First Faculty of Medicine, Charles University in Prague

Relationship between large-scale chromatin organization and nucleolus in human cells

PhD thesis

Markéta Kalmárová

Prague 2008

Poděkování:

Děkuji svému školiteli prof. Ivanu Raškovi za jeho odborné vedení mojí dizertační práce, možnosti vědeckého růstu a nabyté zkušenosti. Mé poděkování patří dr. Evgenyji Smirnovovi za cenné rady a také za přátelské poskytnutí odborné průpravy, a rovněž všem spolupracovníkům z Ústavu buněčné biologie a patologie 1. LF UK za pomoc během mého působení na tomto ústavu.

Poděkování patří také mé rodině, za jejich podporu, trpělivost a toleranci.

Contents:

Abbreviations

BrdU	5´bromo 2´-deoxyuridine
BrUTP	5-bromouridine 5´-triphosphate
CT	Christmas tree
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco modified Eagle's medium
EM	electron microscopy
FISH	fluorescence *in situ* hybridization
GFP	green fluorescent protein
HeLa	human cells derived from adenocarcinoma cervix cells
LEP	diploid human embryonic lung fibroblast cells
LM	light microscopy
NORs	Nucleolus Organizer Regions
PBS	Phosphate Buffered Saline
PCC	premature chromosome condensation
Pol I	RNA polymerase I
rDNA	DNA sequence(s) encompassing ribosomal genes
RT	room temperature
SSC	standard sodium citrate
UBF	Upstream Binding Factor

Chapter 1. Outline of the thesis

The projects of the laboratory that is joining the Institute of Cellular Biology and Pathology of the First Faculty of Medicine, Charles University in Prague and Department of Cell Biology, Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., are focused on the functional organization of the cell nucleus, particularly on the dynamic organization of chromatin in relation to RNA and DNA synthesis as well as mRNA and rRNA maturation. I have participated in several projects concerning the functional organization of the cell nucleus, the results of which were summarized in Koberna et al. (2002), Pliss et al. (2005), Fidlerová et al. (2005), Koberna et al. (2005).

This thesis is devoted to a specific problem of nuclear organization, namely the large-scale order of chromosome positioning and its relation to nucleoli and transcription activity/competence of ribosomal genes. Basic information about cell nucleus, nucleolus and ribosomal genes with a special emphasis on large-scale positioning of the Nucleolus Organizer Regions (NORs) and NOR-bearing chromosomes and maintenance of these chromosome positions after mitosis, is provided in the Chapter 2. The aims of the thesis are defined in the next chapter. The basic methods are surveyed in the Chapter 4. In the next three chapters, proper results of my PhD thesis are presented. A study of the transcription status of NORs during the cell cycle in both primary LEP and immortalized HeLa human cells is described in the Chapter 5. This study was published in Folia Biologica (Smirnov et al., 2006). The Chapter 6 contains findings that expand the results of Smirnov et al., 2006 and analyze nuclear positions of NORs, as well as chromosomes bearing NORs, with respect to nucleoli in HeLa and LEP interphase cells. This work was published in the Journal of Structural Biology (Kalmárová et al., 2007). In Chapter 7, a comparison between the two daughter cells is made with regard to the position of the NOR-bearing chromosomes in relation to nucleoli. This study is in press in Physiological Research, n. 3, vol. 57 (Kalmárová et al., 2008). The final part of the thesis includes summary and general discussion (Chapter 8), conclusions (Chapter 9), and list of references (Chapter 10).

Chapter 2. General introduction

2.1. Cell nucleus

The cell nucleus, discovered by Brown in 1831, continues to be an object of great fascination for scientists today. This is because the nucleus is the repository of the vast majority of hereditary information, and the first steps of gene expression take place in this organelle.

The DNA-protein complex occupying much of the nuclear volume is termed "chromatin"[*]. Chromatin has a specific organization according to the type of cell, stage of cell differentiation and current metabolic status of the cell. This organization is closely related to the gene expression. Gene transcription, replication, and repair are influenced by the underlying chromatin architecture. This arrangement in the nucleus presumably facilitates cellular functions, and exploring the link between transcription and nuclear organization will be an exciting area of future research (Gilbert et al., 2005).

Microscopic observations of interphase nuclei reveal two distinct types of chromatin, heterochromatin and euchromatin, although we have to bear in mind that the distinction between the two types of chromatin is not clear cut. It is generally accepted that euchromatin encompasses transcriptionally active parts of chromatin. Heterochromatin is presumably more condensed than euchromatin, and is stained intensely with DNA-binding dyes such as DAPI. Chromatin which remains condensed throughout the cell cycle is called constitutive. It is considered to be genetically inert and is basically composed of satellite DNA. Facultative heterochromatin is chromatin that will acquire heterochromatic (or euchromatic) properties in a developmentally controlled manner. A classic example of this phenomenon is the inactive X chromosomes of female mammals (Gilbert et al., 2005).

The cell nucleus represents a complex and dynamic structure that includes, beside chromatin, many functionally specialized regions (domains), substructures, or subcompartments (Fig.1). The most prominent nuclear substructures in eucaryotic cells

[*] This widely used chromatin description may not be fully correct as various ribonucleoproteins are also bound to "chromatin".

are: nucleoli, Cajal bodies (CB), nuclear speckles (splicing factor compartments; SFC), Gems, PML bodies (reviewed in Dundr and Misteli, 2001; Spector, 2001; Spector, 2003). These structures can be directly observed in the electron microscope[*]. In the light microscopy, they can be visualized, for instance, by means of immunocytochemistry and/or *in situ* hybridization using specific antibodies and/or specific hybridization probes. Specific macromolecules can be highly enriched in distinct nuclear domains (Fig. 1).

