NORs and Their Transcription Competence during the Cell Cycle

(ribosomal genes / NOR / UBF / silver staining / chromosomes / transcription)

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Abstract. In human cells ribosomal genes are organized as clusters, NORs, situated on the short arms of acrocentric chromosomes. It was found that essential components of the RNA polymerase I transcription machinery, including UBF, can be detected on some NORs, termed "competent" NORs, during mitosis. The competent NORs are believed to be transcriptionally active during interphase. However, since individual NORs were not observed in the cell nucleus, their interphase status remains unclear. To address this problem, we detected the competent NORs by two commonly used methods, UBF immunofluorescence and silver staining, and combined them with FISH for visualization of rDNA and/or specific chromosomes. We found that the numbers of competent NORs on specific chromosomes were largely conserved in the subsequent cell cycles, with certain NOR-bearing homologues displaying a very stable pattern of competence. Importantly, those and only those NORs that were loaded with UBF incorporated bromo-uridine in metaphase after stimulation with roscovitine and in telophase, suggesting that competent and only competent NORs contain ribosomal genes transcriptionally active during interphase. Applying premature chromosome condensation with calyculin A, we visualized individual NORs in interphase cells, and found the same pattern of competence as observed in the mitotic chromosomes.

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Abbreviations: AgNORs – silver-stained NORs, BrU – bromo-uridine, DAPI – 4',6-diamidino-2-phenylindole, NORs – nucleolus organizer regions, PCC – premature chromosome condensation, pol I – RNA polymerase I, SL 1 – promoter selectivity complex, UBF – upstream binding factor.

Ribosomal genes encoding 5.8S, 18S and 28S rRNA are organized in clusters that can be identified in mitotic chromosomes and are called nucleolus organizer regions (NORs). In normal human cells these regions are situated on the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22 (Henderson et al., 1972; Long and David, 1980; Puvion-Dutilleul et al., 1991). Several studies demonstrated that at least some subunits of RNA polymerase I (pol I) along with its main transcription factors, the upstream binding factor (UBF) and promoter selectivity complex (SL1), can be detected on certain NORs even in metaphase, although rDNA transcription is efficiently blocked from prophase to late anaphase (Babu and Verma, 1985; Weisenberger and Scheer, 1995; Jordan et al., 1996; Roussel et al., 1996; Gebrane-Younes et al., 1997; Sirri et al., 1999; Leung et al., 2004; Prieto and McStay, 2005). It is believed that such and only such NORs, termed "transcriptionally competent" or "competent" (Dousset et al., 2000; Savino et al., 2001), are transcribed, while the others, "non-competent" NORs, remain silent throughout the interphase (Weisenberger and Scheer, 1995; Roussel et al., 1996; Gebrane-Younes et al., 1997). The rule provides a simple explanation for the apparently constant number of competent NORs in cycling cells. It also agrees well with the supposed mechanisms of the rDNA silencing during mitosis (Gebrane-Younes et al., 1997). However, the hypothesis claiming identity of active and UBF-loaded NORs has not been directly proved, since individual NORs were not observed in the interphase cell nucleus.

To address this issue, we chose two cell lines of human origin: HeLa, a tumour-derived line with abnormal karyotype, and diploid LEP cells originating from embryo lung fibroblasts. First, we studied regularities

in distribution of the transcriptional competence among different chromosomes on mitotic spreads. Next, the distribution of transcriptional competence was compared with distribution of transcription signals on NORs in telophase cells, and also in metaphase cells after stimulation of rDNA transcription with roscovitine. Finally, employing premature chromosome condensation (PCC) with calyculin A, we analysed the pattern of NOR competence during the interphase. The results obtained in this work expand our knowledge on the fate of NORs in the cell cycle, and strongly argue that the presence of UBF on NORs (or their silver stainability) in mitosis serves as an indicator of their transcription activity in the previous interphase.

Material and Methods

Cell culture and preparation of the chromosomal spreads

HeLa and primary LEP cells were cultivated in flasks or on coverslips at 37°C in Dulbecco modified Eagle's medium (DMEM, Sigma, Saint Louis, MO) containing 10% foetal calf serum, 1% glutamine, 0.1% gentamycin, and 0.85g/l $NaHCO_3$ in atmosphere supplemented with 5% CO_2.

To prepare spreads of the mitotic chromosomes, the cells were cultured with 0.5 ng/ml colcemid (Sigma) for 1 h, hypotonically swollen in 0.075 M KCl for 10 min, harvested by mitotic shake-off and pelleted. The pellet was resuspended in a fixative (methanol and acetic acid, 3 : 1) and centrifuged (5 min at 400 *g*). After threefold repetition of this procedure, the cells were spread on chilled coverslips and dried.

PCC with calyculin A

Calyculin A, a potent and specific inhibitor of protein phosphatases PP1 and PP2A causes PCC through phosphorylation of histones H1 and/or H3 (Bezrookove et al., 2003, Tosuji et al., 2003, Bui et al., 2004). Cells were treated with calyculin A (AG Scientific, Inc., San Diego, CA), which was added into the medium to the final concentration 80 nM for 30, 60, 90 and 120 s at 37°C. The period of 60 s was chosen after the test experiments as optimal for further usage. Following the treatment, cells were either processed as for the chromosomal spread preparation, or fixed with methanol (30 min at -20°C) or 2% paraformaldehyde (10 min at room temperature). In some cases 20 μM BrdU (Sigma) were introduced 5 min before fixation. Replication signal was detected with monoclonal mouse anti-BrDU antibody (Roche, Indianapolis, IN).

Transcription labelling

Transcription sites were visualized by applying a method of hypotonic shift as described earlier by Koberna et al. (Koberna et al., 1999; 2000). In short, cells were washed in KHB (10 mM HEPES, pH 7.4, 30 mM KCl), incubated in 20 mM BrUTP in KHB at 37°C for 5 min, then in DMEM at 37°C for 10 min, and fixed in methanol. Transcription signals were visualized after methanol fixation using mouse antibody against BrdU (Roche).

