a stationary phase created by carbon starvation. Cells were grown in a synthetic medium with a limited amount of glucose, and growth was monitored by measuring cell density. At about 20 h after the cells stopped growing because of the exhaustion of glucose, the nucleolar structure was examined by immunofluorescence microscopy. Although the region stained with anti-A190 or anti-SSB1 was somewhat smaller in size, no fragmentation was seen and both antibodies stained the same region (Fig. 5). Thus, inhibition of rRNA synthesis (and protein synthesis) by carbon starvation does not lead to conversion of the nucleolus to MNBs.

Alterations of nucleolar structure in the *prp20-1* mutant. The *prp20* mutant was originally isolated as a mutant accumulating unspliced mRNA precursors at restrictive temperatures (1). The gene responsible for the mutation, which turned out to be identical to the *SRM* gene (7), was cloned and identified as a homolog of a human gene, *RCC1*, implicated in the negative control of chromosome condensation (14, 30, 32). It was previously observed that the structure of the nucleolus is altered in the temperature-sensitive *prp20-1* mutant after a shift to a nonpermissive temperature (1). The change was noted by microscopic observation of cells stained with nucleolar-specific silver staining as well as electron microscopic observation of thin sections. We reexamined the alteration of the nucleolar structure after a temperature shift in this mutant, using immunofluorescence microscopy for comparison with the MNB pattern observed in the Pol I mutants described above. As can be seen in Fig. 6, double labeling with anti-A190 and anti-SSB1 showed that the nucleolus in the *prp20* mutant cells growing at 23°C was essentially normal, but fragmentation of the nucleolus took place in nearly all of the cells within 1 h after temperature shift to 37°C, and the pattern was very similar to the MNB pattern observed with the Pol I mutants.

DISCUSSION

Role of Pol I in the maintenance of nucleolar structure. Our studies on the Pol I deletion and Pol I temperature-sensitive mutants unambiguously demonstrate that the presence of intact Pol I is essential for the maintenance of an intact crescent nucleolar structure in yeast *S. cerevisiae*. However, not yet settled is the question of whether the requirement for intact Pol I for the maintenance of nucleolar structure is simply because of the requirement for transcription of rRNA genes at the nucleolar organizer or, alternatively, Pol I itself plays a role as a structural element for the maintenance of the nucleolar structure. If the former is the case, inhibition of rRNA synthesis would be expected to have the same consequence as the inactivation of or removal of Pol I, i.e., fragmentation of the nucleolus to form MNBs (in our system) or PNBs (in the previous higher eukaryotic systems). Our experiments using a variety of inhibitors and conditions to inhibit rRNA synthesis failed to confirm this prediction. Of course, these inhibitors and conditions cause inhibition of not only rRNA synthesis but also some other biochemical reactions, including protein synthesis to various extents, and it is possible that those other effects somehow prevent the expected fragmentation, as discussed by Benavente and coworkers in relation to their experiments using actinomycin D (3; see below). Nevertheless, these observations may also suggest that rRNA gene transcription is perhaps not the only function played by Pol I in maintenance of the intact nucleolar structure.

In contrast to the lack of fragmentation of the nucleolus with the various inhibitors and conditions as mentioned above, we have observed nucleolar fragmentation leading to the pattern similar to that of MNBs in two other genetic systems in *S. cerevisiae*. One is the temperature-sensitive *prp20-1* mutant (1), and the results are described in this report. The *PRP20* gene, which is also called *SRM1* (7), was cloned and identified as a homolog of a human gene, *RCC1*, implicated in the negative control of chromosome condensation (14, 30, 32). Although the exact nature of the gene product is unclear, one model proposes that *RCC1* plays a structural role in the decondensation of the chromosomes by holding the chromosomes in their interphase position (13). The yeast PRP20 protein, like the RCC1 protein (31), has the ability to bind (nonspecifically) to double-stranded DNA, and this ability has been shown to be defective in the *prp20-1* mutant (23a). The fragmentation of the nucleolus seen in the *prp20-1* mutant could result from the release of the multiple copies of rDNA from their proper location in the nucleus. The second system is a conditional deprivation of SRP1, the product of *SRP1*, the gene isolated as an allele-specific suppressor of temperature-sensitive *rpa190* mutations (26).