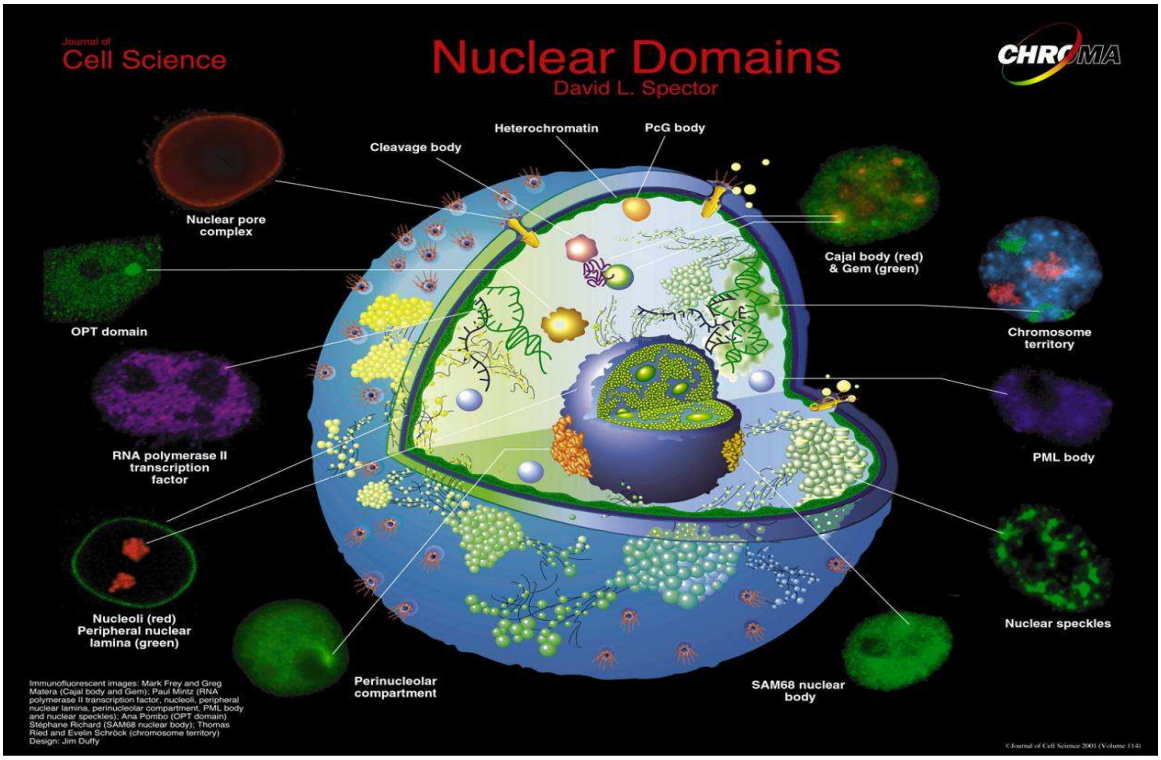


Figure 1. Scheme of mammalian cell nucleus showing large number of various nuclear domains. In the peripheral ring of this Figure, such domains are seen as images in the fluorescence microscope (Spector, 2001).

Much has been learnt about the functional organization of the cell nucleus in the past 15 years, but we are only beginning to understand it. Thus, although the structure of double stranded DNA and core nucleosome are known at the atomic resolution level, and the nucleosomal arrangement of chromatin in 10 nm filament called “string of pearls” is described in general terms, the position of linker histones remains unclear. With regard to the 30 nm filament, we are only at the stage of developing various models, and the zig-zag

[*] Some structures such as nucleoli or mitotic chromosomes can be well discerned in phase contrast or Nomarski microscopy.

model (Bednár et al., 1995; Woodcock and Dimitrov, 2001) is preferred today. Higher levels of DNA arrangement are still basically unknown.

Within the frame of this thesis, two nuclear substructures are in the spotlight: chromosome territories and nucleoli.

2.2. Chromosome territories and their positioning in the interphase nucleus

Chromosomes exist as distinct condensed structures during mitosis. Their organization in the interphase nucleus represents a special problem. In the late 19th and early 20th century Carl Rabl and Theodor Boveri, based on their light microscopy studies, suggested that chromosomes in the interphase nucleus existed as individual separate entities (e.g. Cremer and Cremer, 2006). A territorial organization of the inactive X-chromosome (later termed the Barr body) in human female cells was described by Barr and Bertram in 1949 and Klinger in 1958 (after Foster and Bridger, 2005). The first experimental evidence for existence of interphase chromosomes in the form of the chromosome territories was provided only in 1970s by Cremer and colleagues by inducing focal DNA damage with a laser UV microbeam (Cremer et al., 1974; Lamond and Sleeman, 2003; Cremer and Cremer, 2006). Further structural understanding of these territories was achieved using isotopic and non-isotopic *in situ* hybridization with genomic DNA probes (Manuelidis, 1985). Following the development of fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA libraries, the territorial organization of chromosomes became accepted scientific dogma (Cremer et al., 1988; Lichter et al., 1988) (Fig.2). More recently, introduction of green fluorescent protein (GFP) (Robinett et al., 1996; Belmont and Straight, 1998) and incorporation of fluorescently tagged nucleotides into DNA, have facilitated the visualization of genome dynamics in living cells (Visser et al., 1998; Zink et al., 1998; Manders et al., 1999; Visser and Aten, 1999; Edelmann et al., 2001). It was confirmed that each chromosome does indeed occupy its own territory (Foster and Bridger, 2005). These territories seem to have a porous structure, with numerous channels (Verschure et al., 1999; Visser et al., 2000). According to a speculative model, the interchromatin channels form a compartment distinct from zones of chromatin (Cremer and Cremer, 2001). This model proposes that active genes might reside on the surface of the territory, where transcription and mRNA processing takes

place; while transcriptionally inactive regions would tend to be situated towards the interior of the domain (Baxter et al., 2002).

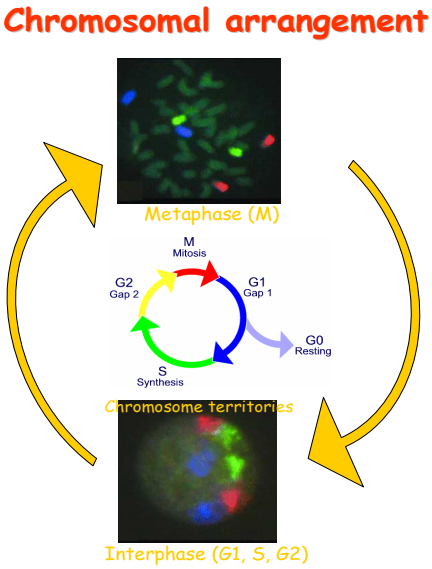


Figure 2. Human chromosomes in the metaphase spread (top) and in the interphase cell (bottom). Fluorescent *in situ* hybridization (FISH) with probes for three different chromosome pairs allows to paint these chromosomes in different colors. The chromosomes appear as highly condensed structures in metaphase, and as less condensed territories in interphase.

Based on statistical evaluation of the chromosome territories positioning, numerous studies have shown that, in vertebrate cells, the chromosome territories are non-randomly arranged in the nuclear space (Cremer and Cremer, 2001; Parada and Misteli, 2002; Cremer and Cremer, 2006). It has been found that gene-rich chromosomes tend to be located towards the nuclear interior, whereas gene-poor chromosomes tend to be located towards the periphery (Bridger and Bickmore, 1998; Parada and Misteli, 2002; Gilbert et al., 2005). Chromosomes also may be positioned in a size-dependent manner, with the larger chromosomes located more peripherally (Sun et al., 2000; Bolzer et al., 2005). It should be mentioned, however, that such regularities in chromosome positioning represent the statistically averaged situation, and individual cells may have different chromosome arrangement. Moreover, there are some cases where the position of chromosomes varies whereas the gene density of chromatin has not changed, for example between proliferating and non-proliferating fibroblasts (Bridger et al., 2000; Meaburn et al., 2007a), during differentiation (Kim et al., 2004; Kuroda et al., 2004, Foster et al.,

2005; Stadler et al., 2004), in cells derived from different tissues and cell types (Boyle et al., 2001; Parada et al., 2004a; Mayer et al., 2005), and, based on analysis of pericentric heterochromatin distribution, in early mouse embryos at the onset of gene transcription (Martin et al., 2006a; Martin et al., 2006b; Meaburn et al., 2007a). Changes in the spatial organization of chromosomes and genes have been observed in several diseases, including cancer (Nikiforova et al., 2000; Cremer et al., 2003; Roix et al., 2003; Murmann et al., 2005; Wiech et al., 2005; Zink et al., 2004), epilepsy (Borden and Manuelidis, 1988) and laminopathies, a group of diseases caused by mutation in the nuclear protein lamin A/C (Meaburn et al., 2007b). It is believed that particular chromosome positioning places genes into special neighborhood favorable for their expression or silencing (Parada and Misteli, 2002). But the situation is complicated by recent data indicating that chromosome territories partially intermingle in human cell nuclei (Branco and Pombo, 2006). Moreover, some genes can be found in loops extended far beyond the area defined as chromosome territory (Baxter et al., 2002; Mahy et al., 2002; Chubb and Bickmore, 2003; Kioussis, 2005; Wegel and Shaw, 2005; Bártová et al., 2008).