Stimulation of transcription in metaphase cells with roscovitine

Roscovitine, being a highly selective inhibitor of Cdc2-cyclin B kinase, can stimulate transcription in mitotic cells apparently through dephosphorylation of the promoter selectivity factor SL1 (Sirri et al., 1999). In our work, colcemid-arrested mitotic HeLa cells were treated with 150 μM roscovitine (BIOMOL Research Laboratories, Plymouth, PA) for 30 min. After that, the transcription hypotonic assay was performed as described above.

In situ hybridization

Cy3- or FITC-labelled DNA probes for human chromosomes 13, 14, 15, 21 and 22 (Oncor, Gaithersburg, MD) were used for visualization of these chromosomes. The chromosomal spreads on coverslips were rinsed in 2x SSC, pH 7, incubated with 100 μg/ml RNAse A (Roche) for 1 h at 37°C, gradually dehydrated in ice-cold 70, 80 and 96% ethanol, and air-dried. The denaturation of the chromosomal DNA was performed in 70% deionized formamide in 2x SSC, pH 7, at 72°C for 3 min. The probe was denatured at 70°C for 10 min. Hybridization ran overnight at 37°C in moisture chamber. Spreads were washed 15 min in 50% formamide in 2x SSC, pH 7, at 43°C; 8 min in 0.1%Tween-20 in 2x SSC at 43°C; and 3x 4 min in 0.1% Igepal (ICN Biomedicals, Inc., Irvine, CA) in 4x SSC.

The biotin-labelled rDNA probe was prepared from a pA plasmid construct (Erickson et al., 1981), kindly donated by James Sylvester (Nemours Children's Clinic Research, Orlando, FL). This construct involved a spanning sequence for downstream 200 nucleotides of 18S rRNA, internal transcribed spacer 1, sequence for 5.8S rRNA, internal transcribed spacer 2 and upstream 4,500 nucleotides of 28S rRNA. The probe was labelled by nick translation using BIONICK Labeling System (GIBCO-BRD, Gaithersburg, MD). Denaturation of the chromosomal spreads, and washing after hybridization, were performed similarly as for the hybridization with chromosomal probes. The probe was denatured in deionized formamide at 70°C for 8 min and kept at 37°C for 30 min. Six μl of a hybridization mix containing 25 μg/ml of the probe, 0.5 mg/ml sonicated salmon sperm DNA, 2 mg/ml *Escherichia coli* tRNA, 70% deionized formamide, 2x SSC, and 10% dextran sulphate were used per each coverslip. The *in situ* hybridization was performed overnight at 37°C in moisture chamber.

Karotypes were studied by multicolour fluorescence *in situ* hybridization (M-FISH). It was performed on the metaphase chromosome spreads using "24*X*Cyte"

probe kit (MetaSystems™ GmbH, Altlussheim, Germany) containing combinatorially labelled painting probes for all autosomes and sex chromosomes of the human karyotype. Hybridization and post-hybridization washes followed standard procedures. Slides were counterstained using DAPI (4′,6-diamidino-2-phenylindole) and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Olympus AX 70 Provis (Olympus, Tokio, Japan) and the *ISIS* M-FISH imaging system (MetaSystems™) were used for image capturing and acquisition.

UBF immunocytochemistry

Fixed cells were rinsed in PBS. Non-specific antibody binding was blocked with 3% BSA in PBS for 30 min at 37°C. Primary antibody against human UBF (antigen NOR 90, kindly provided by U. Scheer, Biocenter of the University of Wurzburg) was applied for 1 h. Secondary anti-human antibodies (Jackson, West Grove, PA) were labelled with Cy3 or FITC. Coverslips were mounted in Mowiol and viewed using Olympus AX70 Provis and Leica TCS NT confocal microscope.

For the cells fixed in methanol/acetic acid, incubation with UBF antibody was performed in moisture chamber for 1 h at 37°C.

Silver staining of NORs

Chromosomal spreads (prepared as described above) were washed in deionized water, air-dried, treated with 2 : 1 v/v mixture of solution I (0.5 g silver nitrate in 1 ml deionized water) and solution II (2 g powdered gelatin in 10 ml deionized water with 1 ml pure formic acid) in dark wet chamber at room temperature for 45 min (after Howell and Black, 1980). The coverslips were then rinsed under running deionized water, and air-dried.

To combine silver impregnation and hybridization with chromosomal probes on the same chromosomes, the silver-stained and rinsed chromosomal spreads were dehydrated in 70%, 80% and 96% ethanol and denatured for the hybridization as above.

In some cases sequential silver staining and rDNA hybridization were performed. For that, the chromosomal spreads stained with $AgNO_3$ were photographed and then silver was removed with 7.5% potassium hexacyanoferrate III for 4 min, followed by 20% sodium thiosulphate for 5 min (Zurita et al., 1998). The spreads were then processed for the hybridization.

Results

Around 13 NORs are regularly revealed on mitotic spreads of HeLa cells

The karyotype of HeLa cells is abnormal but relatively stable (Chen, 1988). In the cell line we used, more than 95% of mitotic spreads contained from 79 to 89 chromosomes, and only such spreads were selected for further study. With respect to acrocentric chromosomes, we have regularly found trisomies of the chromosomes 13, 14, 21, 22 (one chromosome 22 also included material from chromosome 8), two to three chromosomes 15 and one metacentric marker containing the long arms of chromosome 15 (Figure 1), in agreement with the descriptions provided by other authors (see the comparative data in: Macville et al., 1999). After hybridization with the rDNA probe, around 13 signals (12.7 ± 0.5, counted in 100 cells) were observed on the spreads. Combining rDNA and the specific chromosomal probes, we established that the NORs belonged to acrocentric chromosomes 13, 14, 15, 21, 22 and one metacentric painted with the probe for chromosome 15 (Figure 2A, B, E, G, Table 1). All these chromosomes exhibited rDNA signal close to the centromeric region. The metacentric has been described earlier as a marker isochromosome composed of the large arms of chromosome 15 (Ghosh and Ghosh, 1975; Macville et al., 1999). However, the presence of rDNA shows that this marker also includes some material from the short arms of the chromosome 15, so this chromosome may be e.g. dicentric.

