Cytometry 43:110–116 (2001)

Correlation Between Silver-Stained Nucleolar Organizer Region Area and Cell Cycle Time

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Received 2 June 2000; Accepted 4 October 2000

Background: The relationship between the population doubling time and the quantity of silver-stained nucleolar organizer region (AgNOR) interphase proteins was studied in cell culture at three different temperatures used to modulate the cell cycle duration.
Methods: After MIB 1 and AgNOR combined staining, the quantity of AgNOR proteins was measured in cycling cells by image cytometry.
Results: Among the several parameters calculated, the AgNOR relative area showed a strong correlation with the changes of the population doubling time induced by different temperatures.
Conclusions: The results support the hypothesis that the cell cycle time and the size of the ribogenesis machinery are coregulated and that measurements of AgNORs can thus be used as a static evaluation of the cell cycle duration in arbitrary units. Cytometry 43:110–116, 2001.

Key terms: AgNORs; doubling time; MIB 1; Ki-67; MCF-7; image cytometry; cell cycle; SAMBA

The nucleolar organizer regions (NORs) are located at the secondary constriction of human metaphase acrocentric chromosomes 13, 14, 15, 21, and 22. During interphase, the NORs are located in the fibrillar centers surrounding dense fibrillar components of the nucleoli. Loops of ribosomal DNA are responsible for the transcription of the ribosomal RNAs (except 5S rRNA) where the enabling NOR proteins are located (RNA polymerase I, DNA topoisomerase I, B23 and C23 proteins, and fibrillarin). These proteins can be visualized using a silver-staining technique and are called AgNORs (1–3).

The quantity and distribution of interphase AgNORs have been used for differential diagnosis of malignant versus benign cells (4–7) and for cancer prognosis (8–15). Although the correlation between AgNORs and other so-called proliferation markers (MIB 1/Ki-67, PCNA, p53) remains debatable, most authors agreed that AgNOR size or number is related to the proliferation activity (16–18). Using different cell lines that differ by their respective doubling times (Td) in vitro, a linear relationship between interphase AgNOR size and cell Td was observed: the larger the interphase AgNORs, the shorter the cell population Td (16,19–22). Nevertheless, the question remains: Are such differences the result of biological differences between the cell lines used or are they the result of actual differences of the kinetics of respective cell populations? The aim of this study was to analyze the cell cycling time and the quantity of AgNORs in one and the same cell line where the cell cycle duration (CD) was modulated by temperature (23–25).

MATERIALS AND METHODS

Cell Culture

The MCF-7 cell line was used. MCF-7 cells were derived from a pleural metastasis of an estrogen-dependent human breast cancer. The cell line was maintained as a monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamin (200 mM), and streptomycin/penicillin (24 IU/ml) and added to amphotericin (250 μg/ml), insulin (0.2 U/ml), and nonessential amino acids (0.1 mM). All the cells were trypsinized regularly using 0.05% trypsin-EDTA. The culture medium was changed every 3 days.

Population Td and Cell CD

For assessment of cell growth, aliquots of 1×10^5 cells were grown in 66 flasks (26 cm^2). The flasks were placed in a humidified 5% CO_2 incubator at 37°C. After 1 day, one half was placed at 33°C in a humidified 5% CO_2 incubator, which was the time necessary for the cells to adhere. Another range was prepared: 45 flasks were placed at 35°C after 1 day at 37°C. The Td was determined for each aliquot by counting the cells in triplicate samples at reg-

Grant sponsor: "la ligue nationale contre le cancer, comité départemental de la Haute-Savoie."
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Table 1
Number of Analyzed Cells for Each Slide at 33°C, 35°C, and 37°C

33°C		35°C		37°C	
MIB 1-positive cells	Total cells	MIB 1-positive cells	Total cells	MIB 1-positive cells	Total cells
Slide 1					
306	351	190	217	338	377
Slide 2					
350	388	200	224	241	281
Slide 3					
295	335	240	271	318	358
Slide 4					
288	326	181	208	306	349

ular time intervals (24 h) according to the method described by Patterson (26). The valid Td was calculated from the polynomial curve using the polynomial equations.