Thus, the rationales for the large-scale order of chromosome positioning, and its relation to gene activity, remain to a large extent unclear.

2.3. Positioning of chromosomes as compared in the daughter cells after mitosis

Non-random territory organization implies the need for a mechanism to sucessfully convey chromosome positional information to daughter nuclei (cellular memory) (Williams and Fisher, 2003). Inheritance of interphase chromosomal order was considered by Boveri in 1909, on the basis of his studies of nematode chromosomes (e.g. Bickmore and Chubb, 2003). Boveri proposed that chromosome arrangements are similar in the two daughter nuclei. Early studies on some plant species supported this view (Heitz, 1932, after Walter et al., 2003). According to the more recent studies, the chromosome positioning was found to be largely maintained in some species (Abranches et al. 1998; Santos et al., 2002) whereas the results obtained on mammalian cells were controversial. Moreover, FISH experiments suggested a strong correlation of the whole chromosomes and centromeres positioning within each pair of cells derived from the same mother cell

(Sun and Yokota, 1999). In other studies (Gerlich et al., 2003; Walter et al., 2003; Essers et al., 2005), fluorescence live cell imaging techniques were used to investigate whether chromosome position in mammalian cells can be maintained from a mother nucleus, through the events of mitosis, in the two daughter nuclei. Some authors claimed that the chromosome arrangements were similar in daughter cells (Gerlich et al., 2003 Essers et al., 2005), while others observed that positions of chromosomes in daughter nuclei differed significantly from the positions seen in mother cell nucleus (Walter et al., 2003). Although differences in experimental procedure could be responsible for these divergent results (Bickmore and Chubb, 2003), further studies of the chromosome positioning in the daughter cells are needed to elucidate this problem.

2.4. The nucleolus

Nucleolus, the most prominent of all nuclear subcompartments (Fig. 3), is a subject of intensive studies. The initial identification of a nucleolus by Fontana in 1781 (e.g. Olson et al., 2002). The name “nucleolus” was coined by Valentin in 1839 (e.g. Olson et al., 2002) who noticed that most cells had a secondary nucleus or a “nucleus within a nucleus”. These early observations of the nucleolus as a subcellular structure remained largely descriptive until the early 1960s. It was then established that nucleolus contains ribosomal genes that are transcribed, and ribosomal subunits are generated in this organelle (Busch and Smetana, 1970; Hadjiolov 1985; Raška et al., 1990; Scheer and Hock, 1999; Olson et al., 2000; Fatica and Tollervey, 2002).

Three major substructures can be distinguished in the nucleoli at the ultrastructural level: the fibrillar center, the dense fibrillar component, and the granular component (Fig. 4). The fibrillar center (FC) contains ribosomal DNA (rDNA) and at least some of the essential components of Pol I transcription machinery. Dense fibrillar component (DFC) usually forms a rim around the FC and sometimes protrudes into the FC. DFC contains a high concentration of ribonucleoproteins, and is, therefore, electron-dense (Raška, 2003). Transcription of rDNA occurs apparently in this region including the boundary between FC and DFC (Koberna et al., 2002). Also the first steps in the pre-rRNA processing take place in this nucleolar subcompartment (e.g., Shaw and Jordan, 1995). The third nucleolar subcompartment is granular component (GC), where the later steps of pre-rRNA

processing and formation of pre-ribosomal take place. In addition to these substructures, chromatin structures and nucleolar interstices can be seen within nucleoli. Importantly, nucleoli are surrounded with a mass of heterochromatin (Figs. 3, 4). This mass is believed to be composed to a large extent of DNA sequences from the NOR-bearing chromosomes (Stahl, 1976; Kaplan et al., 1993), the corresponding ribosomal genes apparently being encompassed within the nucleolus itself. Some chromosomes, besides NOR-bearing chromosomes, are also known to be regularly associated with nucleolus (Stahl, 1976; Ochs and Press, 1992; Kaplan et al., 1993; Sullivan et al., 2001).

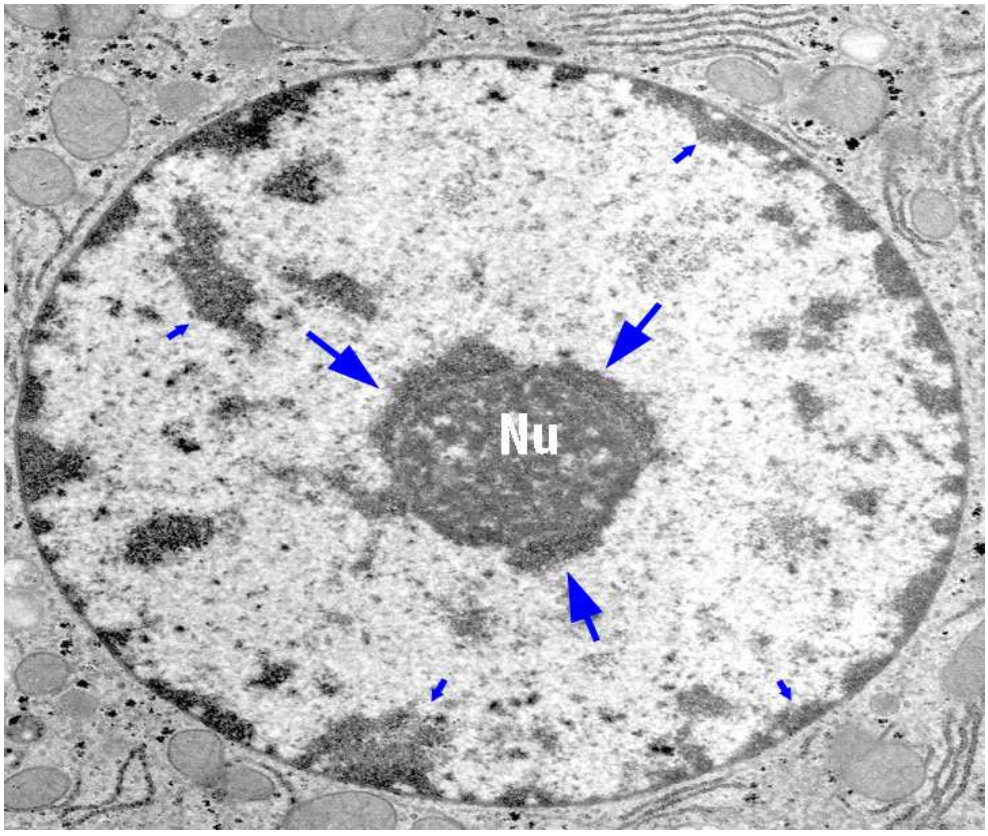


Figure 3. Thin sectioned nucleus as seen in the electron microscope. The prominent nucleolus (Nu) is surrounded by nucleolus-associated heterochromatin (large arrows). Nuclear heterochromatin is indicated by small arrows (Figure provided by Dr. Raška).

In metabolically active mammalian and plant cells as well as in yeast, the nucleolus contains tens to hundreds of active ribosomal genes that are usually characterized by high levels of transcription accounting for about a half of the total cellular RNA production (Raška et al., 2006). This is understandable since the proteins perform most of the everyday work in the cell, and the production of ribosomes, that are protein “manufactures”, is essential for cell metabolism. Ribosome biogenesis thus

requires a tremendous commitment of cellular energy and is tightly regulated by cells in response to various stimuli that immediately affect rRNA synthesis, production of ribosomes, protein synthesis as well as cell growth and proliferation.