In more than 90% cells, NORs were found on all three chromosomes 14, three chromosomes 15, along with the metacentric marker, three chromosomes 22, but only one chromosome 13 and two chromosomes 21. The hybridization signals exhibited considerable chromosome-specific variation in integral intensity (data not shown). Two chromosomes 22 usually carried by far the most intensive signals on the spread (Figure 2 E, G). Since the intensity of rDNA hybridization signal correlates with the number of rDNA repeats (Leitch et al., 1992; Mellink et al., 1994; Suzuki et al., 1996), this result argues for the presence of numerous gene copies in the NORs of two chromosomes 22.

Thus, the rDNA clusters show a stable pattern of distribution among the chromosomes of HeLa cells.

Around nine competent NORs regularly appear on specific mitotic chromosomes in HeLa cells

We used immunocytochemical fluorescence detection of UBF to define the transcriptionally competent NORs on the mitotic spreads. The UBF mapping disclosed 6–12 signals per spread, with average value 8.6 ± 0.2, counted in 100 cells. The signals varied distinctly in shape, size and intensity. Three types were typically seen. The majority of signals (around 90%) belonged to double dots corresponding to the couples of sister chromatids. Elongated, “stick-like” signals (Heliot et al., 2000) appeared in all spreads, though in small numbers. Single dots pertaining to one of the chromatids were the rarest and were absent in some spreads.

Results of several studies showed that silver nitrate solution specifically stains pol I and UBF (Roussel and

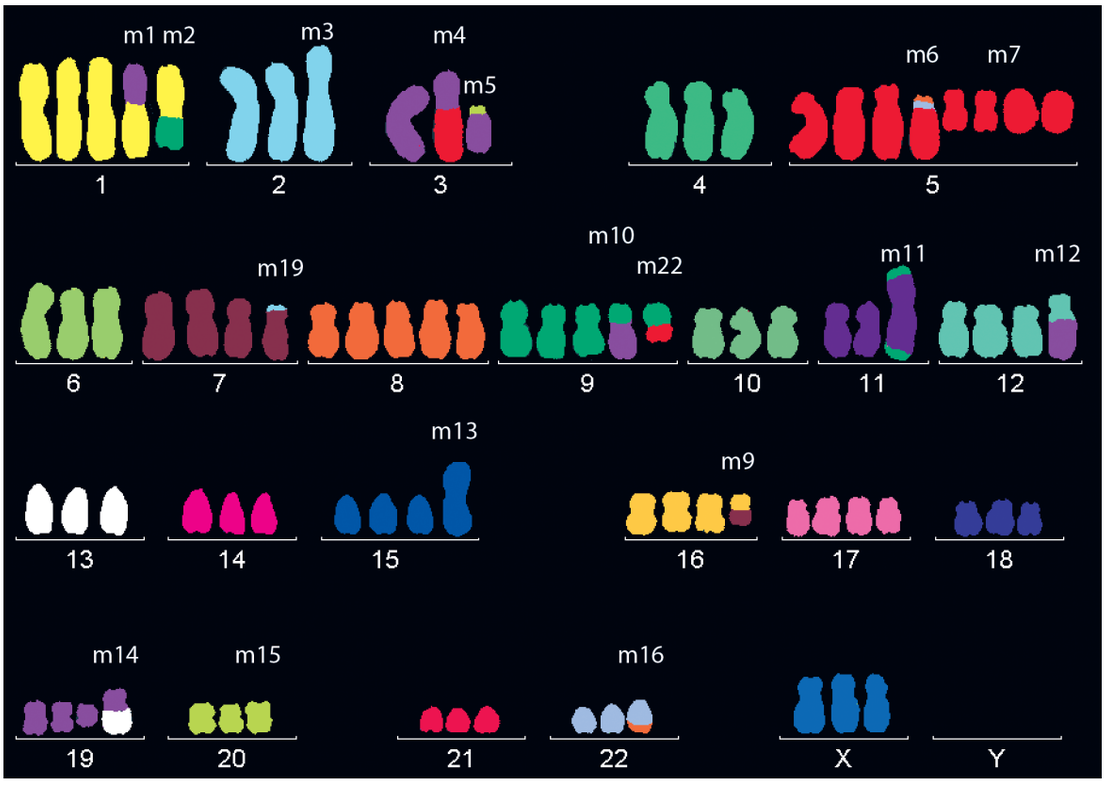

Fig. 1. Karyotype of the HeLa cells assessed by multicolour fluorescence *in situ* hybridization (M-FISH). There are trisomies of acrocentric chromosomes 13, 14, 15, 21, 22. One marker chromosome M13 appears as isochromosome 15; p-arms of the chromosome 13 are included into the marker M14 together with q-arms of the chromosome 19. One of the chromosomes 22 is defined as marker M16 since it includes some material from the chromosome 8.

Hernandez-Verdun, 1994; Roussel et al., 1996; Sirri et al., 2000). Thus, the silver impregnation became the most frequently used method for the labelling of the competent NORs on chromosomal spreads (Goodpasture and Bloom, 1975; Rufas et al., 1982; Ferraro and Prantera, 1988; Smetana and Likovsky, 1982; Smetana et al., 1999; Zurita et al., 1999). We accordingly used the silver staining as an alternative method for revealing competent NORs. In all cases, this procedure afforded statistically identical results (considering the number of signals and incidence of the morphological patterns) as UBF immunostaining, and could be more efficiently coupled with DNA hybridization than UBF labelling. We therefore employed the method of silver impregnation for the following study of NORs on individual chromosomes.