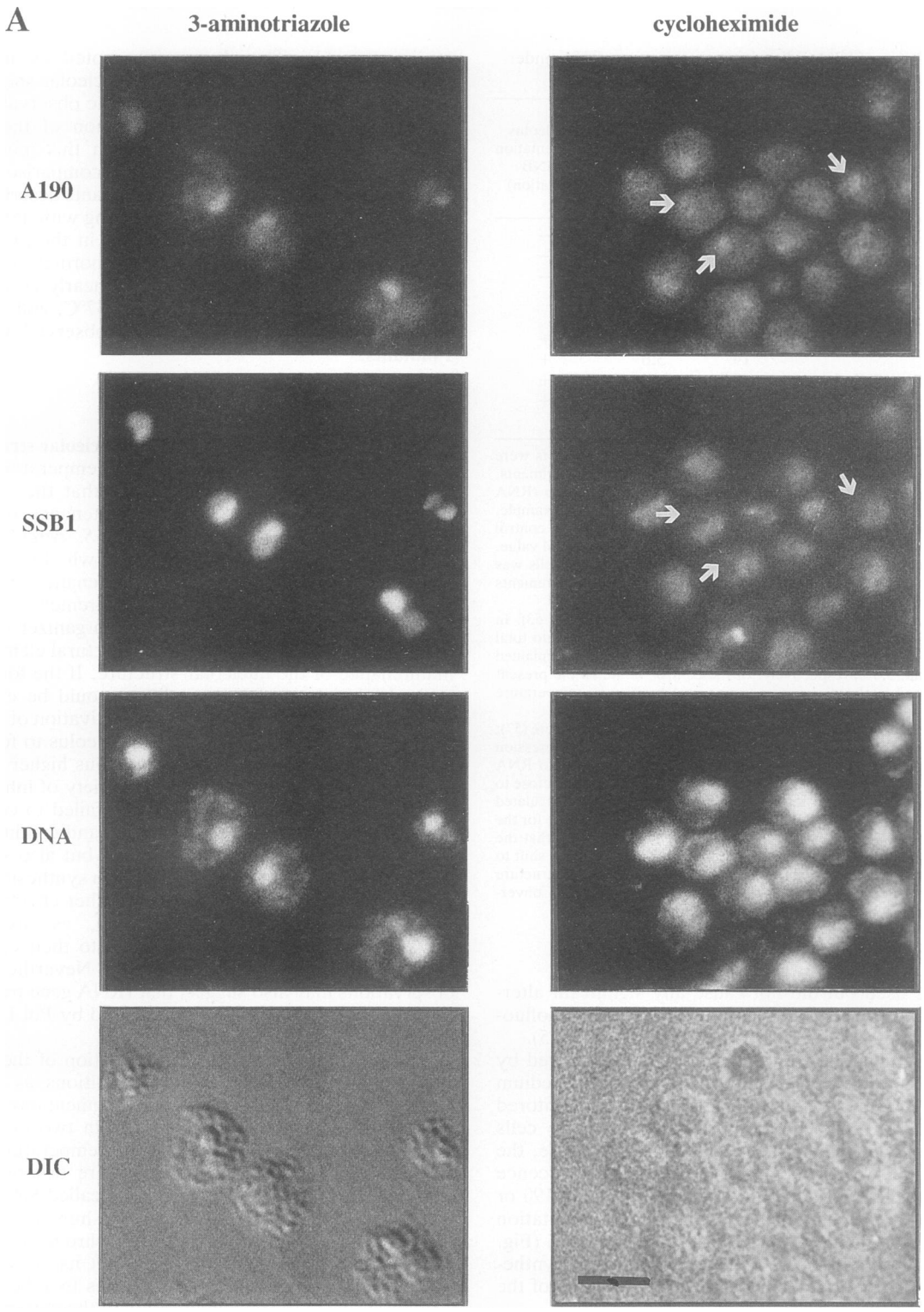
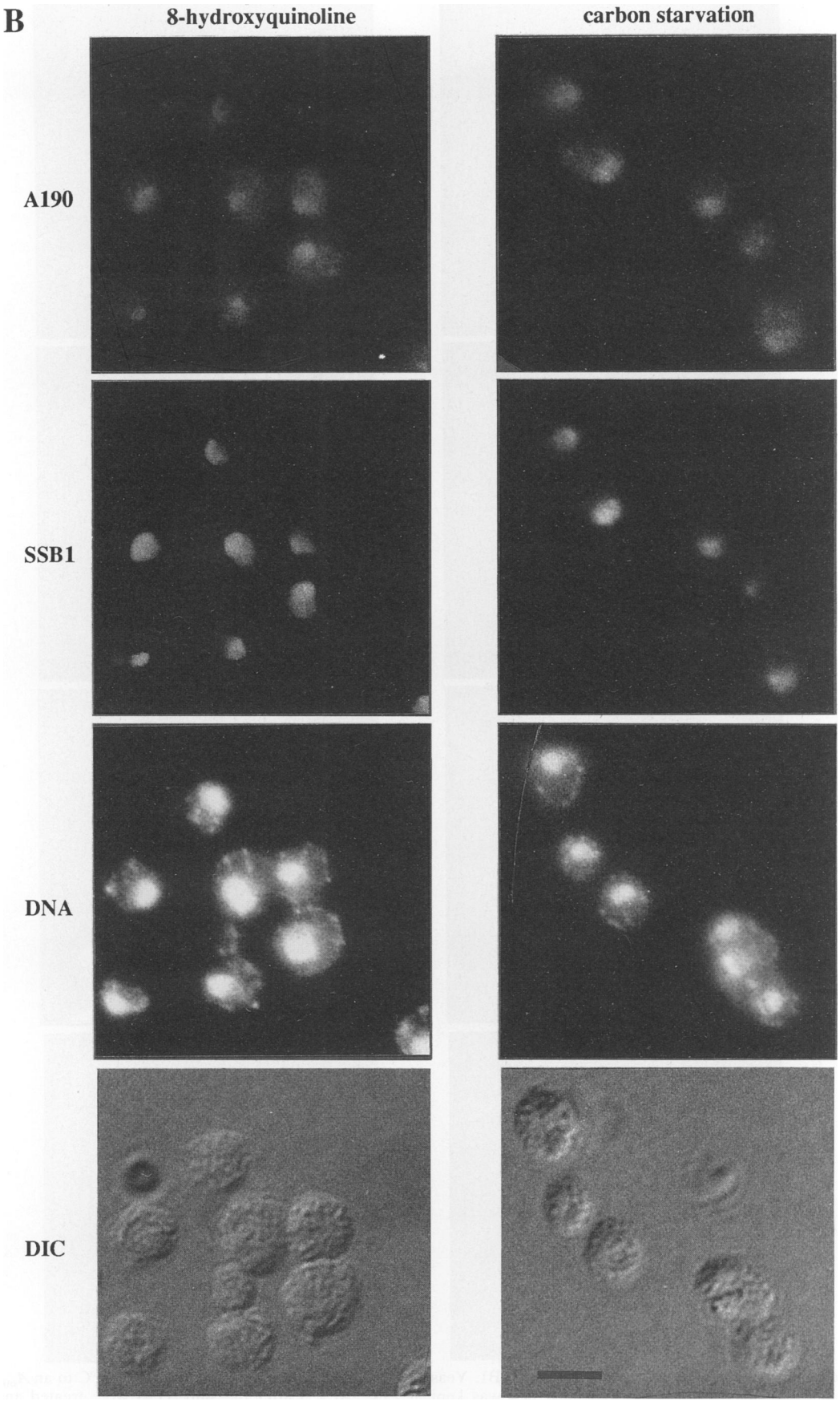