The cell CD was calculated for each slide seeding using the following formula: t = culture time in hours, N_0 = number of cells at time 0, N_t = number of cells at time t, G = growth fraction , i.e., the proportion of cells in cycle:

$$CD = t.\ln (G + 1)/\ln (N_t/N_0)$$

The growth fraction was calculated using the cells quantified for AgNOR quantitation in the proliferating zone of each slide and not all the cells of the slides.

MIB 1 and AgNOR Staining

Parallel to the evaluation of the Td, aliquots of 3.3×10^3 cells per milliliter were seeded on Lab-Tek culture chamber slides (Nunc, Naperville, IL). Eight slides were placed immediately at 37°C, eight slides were placed at 35°C, and eight slides were placed at 33°C after 1 day, the time necessary for the cells to adhere. The slides were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) after 72, 120, 168, and 240 h at 33°C, and after 72, 120, 168, and 216 h at 35°C and 37°C.

MIB 1 Immunostaining

After quenching the endogenous alkaline phosphatase and blocking with normal goat serum, the MIB 1 monoclonal antibody (MIB 1, Immunotech, Marseille, France, reference 0505) at a 1:50 dilution in PBS 1% was added to cover the cells for 60 min at room temperature in a humidified chamber. The cells on the slide were incubated with the second antibody GAM-AP (Jackson, Immunotech, reference 115-056-062/0818) at a 1:30 dilution. The alkaline phosphatase reaction was developed for 20 min with revelation's kit TR/Naphtol AS-MX (Sigma, St. Qentin-Fallavier, France). The MIB 1 monoclonal antibody stained only the nuclei of cells known to be cycling (27). The MIB 1-positive cells were considered in cycle and the growth fraction was calculated as the number of MIB 1 positive cells/total number of quantified cells.

AgNOR Silver Staining

The reference AgNOR silver staining (2) was used but with modifications recommended by The International Workshop on Application of AgNOR in Pathology (1,28,29). The standard AgNOR staining solution—one volume of 2% gelatin in 1% formic acid and two volumes of 50% silver nitrate—was freshly prepared to cover the slides in a dark and humidified chamber at 37°C for 12 min. The cell nuclei were counterstained with methyl green (ICN Biomedicals, Aurora, OH).

AgNOR Measurements

The quantitative analysis of AgNORs was performed using the SAMBA 2005 image cytometry system (SAMBA Technologie, Meylan, France). The system consists of a Tri-CCD color camera (DXC-3000 P Sony) atop a light microscope (Axioscope, Zeiss, West Germany; magnification ×40) interfaced to a PC (Compact Deskpro XL 590). The following parameters were measured only in cycling cells (positive for MIB 1): NA = nuclear area (μm^2), AA = total area of AgNOR dots per nucleus (μm^2), RA = relative AgNOR area (AA/NA), DN = number of AgNOR dots per nucleus. At least 200 cells were analyzed per slide (Table 1).

A semiautomatic application, written in Sambic language using the dynamic library of the SAMBA system, was used to perform sampling and AA measurement. In order to extract NA and AA, their contours were isolated by selecting an appropriate grey-level threshold from the corresponding digitized image. The discrimination between the MIB 1-positive and the MIB 1-negative cells was based on the hue value measured in the nuclei. Figure 1 shows MIB 1 immunostaining, AgNOR silver staining, and methyl green counterstaining.

Statistical Analysis

Results obtained by image cytometry were assessed for statistical significance using Excel 97 SR-2 (Microsoft, Toulouse, France). The confidence interval (95% CI) measures the spread of a distribution around the mean. The correlation coefficient was calculated as the Pearson correlation coefficient, denoted *r*. If there is an exact linear relationship between two variables, the correlation is 1 or −1 depending on whether the variables are positively or neg-