In the hybridization assays with chromosomal probes combined with silver staining (at least 100 cells were studied for each case), the silver signals usually appeared on one chromosome 13, one chromosome 14,

Table 1. NORs and AgNORs on individual chromosomes of the HeLa cells. Mean values and standard errors are indicated for the measured number of chromosomes, NORs and AgNORs. In each measurement, 100 cells were analysed

Chromosome	Number of chromosomes	Number of NORs	Mode number of NORs	Number of AgNORs
13	2.9 ± 0.1	1.1 ± 0.1	1	0.9 ± 0.1
14	3.0 ± 0.0	3.0 ± 0.0	3	0.9 ± 0.1
15	3.8 ± 0.1	3.8 ± 0.1	4	4.0 ± 0.1
21	2.7 ± 0.1	1.9 ± 0.2	2	1.8 ± 0.1
22	2.9 ± 0.1	2.9 ± 0.1[1]	3	2.0 ± 0.2
Total	15.3 ± 0.4	12.7 ± 0.5	13	9.5 ± 0.4[2]

[1] Two of these NORs exhibited the most intensive rDNA signals in the cell.

[2] The silver signals were calculated only in the spreads with the mode number of chromosomes, so the sum of the partial mean values exceeds the observed average number of the silver signals per cell.

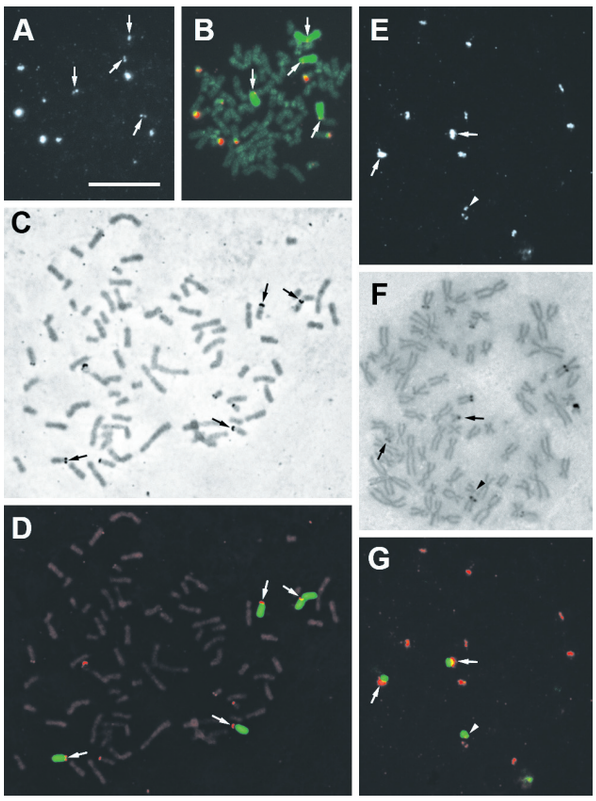

Fig. 2. Localization of NORs and competent NORs on the spread mitotic chromosomes of HeLa cells

A, **B**: simultaneous rDNA (**A**, red in **B**) and chromosome 15 detection (green in **B**). Thirteen rDNA signals are clearly recognized in A. Three acrocentrics and one metacentric chromosome are labelled with a chromosome 15-specific probe (**B**).

C, D: silver staining (**C**, red in **B**) combined with the probe for chromosome 15 (green in **D**). Large silver signals (arrows) are seen on all four painted chromosomes. Compare with the low intensity rDNA signals in **A** and **B**.

E, F, G: detection of rDNA (**E;** red in **G**), silver staining (**F**) and chromosome 22 (green in **G**). Two very large rDNA signals (arrows in **E**, **G**) correspond to relatively small silver signals (arrows in **F**, **G**). The third rDNA signal belonging to a smaller painted chromosome (arrowheads in **E**, **G**) is less intensive and corresponds to a silver-negative NOR (arrowhead in **F**, **G**). Bar: 20 mm.

all chromosomes 15 (3 acrocentrics + metacentric marker), two chromosomes 21, and two chromosomes 22 (Figure 2C, D, F, G). Interestingly, Sirri et al. (1999) observed, as can be assessed from their published figures, six competent NORs in HeLa cells, localized exclusively on the acrocentric chromosomes. In contrast to these data, the HeLa cell line used in our experiments possessed around nine silver or UBF-positive NORs, one of them belonging to the metacentric marker.

To combine silver staining and rDNA hybridization signals on the same spreads, we first carried out silver staining and took pictures. Then, silver was dissolved and hybridization was performed, according to Zurita et al. (1999). In these assays the silver signals always pertained to the regions of ribosomal genes, which rules out the possibility of "false" NORs (Dobigny et al., 2002). To define the competence status of two enlarged NORs in the chromosomes 22, painting of this chromosome was performed simultaneously with rDNA after silver staining. We found that the smaller NOR of the chromosome 22 was usually silver-negative (Figure 2E-G). This chromosome also displayed shorter arms after hybridization with chromosome 22-specific probe. The karyotype analysis suggested that the telomeric parts of its p-arms were replaced by the fragments of the chromosome 8 (Figure 1).

Table 2. NORs and AgNORs on individual chromosomes of the LEP cells. Mean values and standard errors are indicated for the number of AgNORs. In each measurement, 100 cells were analysed

Chromosome	Number of chromosomes	Number of NORs	Number of AgNORs
13	2	2	1.0 ± 0.1
14	2	2	1.7 ± 0.1
15	2	2	1.9 ± 0.1
21	2	2	1.6 ± 0.1
22	2	2	1.6 ± 0.1
Total	10	10	7.8 ± 0.5

The results obtained on mitotic chromosomal spreads are summarized in Table 1 and Figure 4. These data show a high regularity in the number and intensity of the UBF/silver signals on specific chromosomes in HeLa cells. There is a significant discrepancy between the pattern of rDNA distribution and the pattern of NOR competence: even when rDNA and UBF/silver signals coincide on the same chromosome, their intensities do not correlate.