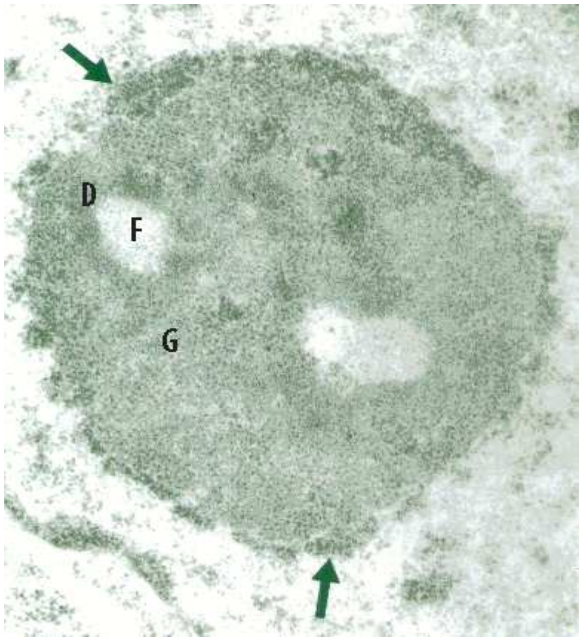


Figure 4. Thin section of nucleolus of phytohemagglutinin stimulated human lymphocytes in the electron microscope. Fibrillar center (F), dense fibrillar component (D) and granular component (G) are seen. The arrows indicate perinucleolar chromatin (Figure provided by Dr. Raška).

The metabolically active and cycling mammalian cells producing a great number of ribosomes usually contain several large nucleoli with numerous tiny fibrillar centers (Bush and Smetana, 1970; Koberna et al., 2002; Raška, 2003). For instance, dormant human lymphocytes frequently contain a single small ring-shaped nucleolus with only one FC, and are characterized by a decreased level of rRNA synthesis. But 24-48 h after phytohemagglutinin stimulation in culture these cells exhibit several large nucleoli with several FCs and intensively produce rRNA (Busch and Smetana 1970; Smetana et al., 1967, Smetana et al., 1968; Biberfeld, 1971; Raška et al., 1983a; Raška et al., 1983b; Ochs and Smetana, 1989). Along these lines, the nucleolar morphology has proved to be a convenient diagnostic marker in human pathology, particulary in cancer cells (Bush and Smetana, 1970).

The functional analysis of nucleolar structures with respect to the metabolic processes has been significantly expanded. The new findings include ultrastructural identification of nascent molecules of ribosomal RNA as well as active ribosomal genes (Koberna et al., 2002). Microscopic approaches enabled to identify, although so far only in yeast, some key steps in maturation of pre-ribosomal RNA (Osheim et al., 2004).

The most spectacular progress in our knowledge of nucleolus was provided by functional proteomic analysis. Less than 200 different proteins were identified in human nucleolus at the very beginning of 21st century. With implementation of the nucleolar proteomics, this number increased to 350 in the year 2002, and in 2005 to almost 700. Current studies of the isolated nucleoli from various types of human cells suggest presence of more than 2000 proteins, although not all these nucleolar proteins were identified in each of the examined cell types (Lam et al., 2007).

Functional proteomic analysis of nucleoli not only helped us to answer a number of "ribosomal" questions, but also confirmed and expanded our knowledge of the non-ribosomal nucleolar functions. It seems that nucleolus plays a role in the control of cell cycle, senescence and regulation of telomerase activity, oncogene activities and tumor suppression, cell stress reactions, metabolism of mRNA molecules, maturation of the "extranucleolar" RNAs and control of viral infections. These functions are not well understood (Olson et al., 2002; Raška et al., 2006, Boisvert et al., 2007). Additionally, importance of the maternal nucleolus in early development was recently established in sheep Dolly-like experiments (Ogushi et al., 2008). It is yet unknown why nucleolus participates in so many non-ribosomal activities, and how these activities are interrelated.

2.5. Nucleolus Organizer Regions (NORs)

Ribosomal genes coding 5.8S, 18S and 28S rRNA form arrays of tandem repeats at specific chromosomal sites termed nucleolus organizer regions (NORs)[*] (Fig. 5). In humans, NORs are located 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).

[*] The original definition of NORs was rather a cytological one.

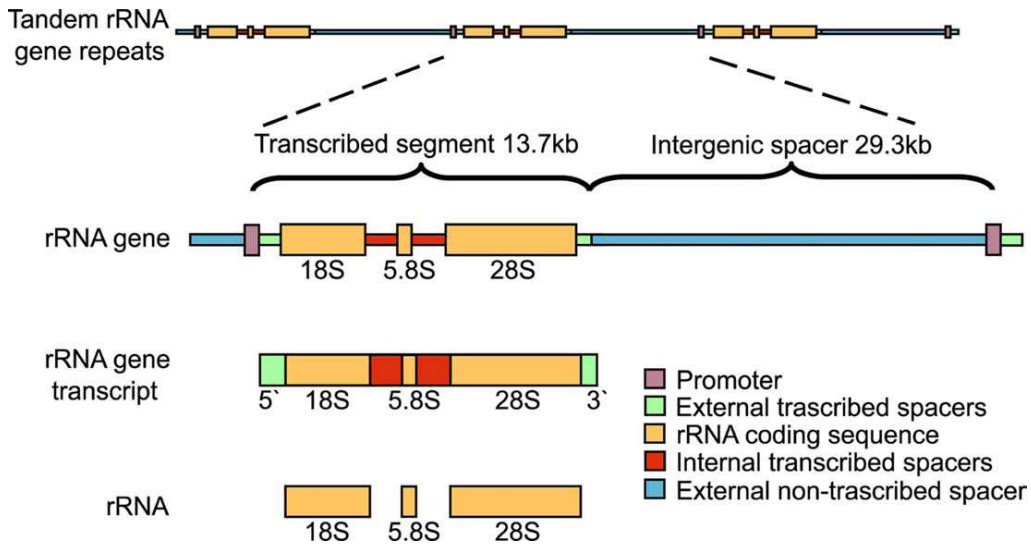


Figure 5. General scheme of human rDNA ribosomal genes and their transcripts. Each ribosomal gene unit usually consists of a transcribed and an external non-transcribed spacer. The transcription is driven by the nucleolar RNA-polymerase I, that synthetizes the long precursor rRNA (pre-rRNA). This precursor molecule contains 18S, 5.8S and 28S rRNA sequences.

Some NORs can be identified as secondary constrictions on metaphase chromosomes, or visualized by immunocytochemistry or silver staining, due to the abudance of specific associated proteins (Goodpasture and Bloom, 1975; Weisenberger and Scheer, 1995; Roussel et al., 1996; Gebrane-Younes et al., 1997). It is generally accepted, but not proven, that such NORs, termed "transcriptionally competent" or "competent" (Dousset et al., 2000; Savino et al., 2001), are transcriptionally active during previous interphase (Weisenberger and Scheer, 1995; Roussel et al., 1996; Gebrane-Younes et al., 1997). The proteins responsible for this unique chromatin structure are components of the RNA polymerase (pol I) transcription machinery, including Upstream Binding Factor (UBF), which remain associated with NOR during mitosis when pol I transcription is repressed (Weisenberger and Scheer, 1995; Jordan et al., 1996; Roussel et al., 1996). UBF binding confers to NORs a unique architecture (e.g. McStay, 2006). The reactivation of pol I transcription starts in late anaphase. Primary nucleoli reform around the active NORs at the end of mitosis (Ochs et al., 1985; Benavente et al., 1987; Jimenez-Garcia et al., 1994) and fuse into final nucleoli in early G1. It should be also noted that short arms of human acrocentric chromosomes contain, apart from ribosomal genes, apparently just repetitive sequences (Kaplan et al., 1993; Sullivan et al., 2001).