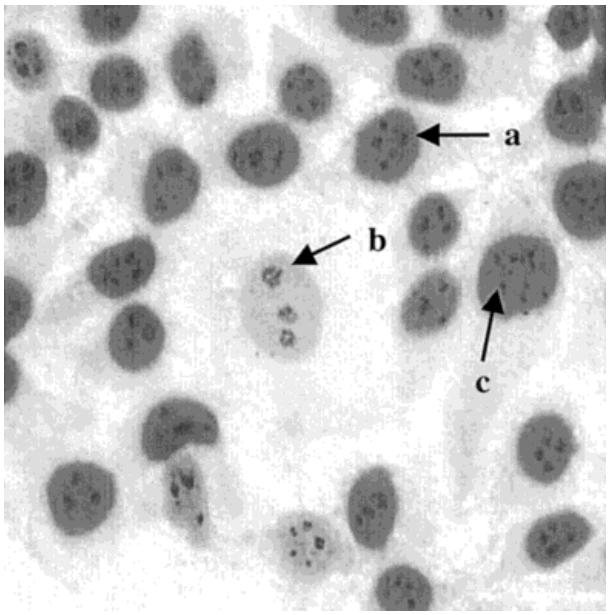

FIG. 1. Digital image of MCF-7 cells stained by MIB 1 immunostaining and AgNOR silver staining and counterstained with methyl green (×40). **a:** MIB 1-positive nucleus. **b:** MIB 1-negative nucleus counterstained with methyl green. **c:** AgNOR dots.

atively related. If there is no relationship, the correlation tends toward zero. The cell population respective Td were derived from a polynomial regression analysis using Excel 97 SR-2. The polynomial curve of degree 2 was tilted to the data points using least-squares regression for the three populations of cells (33°C, 35°C, and 37°C).

RESULTS

Td and CD of Cells Grown at 33°C, 35°C, and 37°C

Figure 2 shows the growth curves of the MCF-7 cells at 33°C, 35°C, and 37°C, respectively. These curves have the same profile although the growth of cells at 33°C was obviously slower compared with 35°C and at 35°C compared with 37°C. The Td was 40.4 h ± 2.02 at 33°C, 36.6 h ± 1.83 at 35°C, and 29.9 h ± 1.5 at 37°C. A 2°C decrease of the temperature thus increased the cell Td by a factor of 1.2 and a 4°C decrease increased the cell Td by a factor of 1.4.

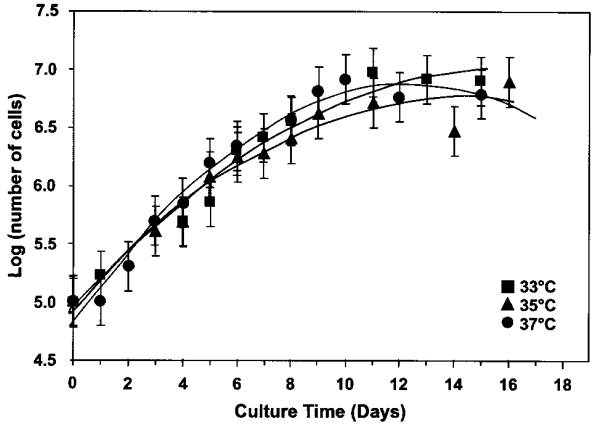

FIG. 2. Growth curves and SD of the MCF-7 cell line at 33°C, 35°C, and 37°C and regression curves for the calculation of the Td. y = -0.0088x2 + 0.515x + 4.9723, R^2 = 0.9496 at 33°C; y = -0.0086x2 + 0.2663x + 4.9227, R^2 = 0.9754 at 35°C; and y = -0.0134x2 + 0.3317x + 4.8112, R^2 = 0.9783 at 37°C.

Table 2
Growth Fractions and Derived Cell CD at 33°C, 35°C, and 37°C

Days	Growth fraction (%)	CD (h)
33°C		
3	87.18	31.21
5	90.21	34.53
7	88.06	37.06
10	88.34	43.36
Mean	88.45 ± 1.2	36.54 ± 1.73
35°C		
3	87.56	30.83
5	89.29	32.95
7	88.56	35.19
9	87.02	37.81
Mean	88.11 ± 1.0	34.20 ± 1.01
37°C		
3	89.66	29.18
5	85.77	28.56
7	88.83	31.41
9	87.68	34.51
Mean	87.68 ± 1.7	30.92 ± 0.91

The growth fractions and the derived cell cycle durations at 33°C, 35°C, and 37°C, respectively, are shown in Table 2. The growth fractions were similar at 33°C, 35°C, and 37°C, but the cell CD at 33°C was significantly higher when compared with 35°C and at 35°C compared with 37°C.