The competent NORs are regularly distributed among the chromosomes of the diploid LEP cells

The data obtained on the transformed aneuploid cell line were complemented by a similar study on the diploid LEP cells (Table 2). After multicolour painting of the chromosomes in the LEP cells we confirmed that these cells had a normal diploid karyotype (data not shown).

Using rDNA hybridization we observed NORs on all chromosomes 13, 14, 15, 21 and 22 (Figure 3A, B). In contrast to HeLa cells, no striking difference in intensity of the hybridization signals was observed. Six to ten of these NORs (most frequently 8, average value 7.8 ± 0.1; counted in 100 cells) were silver- or UBF-positive. At least one chromosome in each pair usually carried Ag-positive NOR. In 95–97% of the spreads, both copies of the chromosome 15 were silver-positive, and one of the chromosomes 13 was negative (Figure 3C, D). Chromosomes 14 were both positive on 85% spreads. Chromosomes 21 and 22 carried competent

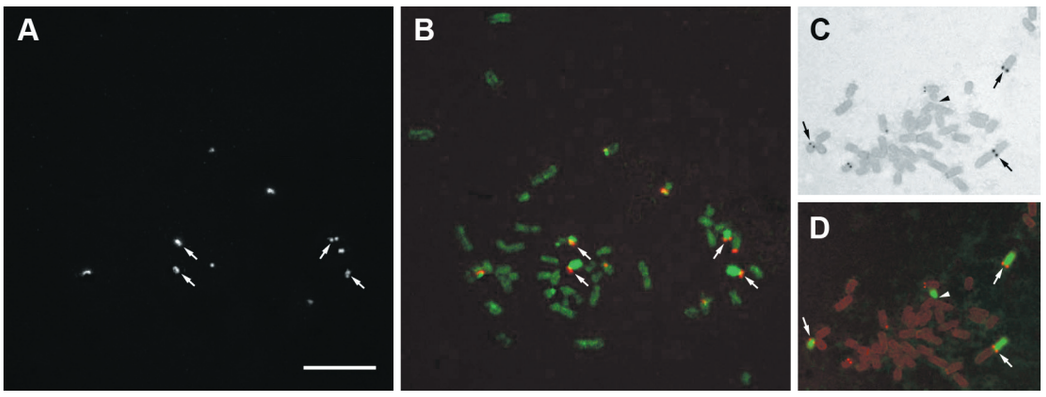

Fig. 3. Localization of NORs and competent NORs on the spread mitotic chromosomes of LEP cells.

A, B: Simultaneous detection of rDNA (**A**, red in **B**), chromosome 13 (**B**, large acrocentrics painted in green) and chromosome 22 (small acrocentrics in green); rDNA signals (arrows) are seen on all four painted chromosomes. Ten NORs can be easily recognized in **A.**

C, D: silver staining (red) combined with the probes for chromosomes 15 (**D**, large acrocentrics painted in green) and 21 (small acrocentrics in green). Silver signals (arrows) are seen on all the painted chromosomes. Seven silver signals are visible in C.

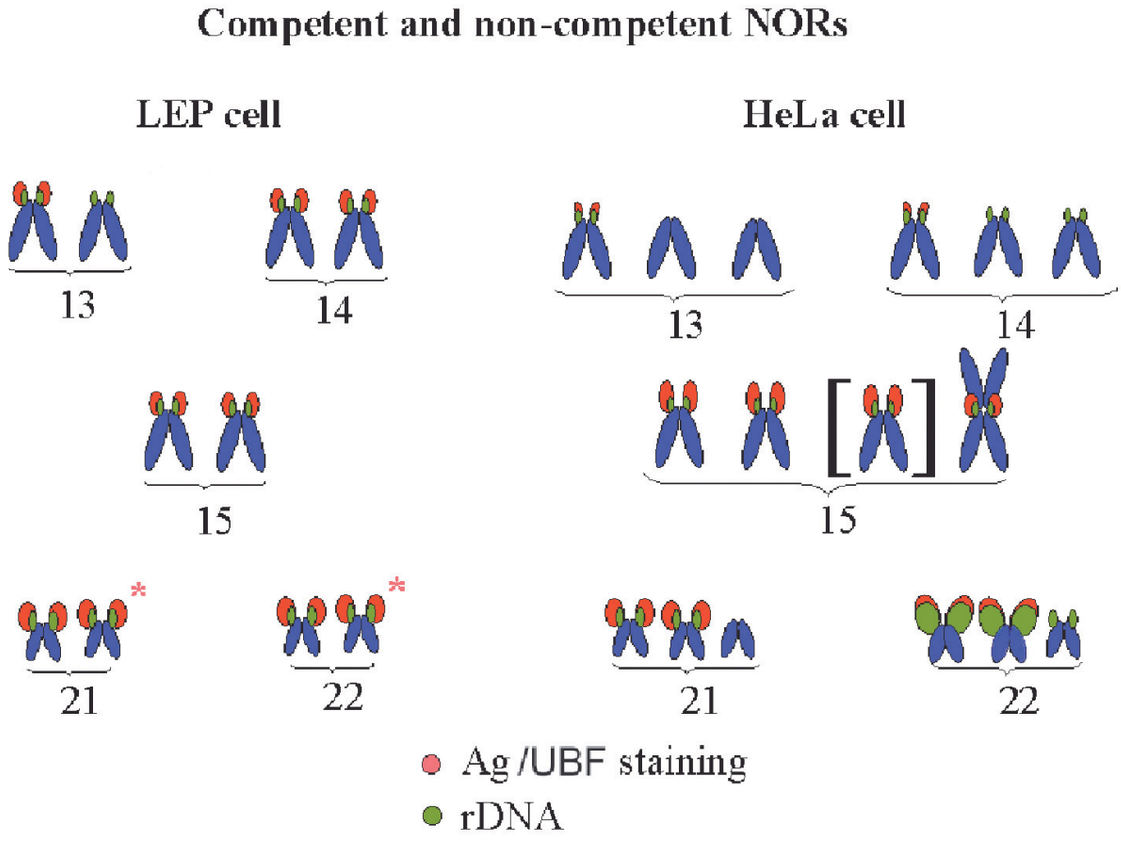

Fig. 4. Distribution of the NORs (green) and competent NORs (red) among the different chromosomes in HeLa and LEP cells. One of the chromosomes 15 of the HeLa cells is taken in parenthesis to show the variability of the chromosome number. Asterisks indicate two chromosomes of LEP cells that may be competent or non-competent with comparable frequencies. For simplicity the competent NORs are always shown on both chromatids. The different size of the red and green dots illustrates the different intensities of the rDNA and UBF/Ag signals.