Transcription activity of rDNA can be regulated at the level of whole NORs, which is particularly seen in the hybrid cells as so called “nucleolar dominance” (McStay, 2006, Stefanovsky and Moss, 2006; Lewis et al., 2007; Preuss and Picaard, 2007). At the level of individual ribosomal genes within a given NOR, despite enormous progress (Grummt, 2007), a synthetic view on the regulation of their transcription is not yet achieved. The expression of the individual ribosomal genes within a given NOR can be studied at best on DNA spreads (“Christmas trees”) using electron microscopy (Fig. 6). However, this unique approach was successfully applied so far only for the yeast, and insect as well as amphibian oocytes (Miller and Beatty, 1969; Trendelenburg, 1974; Puvion-Dutilleul et al., 1977; Franke et al., 1979; Scheer et al., 1981; Mougey et al., 1993; Dragon et al., 2002). Only a few pictures documenting mammalian “Christmas trees” were published in the literature (e.g. Scheer and Benavente, 1990).

Relationships among the NORs, nucleoli and chromosome territories apparently play important role in the functioning of nucleus, and all these relationships are not sufficiently understood. Also, a synthetic view on the regulation of the expression of ribosomal genes has not been yet elaborated.

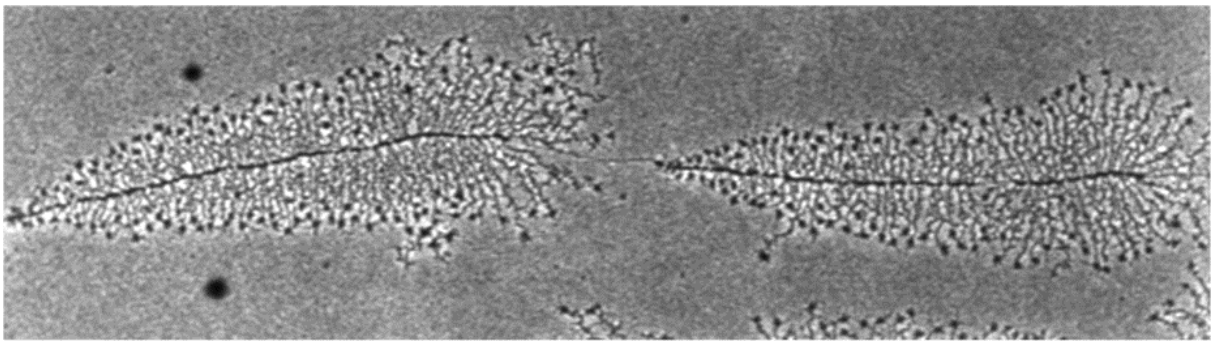


Figure 6. The molecular organization of ribosomal transcripts in the form of so-called “Christmas trees” (CTs) (Figure published in Alberts et al., Molecular Biology of the Cell, Fourth Edition, 2002). Spread active ribosomal genes associated with RNA polymerase I and nascent pre-rRNAs was described in amphibian oocytes more than 30 years ago (Miller and Beatty, 1969). Similar structures were also reported later in other species (e.g., Trendelenburg, 1974; Puvion-Dutilleul et al., 1977; Franke et al., 1979; Scheer et al., 1981; Mougey et al., 1993; Dragon et al., 2002). However, due to the presence of interfering chromatin structures, only a very few pictures documenting spread CTs from mammalian cells were published in the literature (e.g. Scheer and Benavente, 1990).

2.6. Survey of the introduction

The introduction contained brief comments on the cell nucleus, chromosome positioning, its maintenance in the daughter cells after mitosis, nucleolus and NORs. Despite the exciting progress in this field, our knowledge is only partial and often controversial. It is known that ribosomal genes are transcribed in the nucleoli, but rationale behind the existence of nucleoli remains elusive. Until recently, it was believed that nucleolus originates from transcription activity of ribosomal genes exclusively. New data urge us to reconsider this hypothesis (Gonda et al., 2003). Relationships between spatial organization of NOR-bearing chromosomes and nucleoli are also not clear, particularly with respect to chromosomes bearing non-competent NORs. Although it is widely accepted that arrangement of chromosomes in the cell nucleus is non-random, there are basically no data on the position maintenance of the NOR-bearing chromosomes in relation to nucleoli in the daughter cells after mitosis.

Therefore, it has been important for my work to focus on chosen specific aspects of these challenging problems, namely: transcription status of NORs during the cell cycle; positioning of NORs and NOR-bearing chromosomes with regard to nucleolus; and maintenance of the NOR-bearing chromosome positions with respect to nucleoli after mitosis.

In our future project we wish also to apply cell biological approaches such as *in vivo* studies of the dynamics of NORs and chromatin components using GFP (and related) techniques, particularly:

- with respect to maintenance of positioning of chromosomes bearing ribosomal genes, to define to what extent the same chromosomes are associated with a given nucleolus in daughter cells after mitosis;
- To study maintenance of the epigenetic information on individual competent NORs through mitosis using a cell line stably expressing UBF-GFP.

Chapter 3. Specific aims of the thesis

In this thesis, I wished to expand our knowledge of the large-scale order of the NOR-chromosome positioning with respect to nucleoli, while assessing the competence of their NORs, as well as transcription activity of ribosomal genes. Accordingly, we first characterized the chosen model cell lines with respect to their NORs and competent NORs. The obtained data were used in subsequent studies of nucleolar associations of the NOR-bearing chromosomes, and localization of the transcriptionally competent and non-competent NORs. In the pairs of daughter cells, we also compared nucleolar associations of chosen NOR-bearing homologues with nucleoli, as well as numbers of nucleoli.

The specific aims of the thesis are:

- To establish the regularities in distribution of competent (active) as well as non-competent (inactive) NORs among the NOR-chromosomes in two human-derived cell lines, transformed HeLa and primary LEP cells.
- To establish the fate of competent NORs during the cell cycle in the two cell lines.
- To analyze nucleolar association of the NOR-bearing chromosomes in HeLa and LEP cells, with respect to the established occurrence of competent/non-competent NORs in different NOR-bearing chromosomes.
- To investigate nuclear/nucleolar location of competent and non-competent NORs in the two cell lines.
- In the pairs of daughter HeLa cells, to compare positioning of the NOR-bearing chromosomes in relation to nucleoli.
- To establish whether the same number of nucleoli is seen in the pairs of daughter HeLa cells after mitosis.

Chapter 4. Materials and Methods

This chapter surveys the material and methods used in the thesis. The detailed description is provided in chapters 5.2, 6.2 and 7.2.

4.1. Cell culture

Human HeLa cells, derived from adenocarcinoma cervix cells, and diploid human embryo lung fibroblasts (LEP) were cultivated as described in chapters 5, 6 and 7.

4.2. DNA probes and antibodies

We used commercial Cy3- and FITC- labeled whole chromosome painting probes for human chromosomes 13, 14, 15, 21 and 22 (Appligene Oncor, USA), rDNA probes and antibodies are described in detail in chapters 5, 6 and 7.

4.3. Immunofluorescence

Cells growing on coverslips were washed in phosphate-buffered saline (PBS), fixed in methanol at -20°C for 30 min and air-dried. Following three washes in PBS, the cells were incubated with anti-fibrillarin antibody, washed in PBS and incubated with secondary antibodies conjugated with either FITC or Cy3.