FIG. 5. Double-label immunofluorescence of yeast cells under conditions which affect RNA synthesis. (A) Treatments with 3-aminotriazole and cycloheximide; (B) treatment with 8-hydroxyquinoline and carbon starvation. NOY460 was grown in SGal at 30°C to an A_{600} of 0.2. 3-Aminotriazole or cycloheximide was added to half of the cultures to a final concentration of 10 mM or 10 μg/ml, respectively. After 1 h, cells were collected and prepared for immunofluorescence microscopy as for Fig. 1A. NOY260 was grown in SD-His-Leu-Trp at 23°C to an A_{600} of 0.2. 8-Hydroxyquinoline was added to half of the culture for a final concentration of 20 μg/ml. After 1 h, cells were collected and prepared for immunofluorescence microscopy as for Fig. 1A. NOY460 was grown in SD-Casamino Acids-Trp-Ade at 30°C to an A_{600} of 0.2. The cells were removed from the media by filtration and resuspended in the same media containing 0.02% glucose, and cell growth was monitored by measuring cell density of the culture. After 4 h, cell growth ceased, and after an additional 20 h of incubation (carbon starvation), the cells were prepared for immunofluorescence microscopy as for Fig. 1A. Arrows in panel A point to the regions stained with anti-A190 which are clearly displaced from the regions stained with anti-SSB1. These are shown as examples of nucleolar segregation (see the text). DIC, differential interference contrast. Bar, 5 μm.