Evaluation of the AgNOR Parameters

The AgNOR parameters are reported in Table 3. The NA, AA, and RA decreased with the culture time according to a significant correlation as shown in Table 4.

AgNOR RA

The relationship between the AgNOR RA and the duration of culture is shown in Figure 3. During the 5 first days of culture at 33°C and 37°C, a similar decrease of the mean RA was observed. The decrease was greater during days 5 and 6. After 7 days for cells at 37°C and 33°C, the decrease of RA stabilized at a significantly lower value at 33°C compared with 37°C. For cells at 35°C, the decrease of RA is regularly compared with 33°C and 37°C.

For the culture temperature at 35°C, a similar decrease of the mean RA was observed during days 4 and 5, respectively. However, the linear trend curves at three temperatures show the same slope but the decrease of RA is higher at 35°C and 33°C than at 37°C.

AA

The relationship between the AA and the culture duration is shown in Figure 4. A similar decrease was observed

Table 3
Mean Values and 95% CI of the AgNOR Parameters Measured by Image Analysis

Days	NA (μm^2)	AA (μm^2)	RA (%)	DN
33°C				
3	95.20 ± 3.05	6.53 ± 0.42	6.91 ± 0.38	5.61 ± 0.24
5	109.48 ± 4.02	7.08 ± 0.68	6.46 ± 0.39	5.93 ± 0.26
7	97.17 ± 2.80	4.60 ± 0.34	4.92 ± 0.36	5.95 ± 0.23
10	94.05 ± 4.10	4.42 ± 0.33	4.88 ± 0.33	6.43 ± 0.38
Mean	98.97 ± 2.40	5.66 ± 0.45	5.79 ± 0.35	5.98 ± 0.11
35°C				
3	135.39 ± 4.62	9.81 ± 0.58	7.26 ± 0.33	9.79 ± 0.66
5	88.25 ± 3.82	5.36 ± 1.10	6.06 ± 0.94	5.75 ± 0.31
7	79.64 ± 3.92	4.26 ± 0.26	5.60 ± 0.32	5.69 ± 0.26
9	72.41 ± 3.35	3.74 ± 0.23	5.25 ± 0.27	5.56 ± 0.42
Mean	93.92 ± 9.58	5.79 ± 0.93	6.04 ± 0.30	5.70 ± 0.04
37°C				
3	106.67 ± 3.90	7.63 ± 0.42	7.35 ± 0.33	5.36 ± 0.25
5	100.22 ± 4.53	6.94 ± 0.52	7.05 ± 0.41	5.34 ± 0.32
7	107.02 ± 3.62	6.13 ± 0.43	5.88 ± 0.40	5.27 ± 0.32
9	93.16 ± 2.84	5.31 ± 0.36	5.90 ± 0.38	5.53 ± 0.27
Mean	101.77 ± 2.20	6.50 ± 0.34	6.54 ± 0.26	5.37 ± 0.04

at 33°C and 37°C after 4 culture days but the decrease was greater at 33°C. A decrease was observed for the culture at 35°C and was greater than at 33°C and 37°C.

Number of AgNOR DN

Figure 5 shows the increase of the number of AgNOR DN plotted against the culture duration. Because of poor intraobserver reproducibility due to the high subjectivity of the counting criteria of the AgNOR dots (30), the DN parameter was disregarded from further analysis although it showed a high correlation with the culture duration.

Figure 6 summarizes the mean values of the AgNOR parameters (AA, DN, and RA) according to temperature. A decrease was observed for the number of AgNOR DN with the increase of culture temperature. A significant increase was observed for the AA and the RA.