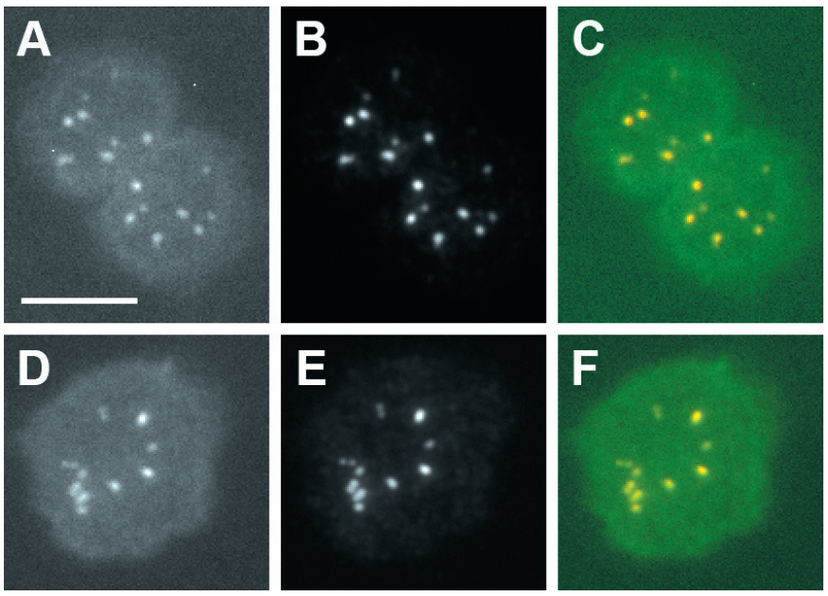

Fig. 5. Co-localization of transcriptionally active and competent NORs in telophase (**A-C**) and metaphase after roscovitine treatment (**D-F**). **A**, **D**: transcription signal (BrU); **B**, **E**: UBF. **C**, **F** co-localization of transcription (green) and UBF (red) signals.

NORs on one or both chromosome copies with similar frequencies (Table 2, Figure 4).

These data indicate that the LEP cells also show quite a regular pattern in the distribution of NOR transcriptional competence among the different chromosomes.

Transcription activity of the NORs correlates with their transcription competence in metaphase after roscovitine stimulation and in telophase

Using confocal microscopy, we counted the numbers of bromo-uridine (BrU) transcription signals in telophase cells and also in metaphase cells following stimulation of rDNA transcription with roscovitine (not shown). In both cases, the average number of BrU incorporating NORs per cell (calculated in 100 cells) corresponded to the average number of competent NORs labelled with antibody against UBF, i.e. nine in HeLa and eight in LEP cells. In co-localization experiments, in more than 95% of cells, the intensities of UBF and BrU signals obviously correlated (Figure 5A-C, D-F). These results strengthen the view that transcriptional competence of NORs identified in mitosis through UBF immunofluorescence serves as a reliable indicator of their transcription activity in interphase.

In the interphase, the same number of competent NORs can be observed as in the mitosis

In the following experiments we asked whether the same pattern of NOR competence may be present during the interphase. To answer this, we employed the PCC induced by calyculin A. This drug, being an efficient inhibitor of protein phosphatases, induces chromosome condensation through phosphorylation of the histones (Tosuji et al., 2003; Bui et al., 2004).

After calyculin A treatment the cells still had nuclei and some fibrillarin-positive remnants of nucleoli (data not shown). The cells lost their regular shape, developed bulky pseudopodia and lost their capacity to incorporate BrU. These changes could not be thoroughly reversed after cultivation in fresh medium. On the spread preparations (Figure 6), complete PCC was observed in G2 phase (Figure 6F). Only weak condensation was perceived in G1 (Figure 6B). In S phase the chromosomes were “pulverized”, i.e. fully condensed segments alternated with the non-condensed ones (Figure 6D); the latter included BrdU if it had been introduced for 5 min before the calyculin treatment in normal medium (data not shown). Such pattern can also be observed in PCC produced by other agents (Hameister and Sperling, 1984) and apparently results from different organization of chromatin in the replicating regions of chromosomes.

In the cells treated with calyculin, NORs appeared as separate units at all stages of interphase, even when the chromosome condensation was poor (Figure 6A-F). At least 100 cells were studied for each assay. As in the case of mitotic spreads, in G1 and G2 phases we most frequently revealed nine UBF signals for the HeLa cells, eight for the LEP cells. In S phase the average number of the UBF signals was the same as in the other phases, i.e. the difference never exceeded a statistical error, but in few cells (no more than 5%) this number decreased to less than 6. In G1 all the signals were single. In G2 most of them (around 90%) became doublets, like in mitosis. To examine whether the highly