B
8-hydroxyquinoline
carbon starvation
A190
SSB1
DNA
DIC

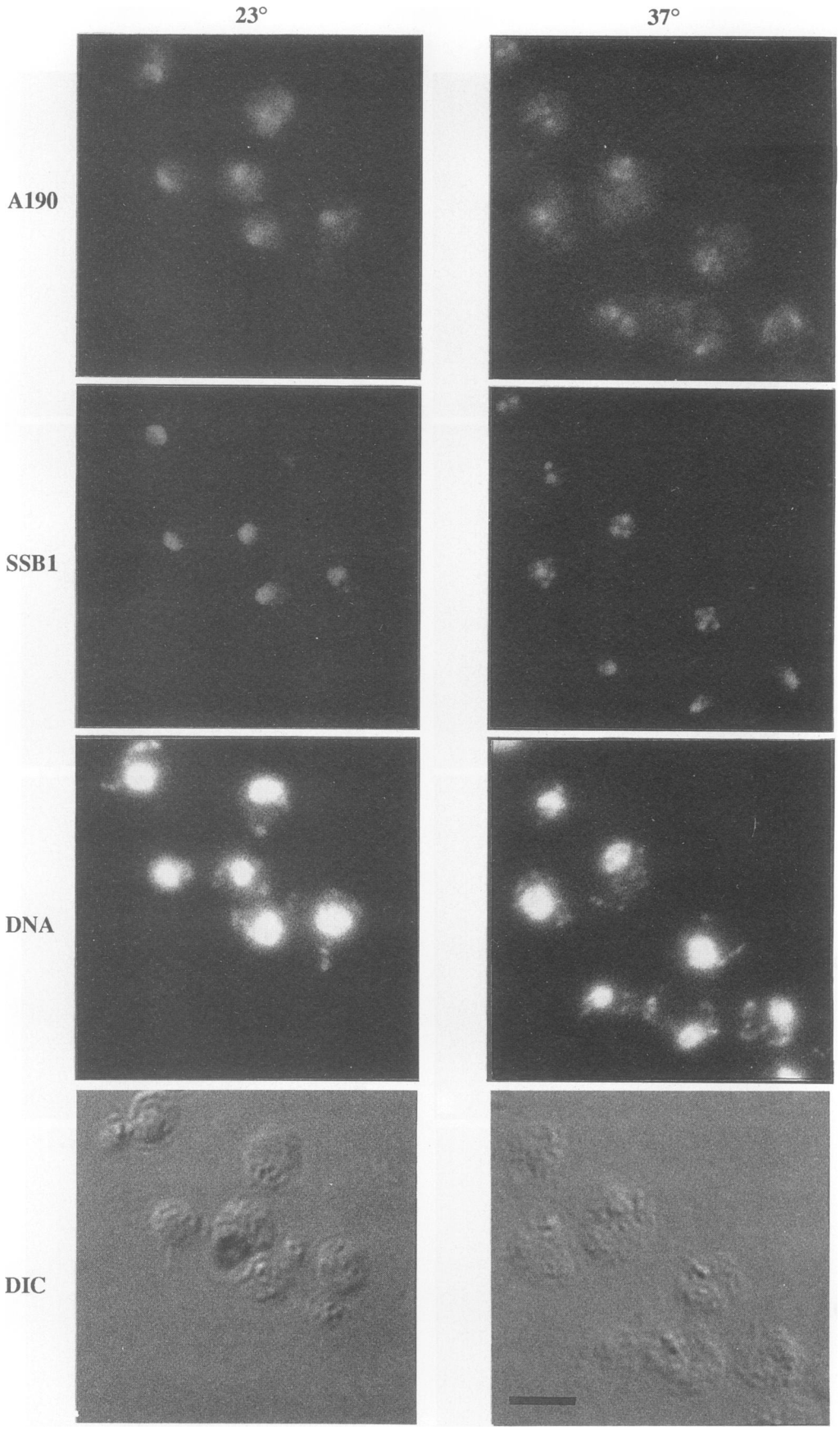


FIG. 6. Double-label immunofluorescence of A190 and SSB1. Yeast strain *prp20*/6A was grown in YEPD at 23°C to an A_{600} of 0.2. Half of the culture was shifted to 37°C, and the remaining half was kept at 23°C. At 1 h after the shift, cells were treated and stained for immunofluorescence microscopy as for Fig. 1A. DIC, differential interference contrast. Bar, 5 μm.