Relationship Between the cell CD and AgNOR Size

In order to evaluate whether the AgNOR absolute (AA) and relative (RA) areas were as high as the cell CD was long, the product CD × AA and CD × RA were calculated (Table 5). Both these values were not significantly differ-

Table 4
Correlation Coefficients (r) Between the AgNOR Morphometric Parameters and Culture Duration

Temperature	NA	DN	AA	RA	Culture time	CD	Td
33°C							
NA	1						
DN	−0.17	1					
AA	0.68	−0.67	1				
RA	0.40	−0.78	0.94	1			
t	−0.32	0.97	−0.83	−0.91	1		
CD	−0.32	0.98	−0.80	−0.87	0.99	1	
Td	−0.27	0.8	−0.89	−0.99	0.91	0.87	1
35°C							
NA	1						
DN	0.79	1					
AA	1.00	0.79	1				
RA	0.99	0.87	0.99	1			
t	−0.90	−0.97	−0.90	−0.96	1		
CD	−0.88	−0.98	−0.88	−0.94	1.00	1	
Td	−0.78	−1.00	−0.78	−0.87	0.97	0.98	1
37°C							
NA	1						
DN	−0.86	1					
AA	0.67	−0.51	1				
RA	0.35	−0.22	0.93	1			
t	−0.67	0.49	−1.00	−0.93	1		
CD	−0.63	0.68	−0.92	−0.85	0.90	1	
Td	−0.82	0.79	−0.93	−0.76	0.91	0.96	1

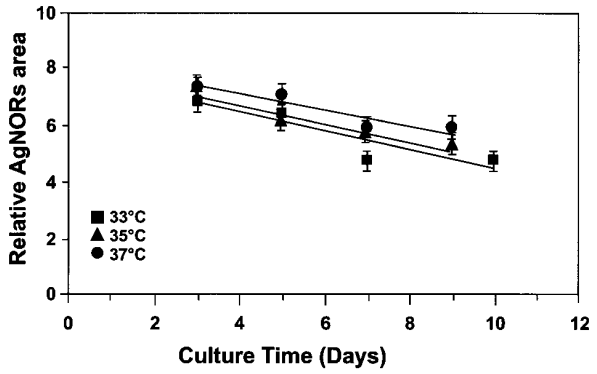

FIG. 3. AgNOR RA plotted against culture duration at 33°C, 35°C, and 37°C. The mean RA and the 95% CI were derived from three separate experiments: y = -0.3189x + 7.7865, R^2 = 0.8301 at 33°C; y = -0.3243x + 7.9894, R^2 = 0.9129 at 35°C; and y = -0.2771x + 8.2068, R^2 = 0.8679 at 37°C.

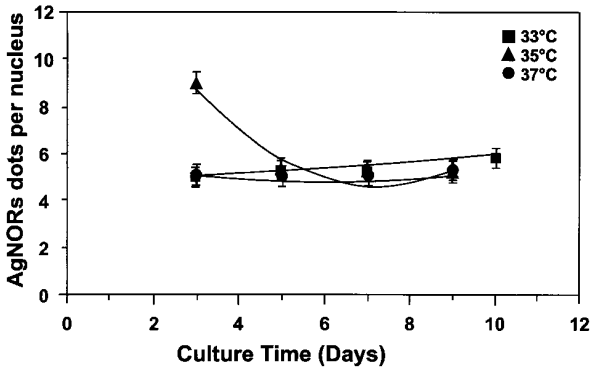

FIG. 5. Number of AgNOR DN plotted against culture duration at 33°C, 35°C, and 37°C. The mean area and CIs were derived from three separate experiments. The equations of the polynomial curves are y = 0.0038x2 + 0.0584x + 5.4412, R^2 = 0.9386 at 33°C; y = -0.0051x2 + 0.0223x + 5.7761, R^2 = 0.9939 at 35°C; y = 0.018x2 - 0.1949x + 5.8049, R^2 = 0.8154 at 37°C.

ent whatever the temperature. The longer the cell cycle, the smaller the AgNOR size.

DISCUSSION

Temperature Modulates Cell CD

The decrease of the temperature by 10°C in vivo, as analyzed in Amphibia (25), lengthened the cell cycle phases by a factor of two. In mammals, the temperature cannot vary from more than a few degrees around the optimal. However, in Amphibian cells, the CD was also reported to increase as the temperature decreased (23,24).