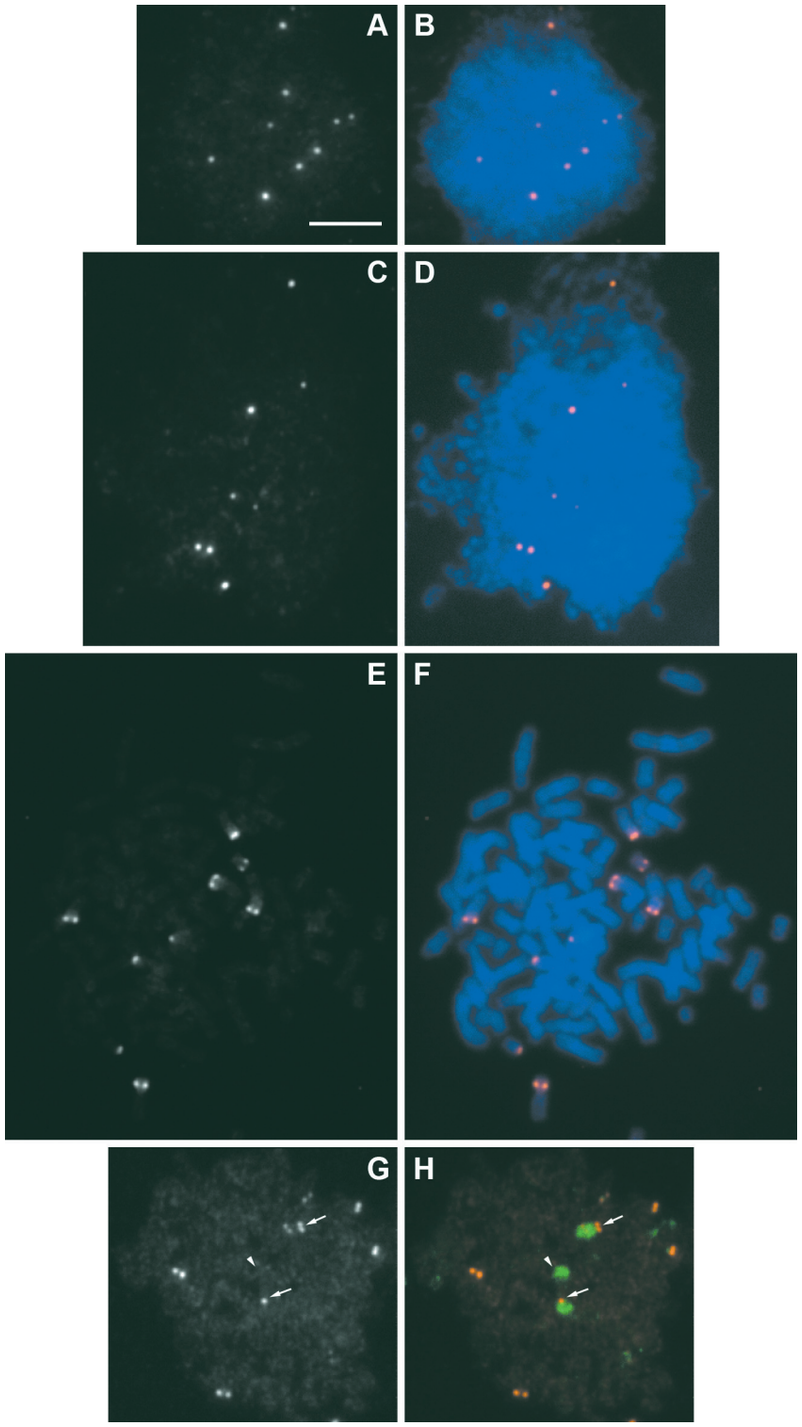

Figure 6. **A-F.** UBF-positive NORs (**A-E**, red in **B-F**) in the interphase HeLa cells after calyculin A treatment
A, **B**: G1 phase; **C**, **D**: S phase; **E**, **F**: G2 phase. DAPI counterstaining is in blue. Nine competent NORs appear as single in G1, mostly double in G2. Condensation of the chromosomes is complete only in G2 phase.
G, **H**: Simultaneous detection of UBF (**G**, red in **H**) and chromosome 22 (green in H) in G2 cells. Two chromosomes 22 carry UBF signals (arrows); the third chromosome is UBF-negative (compare with the Fig. 3F, G).

specific and regular pattern of competence found for HeLa cells in mitosis also persists in interphase, we combined the UBF immunocytochemistry with the chromosomal probes after calyculin treatment. In the case of G1 and S phases, due to the incomplete condensation, it was impossible to decide which NOR belonged to a given chromosome. However, in G2 phase we observed the same number of competent NORs for all chromosomes 13, 14, 15, 21 and 22 as in mitosis (Figure 6G, H). Also in agreement with the data obtained on metaphase chromosomes, the most intensive signals belonged to the chromosomes 15 (not shown), and chromosomes 22 usually carried signals of low intensity (Figure 6G, H).

We thus demonstrated that the pattern of transcriptional competence revealed in G2 phase is also conserved in mitosis. We infer that a uniform pattern of transcription competence persists throughout the whole cell cycle, with only rare loss of competent NORs in the S phase.

Discussion

a) NORs in mitosis: regular distribution of the competence signal among the mitotic chromosomes

No data are available in the literature on the distribution of NORs among the chromosomes of the HeLa cell line. We found in these cells an abnormal but stable pattern of rDNA distribution (Figure 4): not all acrocentric chromosomes carried ribosomal genes; one NOR was always present on a metacentric; two NORs, confined to the chromosomes 22, displayed a particularly high intensity of the hybridization signal. In LEP cells 10 acrocentric chromosomes were rDNA-positive, similarly as in normal human diploid cells.

Around 70 or 80% of the NORs, in HeLa and LEP cells respectively, were transcriptionally competent. In both HeLa and LEP cells, the transcription competence was non-randomly distributed among the NOR-bearing chromosomes (Tables 1 and 2, Figure 4). A regular pattern of the NOR competence, though quite different from what we found in the LEP cells, was observed previously in human lymphocytes (Heliot et al., 2000). Thus, the presence of such a regular pattern may be a common feature of human-derived cells, although it varies depending on the cell type.