4.4. Immuno-FISH and FISH

The combined detection of fibrillarin and *in situ* hybridization (immuno-FISH) was performed after Pliss et al., 2005 and described in chapter 6 and 7.

The results of all single labeling (fibrillarin immuno-labeling and FISH), double labeling (fibrillarin immuno-labeling combined with FISH and double-FISH) and triple labeling experiments (fibrillarin immuno-labeling and double FISH) were compatible.

4.5. Microscopes used

Coverslips were mounted in Mowiol supplemented with DABCO and viewed using Olympus AX70 Provis microscope equipped with the Photometrics CCD camera or Leica TCS NT confocal microscope.

4.6. Statistical evaluation

All statistical evaluations were obtained by analysis of 100 HeLa and LEP cells.

Chapter 5. NORs and their transcription competence during the cell cycle.

5.1. Introduction

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.

5.2. Materials and Methods

5.2.1. 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% fetal 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 hour, hypotonically swollen in 0.075 M KCl for 10 minutes, harvested by mitotic shake-off and pelleted. The pellet was resuspended in fixative (methanol and acetic acid, 3:1) and centrifuged (5 minutes at 400g). After threefold repetition of this procedure, cells were spread on chilled coverslips and dried.

5.2.2. 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 seconds at 37°C. The period of 60 seconds was chosen after 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 minutes at -20°C) or 2% paraformaldehyde (10 minutes at room temperature). In some cases 20uM BrdU (Sigma) was introduced 5 minutes before fixation. Replication signal was detected with monoclonal mouse anti-BrDU antibody (Roche, Indianapolis, IN)

5.2.3. Transcription labelling

Transcription sites were visualized by applying a method of hypotonic shift as described earlier by Koberna et al. (Koberna et al., 1999; Koberna et al., 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 minutes, then in DMEM at 37°C for 10 minutes, and fixed in methanol. Transcription signals were visualized after methanol fixation using mouse antibody against BrdU (Roche).

5.2.4. 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 promotor selectivity factor SL1 (Sirri et al., 1999). In our work, colcemid-arrested mitotic HeLa cells were treated with 150µM roscovitine (BIOMOL Research Laboratories, Plymouth, PA) for 30 minutes. After that, the transcription hypotonic assay was performed as described above.

5.2.5. In situ hybridization

Cy3- or FITC- labeled 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 2xSSC, pH 7, incubated with 100 µg/ml RNAse A (Roche) for 1 hours 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 2xSSC, pH 7, at 72°C for 3 minutes. The probe was denaturated at 70°C for 10 minutes. Hybridization ran overnight at 37°C in moisture chamber. Spreads were washed 15 minutes in 50% formamide in 2xSSC, pH 7, at 43°C; 8 minutes in 0.1%Tween-20 in 2xSSC at 43°C; and 3x 4 minutes in 0.1% Igepal (ICN Biomedicals, Inc., Irvine, CA) in 4xSSC.

The biotin-labeled 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 18 S rRNA, internal transcribed spacer 1, sequence for 5.8 S rRNA, internal transcribed spacer 2 and upstream 4,500 nucleotides of 28 S rRNA. The probe was labeled 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 minutes and kept at 37°C for 30 minutes. 6 µl of a hybridization mix containing 25 µg/ml of probe, 0.5 mg/ml sonicated salmon sperm DNA, 2 mg/ml *Escherichia coli* tRNA, 70% deionized formamide, 2xSSC and 10% dextran sulfate, was used per each coverslip. The ISH was performed overnight at 37°C in moisture chamber.

Karotypes were studied by multicolor 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 labeled painting probes for all autosomes and sex chromosomes of the human karyotype. Hybridization and posthybridization 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™ GmbH, Altlussheim, Germany) were used for image capturing and acquisition.

5.2.6. UBF immunocytochemistry

Fixed cells were rinsed in PBS. Non-specific antibody binding was blocked with 3% BSA in PBS for 30 minutes 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 1hour. Secondary anti-human antibodies (Jackson, West Grove, PA) were labeled 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 hour at 37°C.

5.2.7. Silver staining of NORs

Chromosomal spreads (prepared as descibed 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 1ml pure formic acid) in dark wet chamber at room temperature for 45 minutes (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% ethanols and denaturated 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 minutes, followed by 20% sodium thiosulfate for 5 minutes (Zurita et al., 1998). Where upon the spreads were processed for the hybridization.

5.3. Results

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

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 the further study. With respect to acrocentric chromosomes, we have regularly found trisomies of the chromosomes 13, 14, 21, 22 (one chromosome 22 included also material from chromosome 8), two to three chromosomes 15 and one metacentric marker containing the long arms of chromosome 15 (Fig. 1), in agreement with the descriptions provided by other authors (see the comparative data in: Macville et al., 1999). After the hybridization with 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 probe for the chromosome 15 (Fig. 2A,B,E,G, Table 1). All these chromosomes exhibited rDNA signal close to centromeric region. The metacentric has been earlier described as 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 includes also some material from the short arms of the chromosome 15, so this chromosome may be, for example, dicentric.

In more than 90% cells, NORs were found on all three chromosomes 14, three chromosomes 15, as well as 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 (Fig. 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.

5.3.2. Around 9 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 per cent) 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 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; Zurita et al., 1999). We accordingly used the silver staining as alternative method for revealing competent NORs. This procedure afforded in all cases 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 labeling. Therefore we 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, all chromosomes 15 (3 acrocentrics + metacentric marker), two chromosomes 21, and two chromosomes 22 (Fig. 2C,D,F,G). Interestingly, Sirri et al. (1999) observed, as can be assessed from their published figures, 6 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 9 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 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 (Fig. 2 E-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 (Fig. 1).

The results obtained on mitotic chromosomal spreads are summarized in Table1 and Fig. 4. These data show a high regularity in 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.

5.3.3. 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 normal diploid karyotype (data not shown).

Using rDNA hybridization we observed NORs on all chromosomes 13, 14, 15, 21 and 22 (Fig. 3 A,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 (Fig. 3C,D). Chromosomes 14 were both positive on 85% spreads. Chromosomes 21 and 22 carried competent NORs on one or both chromosome copies with similar frequencies (Table 2, Fig. 4).

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

5.3.4. 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 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. 9 in HeLa and 8 in LEP cells. In colocalization experiments, in more than 95 per cent of cells, the intensities of UBF and bromo-uridine signals obviously correlated (Fig. 5 A-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.

5.3.5. 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 premature chromosomal condensation (PCC) induced by calyculin A. This drug, being 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 bromo-uridine. These changes could not be thoroughly reversed after cultivation in fresh medium. On the spread preparations (Fig. 6), complete PCC was observed in G2 phase (Fig. 6,F). Only a weak condensation was perceived in G1 (Fig. 6,B). In S-phase the chromosomes were "pulverized", i.e. fully condensed segments alternated with non-condensed (Fig. 6,D); the latter included BrdU if

it had been introduced for 5 minutes 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 a 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 (Fig. 6,A-F). At least 100 cells were studied for each assay. As in the case of mitotic spreads, we revealed in G1 and G2 phases most frequently 9 UBF signals for the HeLa cells, 8 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 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 per cent) became doublets, like in mitosis. To examine if the highly specific and regular pattern of competence found for HeLa cells in mitosis persists also 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. But in G2 phase we observed the same number of competent NORs for all chromosomes 13, 14, 15, 21 and 22 as in mitosis (Fig. 6 G,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 (Fig. 6G, H).

We thus demonstrated that that the pattern of transcriptional competence revealed in G2 phase is conserved also 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 S phase.