SRP1 was found to be a protein associated with the nuclear envelope/matrix (58). Using a yest strain in which the expression of *SRP1* was under control of the *GAL7* promoter, we have observed conversion of the crescent-shaped nucleolar structure into MNB-like bodies several hours after a transfer from galactose to glucose media (unpublished data cited in reference 58; for the effects of SRP1 depletion on RNA synthesis, see Table 2). Although the change in nucleolar morphology started to take place before the onset of any significant decline of growth rate or inhibition of rRNA synthesis, a causal relationship between rRNA synthesis inhibition measured biochemically and nucleolar structural alteration observed by immunofluorescence microscopy was difficult to establish with confidence. Nevertheless, it is possible that other nuclear proteins such as SRP1 also play an important role as a structural element for the maintenance of the nucleolar structure (58), and the role of Pol I, which interacts with SRP1 directly or indirectly through other components, might also include a role as a structural element in addition to (or instead of) a functional role to produce rRNA transcripts. In any event, future study should be undertaken to clarify this question.

Resemblance of the MNB to the PNB in higher eukaryotes. MNBs observed in the Pol I deletion mutants resemble PNBs observed in higher eukaryotic cells. In higher eukaryotes, the nucleolus disappears upon the onset of mitosis and then reappears at the end of mitosis. Re-formation of the nucleolus begins with aggregation of nucleolar material into several discrete structures called PNBs. These PNBs are subsequently fused together at the nucleolar organizer in telophase, forming the characteristic nucleolus structure as recognized in interphase (for a review, see reference 11). PNBs were shown to contain fibrillarin, nucleolin (also called C23), and other nucleolar proteins such as NO38 (also called B23) but apparently lack Pol I, topoisomerase, and rDNA (28, 37). By microinjecting anti-Pol I antibodies into cells undergoing mitosis, Benavente et al. (4) demonstrated that the antibodies inhibit conversion of PNBs to the nucleolus. The same inhibition was also observed by treatment of cells undergoing mitosis with an inhibitor of RNA synthesis, actinomycin D. Similarly, Benavente and coworkers (3) showed that microinjection of anti-Pol I antibodies into interphase cells causes breakdown of the nucleolus and appearance of many PNB-like aggregate structures. On the other hand, treatment of interphase cells with actinomycin D caused only separation of the DFC from the rest of nucleolar components, a phenomenon termed nucleolar segregation (for reviews, see references 5 and 43), but not formation of PNB-like structures (3). Other treatment that are known to inhibit rRNA synthesis, such as cycloheximide treatment, also caused nucleolar segregation but not formation of PNBs (see, e.g., reference 47). There is a striking correspondence between these observations and those in our studies on the yeast nucleolus. In our experiments, temperature shift-up of a temperature-sensitive Pol I mutant strain caused breakdown of the nucleolus and formation of MNBs, resembling the situation of anti-Pol I antibody injection into cultured mammalian cells in interphase. In contrast, other treatments with inhibitors such as 3-aminotriazole and cycloheximide did not cause formation of MNBs but quite often caused separation of the region stained with anti-SSB1 antibodies from that stained with anti-A190 antibodies, as mentioned in Results (Fig. 5 and legend). Both 3-aminotriazole and cycloheximide inhibit the synthesis of not only RNA but also protein, and the observed separation of the two nucleolar regions resembles nucleolar segregation observed after treatment of mammalian cells with actinomycin D or cycloheximide. The region occupied by SSB1 (and fibrillarin) and that occupied by Pol I in yeast cells in such instances might correspond to the DFC and FC in higher eukaryotes, respectively.

Both PNBs in higher eukaryotes and MNBs in yeast *S. cerevisiae* are discrete structural entities and exist in the absence of rRNA synthesis, that is, without association with growing rRNA chains. Both of them contain fibrillarin, which is known to be a component of U3 snoRNP (and several other snoRNPs) that function in rRNA processing. The interaction of fibrillarin containing snoRNP with growing rRNA chains is well established (24, 37) and was previously suggested to play a role in the coalescence of PNBs at the nucleolar organizers to form the nucleolus in telophase (37). This interaction does not appear to play any role in maintaining large PNB or MNB structures, although the absence of (nongrowing) rRNA or rRNA fragments in PNBs or MNBs is not experimentally demonstrated.