Remarkably, in the transformed HeLa cells, we found even a more distinct pattern of competence than in diploid cells; in the former, each type of the acrocentric chromosome had a specific individual characteristic of NORs. Accordingly, the chromosomes 15, including the metacentric marker, are the major providers of the competent NORs, since all four of these chromosomes exhibit the silver/UBF signals that also are the most intensive; the only NOR of the chromosomes 13, as well as both NORs of the chromosomes 21, are usually competent; chromosomes 14 and 22, in contrast to the rest, regularly bear non-competent NORs.

In both HeLa and LEP cells, specific NOR chromosomes vary in regularity with which they follow a certain pattern of the NOR competence. This variability is especially striking in LEP cells, where chromosomes 13 and 15 are by far more “regular” than chromosomes 21 and 22 (asterisks in Figure 4). It should be mentioned that we did not follow each individual chromosome, so we cannot rule out a possibility of exchanging competence among the homologous chromosomes.

According to some data obtained on the cells of an Insectivora (Warburton and Henderson, 1979) and of human patients (Wachtler et al., 1986, Zurita et al., 1998, 1999), the level of Ag impregnation of the competent NORs correlates with the intensity of their rDNA signal, and thus rDNA contents. In our study of the HeLa cells, such correlation could be traced at least in one case: the smaller NOR of the chromosomes 22 was inactive. On the other hand, comparing different types of chromosomes, we observed that the huge rDNA signals on two copies of the chromosome 22 had relatively small counterparts in silver-stained NORs (AgNORs), whereas quite weak hybridization signals corresponded to the prominent silver staining of the chromosomes 15 (Figure 2A-G). The correlation between the gene number, as judged from the hybridization signal, and the competence is therefore breached in HeLa cells. These data agree with the view that the activity of the NORs is not proportional to their size (de Capoa et al., 1988; French et al., 2003).

We conclude that both studied cell lines exhibit a characteristic pattern of transcription competence, which remains stable in subsequent cell divisions.

b) NORs during interphase

In the human interphase cells, individual NORs do not correspond to the nucleoli, fibrillar centres or nucleolar silver-stained granules (Busch and Smetana, 1970) and so have never been visualized as separate entities in the cell nuclei. For this reason, the status of individual interphase NORs was not uncovered even in the *in vivo* experiments with UBF-GFP constructs. Here, we employed PCC to assess persistence of the transcription competence on the different NORs during the cell cycle. Upon a short treatment of interphase cells with calyculin A, which enabled us to visualize individual interphase NORs in the non-transfected cells, we have observed the same average numbers (eight for LEP and nine for HeLa cells) of dot-like UBF signals at all stages of interphase. In G1 all the NORs are represented as single dots. In the course of S phase the NORs become duplicated, and so do most of the competent NORs. A small proportion of S phase cells contain a decreased number of UBF signals. This occasional disappearance of signal seems to be a result of NOR reor-

ganization in the course of rDNA replication (Pliss et al., 2005). We can suppose that the competence status of each chromatid is established following the replication and does not change until the next S phase. In G2, the distribution of UBF signals on the specific chromosomes exactly corresponds to the pattern revealed on the mitotic spreads, regarding both the number and intensity of the signals. These data strongly indicate that the pattern of transcription competence observed on mitotic chromosomal spreads persists throughout the interphase, except for a short period in S phase.

Additionally, our results contribute to understanding the role of UBF in rDNA transcription. UBF is described as an architectural element maintaining a chromatin structure accessible for the pol I, due to its ability of binding to the rDNA promoter, bending DNA and sequestering the pol I transcription machinery (Jantzen et al., 1990; Bazett-Jones et al., 1994; Mais et al., 2005). This seems to provide a crucial argument in favour of the conception claiming that the competent and only competent NORs are transcriptionally active. However, the idea of UBF as an indispensable factor in the initiation of rDNA transcription (Stefanovsky et al., 2001) has been recently questioned, since:

– UBF binds to rDNA indiscriminately, i.e. not only at the upstream control element and core element of the promoter (Copenhaver et al., 1994; Hu et al., 1994; O'Sullivan et al., 2002);
– UBF does not bind stably to rDNA, perhaps even on the promoter, but rapidly associates and dissociates (Dundr et al., 2002; Chen et al., 2005; Friedrich et al., 2005);
– Some *in vitro* experiments indicate that the selectivity factor, SL1, rather than UBF, nucleates the pol I activity. UBF is not necessary for formation of the pre-initiation complex, and SL1 can interact with rDNA independently (Friedrich et al., 2005);
– The SL1/TIF-1B complex, but not UBF, is responsible for the promoter selectivity and species specificity of pol I transcription (Learned et al., 1985; Sullivan et al., 2001).

Thus, UBF may only be an activator of the rDNA transcription. If so, can UBF be a marker of the transcribed NORs? Qualitative data on co-localization of transcription and UBF signals at the telophase have served as the only experimental evidence for this hypothesis (Roussel et al., 1996; Gebrane-Younes et al., 1997; Sirri et al., 1999). Here we applied a quantitative approach. Using confocal images, we found equal numbers of the transcription and competence signals in telophase. Moreover, the intensities of UBF and BrU signals on the same NOR positively correlated (Figure 5A-C). Similar correlation of UBF and transcription signals was also observed on metaphase cells after stimulation with roscovitine (Figure 5D-F). Thus, we demonstrate that the incipient or stimulated transcription activity of the NORs exactly follows the pattern of transcription competence that persists over the entire cell cycle, except probably for a short period in S phase. This result strengthens the conception claiming one to one correspondence between the mitotic transcription competence and interphase transcription activity of NORs in the stable physiological state.

The results of the present study, performed on such different objects as transformed HeLa cells and the diploid LEP cells, indicate that the essential components of the pol I transcription machinery, such as UBF, remain associated with the same NORs throughout the cell cycle. Our data strongly argue that the presence of UBF on NORs (or their silver stainability) in mitosis serves as a marker of their transcription activity in the previous interphase.

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