5.4. Discussion

5.4.1. NORs in mitosis: regular distribution of the competence signal among the mitotic chromosomes.

No data are available in 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 (Fig. 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 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, Fig. 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 regular pattern may be a common feature of human-derived cells, though 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 Fig. 4). It should be mentioned that we did not follow each individual chromosome, so we cannot rule out a posibility 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; Zurita et al., 1999), the level of Ag impregnation of the competent NORs correlates with 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 AgNORs, whereas quite a weak hybridization signals corresponded to the prominent silver staining of the chromosomes 15 (Fig. 2,A-G). So the correlation between the gene number, as judged from the hybridization signal, and the competence is 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.

5.4.2. NORs during interphase

In the human interphase cells, individual NORs do not correspond to the nucleoli, fibrillar centers 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 (8 for LEP and 9 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 reorganization 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 Upstream Binding Factor 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. But 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 the formation of pre-initiation complex, and SL1 can interact with rDNA independently (Friedrich et al., 2005);

- 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 be only an activator of the rDNA transcription. If so, can UBF be a marker of the transcribed NORs? Qualitative data on colocalization 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 bromo-uridine signals on the same NOR positively correlated (Fig. 5A-C). Similar

correlation of UBF and transcription signals was observed also on metaphase cells after stimulation with roscovitine (Fig. 5D-F). Thus, we demonstrate that the incipient or stimulated transcription activity of the NORs exactly follows the pattern of transcription competence, which persists over the whole 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.

5.5. Tables and figures

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 analyzed.

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.

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 analyzed.

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

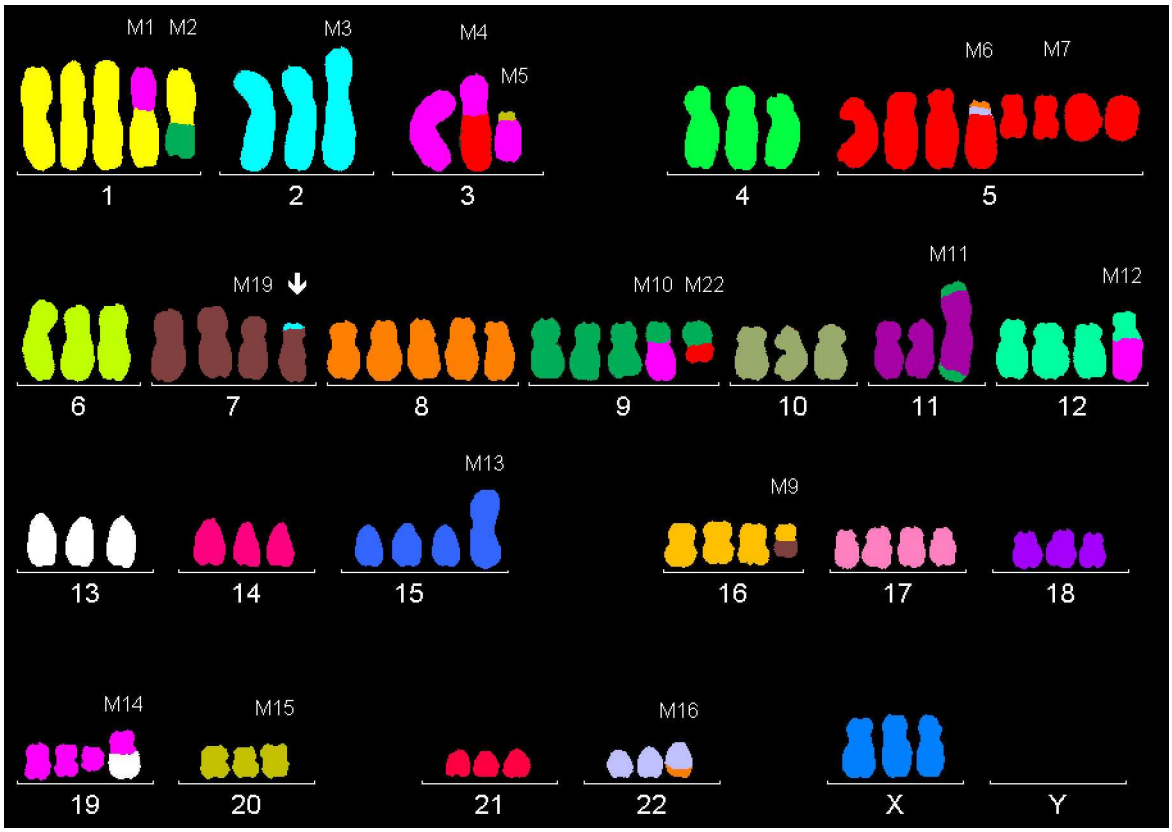


Fig. 1. Karyotype of the HeLa cells assessed by multicolor fluorescence *in situ* hybridization (M-FISH). There are trisomies of acrocentric chromosomes 13, 14, 15, 21, 22. One marker chromosome M13 appears as isochromosome 15; p-arms of the chromosome 13 are included into the marker M14 together with 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.

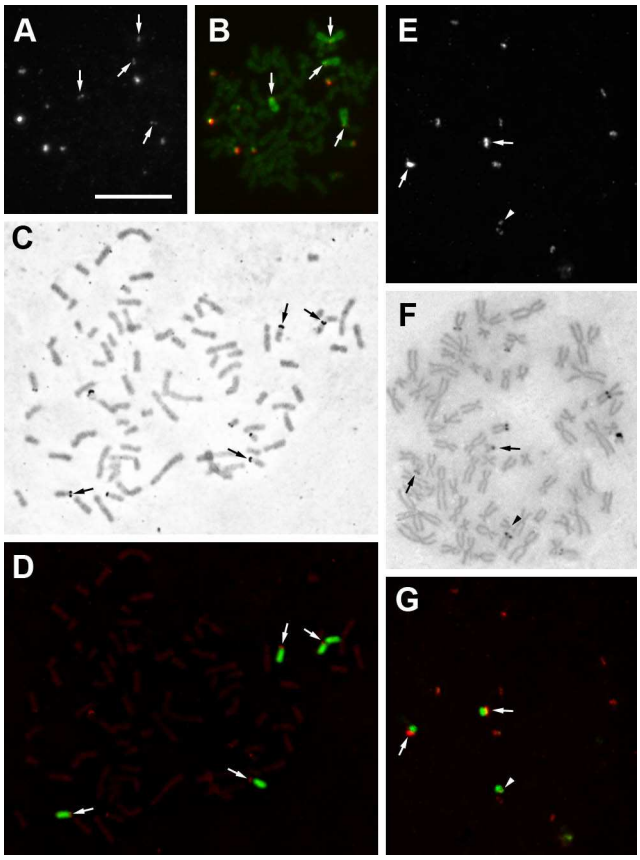


Fig. 2. Localization of NORs and competent NORs on the spread mitotic chromosomes of HeLa cells.

A, **B**: simultaneous rDNA (**A**, red in **B**) and chromosome 15 detection (green in **B**). 13 rDNA signals are clearly recognized in A. Three acrocentrics and one metacentric chromosome are labeled with 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 4 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 silver-negative NOR (arrowhead in **F**, **G**). Bar: 20μm.