Possible functions of MNBs in Pol I deletion strains. We have found that in Pol I deletion strains synthesizing rRNA by Pol II from the *GAL7* promoter, the nucleolus does not have the normal crescent structure and yet the synthesis of rRNA and its processing and assembly into mature ribosomes take place. In these strains, we have found that several granules (MNBs) containing nucleolar proteins such as SSB1 and fibrillarin exist in the nucleus. These nuclear proteins have been shown to function in rRNA processing (10, 50). Since the subnuclear structure found in the Pol I deletion mutant contains SSB1 and fibrillarin, both of which are known to be complexed with several small nucleolar RNAs together with other protein components (10, 17, 39), we infer that this structure recognized as dots by immunofluorescence microscopy also contains these small nucleolar RNAs and associated nucleolar proteins. Thus, the normal nucleolar components found in MNBs may carry out nucleolar functions required for the growth in these deletion strains, such as rRNA processing and perhaps aiding ribosome assembly. However, we do not have any information regarding the efficiency of rRNA processing (or ribosome assembly) in the Pol I deletion strain missing the intact crescent-shaped nucleolus compared with the normal strains. The Pol I deletion cells do grow in galactose media, although the growth rate is reduced to about 40 to 50% of the rate of control strains with the intact nucleolus and Pol I (doubling time of 4 to 5 h, compared with 2 h for the control strain). The slow growth of the Pol I deletion strains may be caused by the insufficient transcription of rRNA genes from the p*GAL7*-35S rDNA or by the possible inefficient rRNA processing/ribosome assembly or both.

An important aspect of this study is the demonstration of MNBs in growing cells synthesizing ribosomes in the absence of the intact nucleolus. In previous studies in higher eukaryotic systems (and in our studies using temperature-sensitive mutants or conditions of SRP1 depletion), it was difficult to show the synthesis of rRNA and its assembly into functional ribosomes in the absence of the intact nucleolar structure. In the Pol I deletion system, rRNA is transcribed by Pol II from the p*GAL7*-35S rDNA hybrid gene on plasmids present in multiple copies in the nucleus. Several possibilities can be considered regarding the processing of rRNA transcripts and their assembly into mature ribosomes. First, the plasmid template is associated with MNBs, and both rRNA processing and ribosome assembly take place within the MNBs. The results of glucose repression experiments show that the pattern of MNBs does not change after

specific inhibition of rRNA synthesis, and the possibility is perhaps less likely that MNBs are associated with growing rRNA chains. However, we do not know the nuclear location of the plasmids (our attempts to localize the plasmids being unsuccessful so far), and we cannot exclude this possibility. Second, the plasmid templates are not associated with MNBs, and the rRNA transcripts are somehow transported to the MNBs and then processed within the MNBs, after which they are assembled into ribosomes either at the same place or outside the MNBs. Third, the plasmid templates are not associated with MNBs but are present in some other nuclear locations, for example at the nuclear envelope, and both rRNA processing and ribosome assembly take place at the site of rRNA transcription, i.e., outside the MNBs. In this case, MNBs represent structural entities containing many various kinds of snoRNP, but their function is to store (or assemble) these particles and other components, which shuttle between MNBs and the site of rRNA transcription. A similar possibility was considered for the role of large structures containing snRNP (snurposomes in oocytes and speckles in mitotic cells) with respect to the mRNA splicing process in higher eukaryotes (18, 45, 56). It should be pointed out that distinguishing among these possibilities is important in connection with the structure and function of the normal nucleolus in yeast as well as higher eukaryotic cells. For example, as mentioned in the introduction, we do not know whether all regions of the FC containing Pol I participate in rRNA transcription in the nucleolus in higher eukaryotes. In fact, the common interpretation of autoradiographic studies of cells pulse-labeled with [^{3}H]uridine is that the site of rRNA synthesis is probably at the periphery of the FC adjacent to DFC or at the DFC itself (see, e.g., reference 23). Similarly, it is not known whether the region called the DFC, which contains fibrillarin (36), is uniformly engaged in rRNA processing and ribosome assembly. Thus, further studies on the present Pol I deletion system as well as other defined mutant systems in *S. cerevisiae* should be useful for our understanding of the structure and function of the nucleolus in general.

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