Our results showed that the Td of cells grown at 33°C and 35°C is significantly longer than at 37°C. This might result from either a smaller growth fraction or a longer cell cycle duration or both. Because we demonstrated that the growth fraction was similar at 33°C, 35°C, and 37°C, these results confirm the previous findings of Rao and Enngelberg (24) and Brugal (25) that the cell CD decreases as the temperature increases and that the growth fraction is independent from the temperature.

The mean cell CD, which could be derived from the cell CD at different time intervals during the culture, was 36.54 h ± 1.73 at 33°C, 34.20 h ± 1.01 at 35°C, and 30.92 h ± 0.91 at 37°C. This demonstrated that decreasing the temperature of MCF-7 cells in culture by 4°C increased the cell CD by a factor of 1.2 and decreasing it by 2°C by a factor of 1.1.

Td and AgNOR Size

The AgNOR RA decreased with the culture duration, the decrease being higher in cells grown at 33°C and 35°C compared with cells grown at 37°C. This decrease might result from either a decrease of the RA versus a constant NA or an increase of the NA versus a constant AgNOR RA, or both. Morphometric analysis showed that both the AgNOR size and the NA decreased, the decrease being sharper for the AgNOR size. The AgNOR RA stabilized for

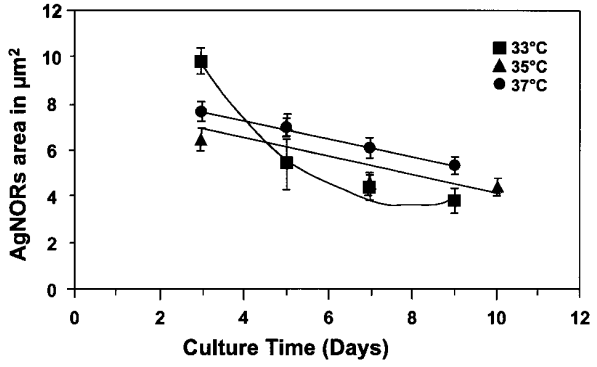

FIG. 4. AA plotted against culture duration at 33°C, 35°C, and 37°C. The mean area and the CIs were derived from three separate experiments. The equations of polynomial curves are y = -0.0014x2 - 0.3582x + 7.9584, R^2 = 0.6961 at 33°C; y = 0.2461x2 - 3.9194x + 19.219, R^2 = 0.9834 at 35°C; and y = -0.008x2 - 0.2931x + 8.5858, R^2 = 0.9998 at 37°C.

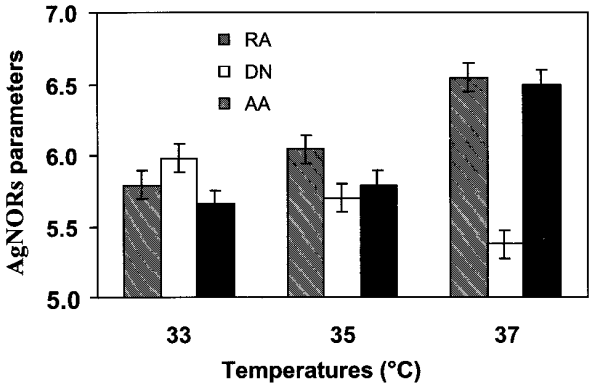

FIG. 6. Relationship between the mean AgNOR parameters (RA, DN, AA) and the culture temperatures. The mean area and CIs were derived from four experiments per temperature.

Table 5
Relation Between the Cell CD and AA and RA

Temperature	CD (h)	AA (μm^2)	RA (%)	CD × AA	CD × RA
33°C	36.54 ± 1.73	5.66 ± 0.45	5.79 ± 0.35	207	212
35°C	34.20 ± 1.01	5.79 ± 0.93	6.04 ± 0.30	198	207
37°C	30.92 ± 0.91	6.50 ± 0.34	6.54 ± 0.26	201	202

both temperatures (33°C and 37°C) after 7 days of culture and was about 1.12 higher at 37°C compared with 33°C. At 35°C, the AgNOR RA decreased regularly after 5 culture days compared with 37°C, but the AgNOR RA was about 1.08 higher at 37°C compared with 35°C.

These results are consistent with previous reports concerning the relation between the population Td and the quantity of AgNOR proteins (17–21,28,29,31) and which established that interphase AgNOR quantity was strictly related to the cell population growth speed: the faster the proliferation speed, the larger the interphase AgNORs. Trerè et al. (17) reported that a significant difference in the quantity of AgNORs could be found in cell lines for which the Td differed by at least 4 h. Using 13 neuroblastoma cell lines characterized by different Td, Derenzini et al. (20) found a decrease of AgNOR size by a factor of 1.5 as the Td increased by 20 h. Using established carcinoma cell lines, Öfner et al. (21) reported a decrease of AgNOR size by a factor of 1.47 as the Td increased by 10 h. The relation between an increase of the cell population Td and a decrease of the AgNOR size reported by several authors can be uniformly expressed in percentage and compared with our results (Table 6). The relations obtained by different authors on different cell lines and in various experimental conditions were between 82% and 121%. Interestingly, the average 100% relation would mean that AgNOR size is inversely proportional to the Td. The values reported in Table 6 are close to such a 100% ratio.

Cell CD

Regarding the biological meaning of the increase of AgNOR size parallel to the increase in temperature, the core relation with cell CD might be more straightforward than the relation with the Td, which depends on both the growth fraction and the cell CD. The product between AgNOR size and cell CD at 33°C, 35°C, and 37°C reported in Table 5 was not significantly different, thus showing that AgNOR × CD is constant whatever the temperature. In other words, AgNORs = 1/CD when expressed in arbitrary units. The hypothesis that the AgNOR size of proliferating cells is as small as the cell cycle is long is thus confirmed by our results. The definitive advantage of our approach is that this relationship was obtained in one and the same cell line for which the cell cycle time was experimentally modified instead of comparing different cell lines with different cell cycles where AgNOR size may differ on account of unexpected biological differences.

Table 6
Comparison of the Relation Between the Increase of the Cell Population Td and the Decrease of AgNOR Size Reported by Several Authors and Uniformly Expressed in Percentage

Td (h)	AgNOR size (au)	Ratio of Td × AgNORs
Derenzini et al. (20)		
22	18.1	82
42	11.5	
Öfner et al. (21)		
48	841.5	121
58	576.3	
Canet et al. (this paper)		
29.9	6.5	92
36.6	5.79	
29.9	6.5	85
40.4	5.66	

It is still questionable why the AgNOR size of proliferating cells is as small as the cell cycle is long. At least two types of mechanisms may account for this: (1) a temperature decrease would trigger a general slowing down of the cell metabolism, including ribogenesis and the synthesis of the proteins necessary for ribogenesis (numatrin, nucleolin, among others) and for the cell cycle (e.g., cyclin, DNA polymerase). In this case, the decrease of AgNORs (lower ribogenesis) and the lengthening of cycle (delayed availability of cyclins among others regulating proteins) would only be two parallel independent effects of the same cause. (2) A temperature decrease would trigger a decrease of ribogenesis. The critical size of the cell would then be reached later, thus delaying the progression of cells through the cycle. In this case, the lengthening of the cell cycle would directly result from a decrease of the AgNOR quantity.

The first mechanism is the most likely. Recent studies suggested that the ribogenesis and the overall cell cycle regulatory mechanisms are coregulated so that the nucleolus can provide the cells with the required number of ribosomes in order to optimize the overall energy consumption required for cell division. Among the possible regulatory factors are the intron derived small nucleolar ribonucleoproteins and the myc/max dependent transcription of the nucleolin.

CONCLUSION

Assessing interphase AgNOR quantity for cancer prognosis in clinical practice is very promising. It would be the only static marker of cell cycle time which, in combination with the Ki-67/MIB 1 antibody, would provide pathologists with a direct measurement of the two parameters responsible for cell proliferation in normal and malignant tissues. The remarkable correlation between the survival of cancer patients and the AgNOR content of their malig-

nant proliferating cells based on such an approach is a paradigm of tumor proliferation marker assessment in medical practice. This study provides experimental evidence supporting the correlation between the cell cycle time and the size of the ribogenesis factory assessed by the quantity of its specific proteins revealed by silver affinity.

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