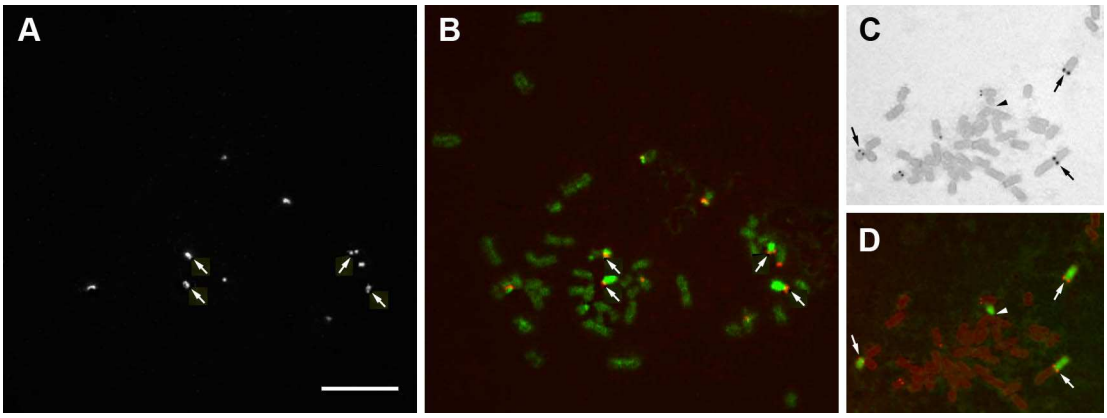


Fig. 3. Localization of NORs and competent NORs on the spread mitotic chromosomes of LEP cells.

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.

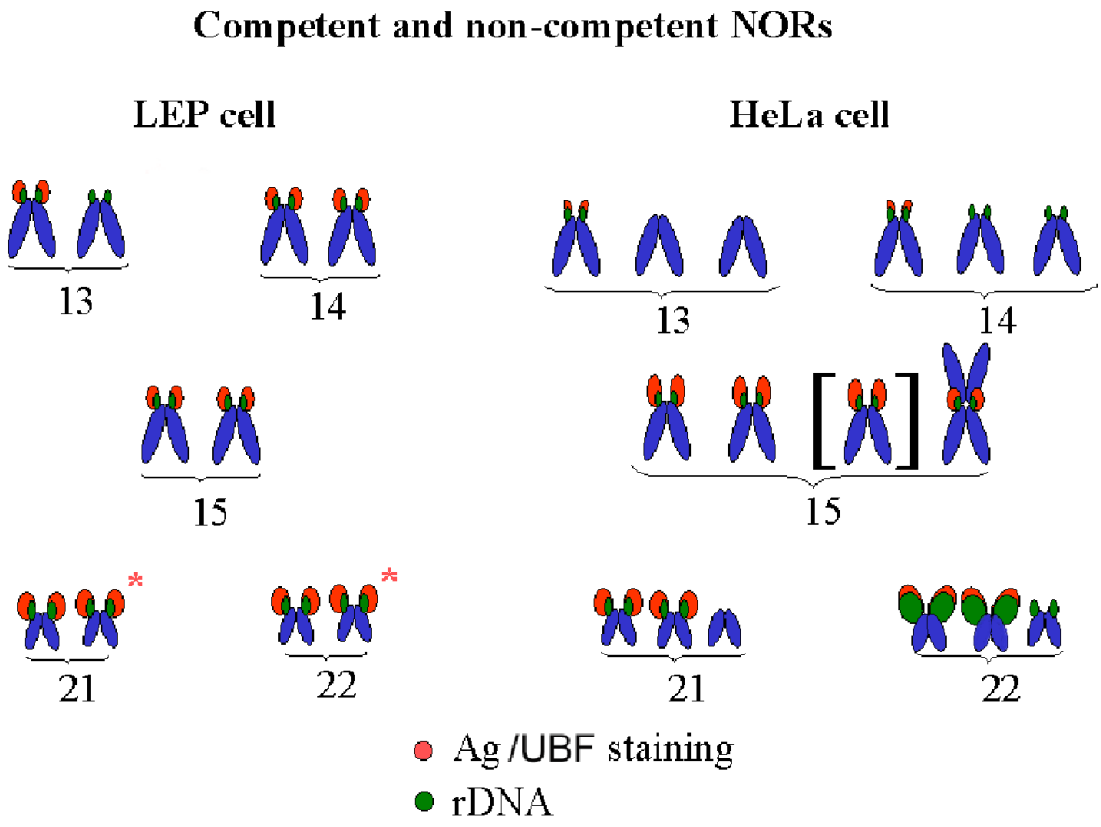


Fig. 4. Distribution of the NORs (green) and competent NORs (red) among the different chromosomes in HeLa and LEP cells. One of the chromosomes 15 of the HeLa cells is taken in parenthesis to show the variability of the chromosome number. Asterisks indicate two chromosomes of LEP cells which may be competent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

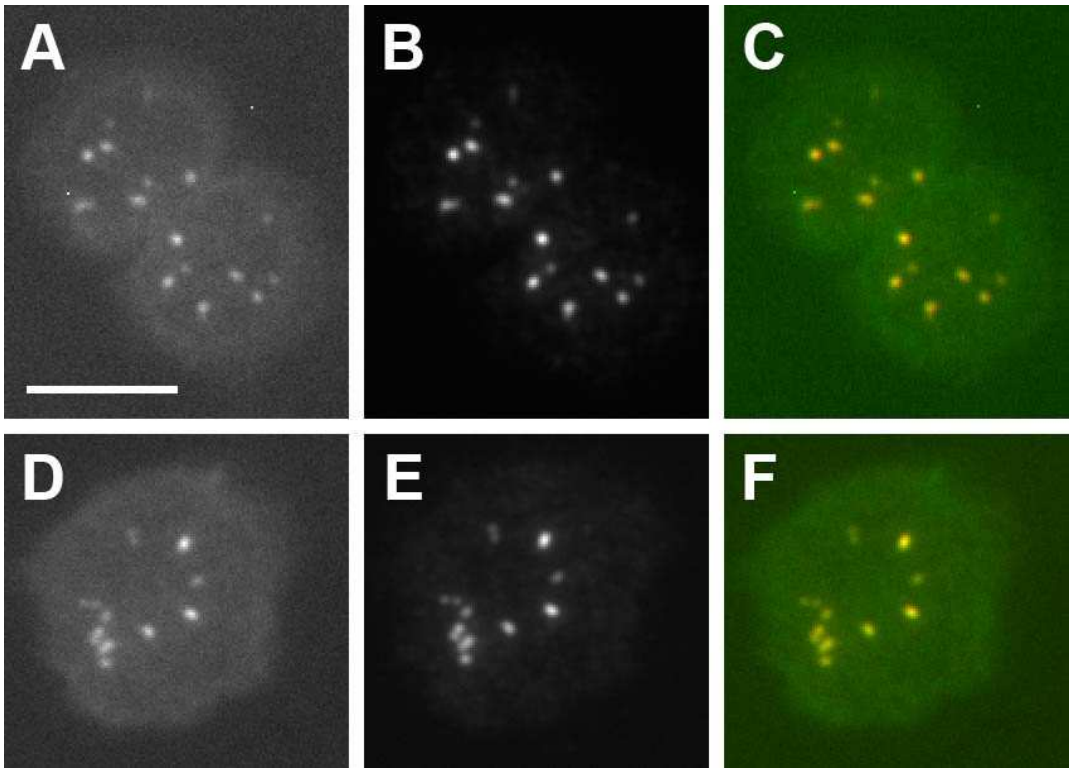


Fig. 5. Colocalization of transcriptionally active and competent NORs in telophase (**A - C**) and metaphase after roscovitine treatment (**D - F**). **A**, **D**: transcription signal (bromo-uridine); **B**, **E**: UBF. **C**, **F** colocalization of transcription (green) and UBF (red) signals.

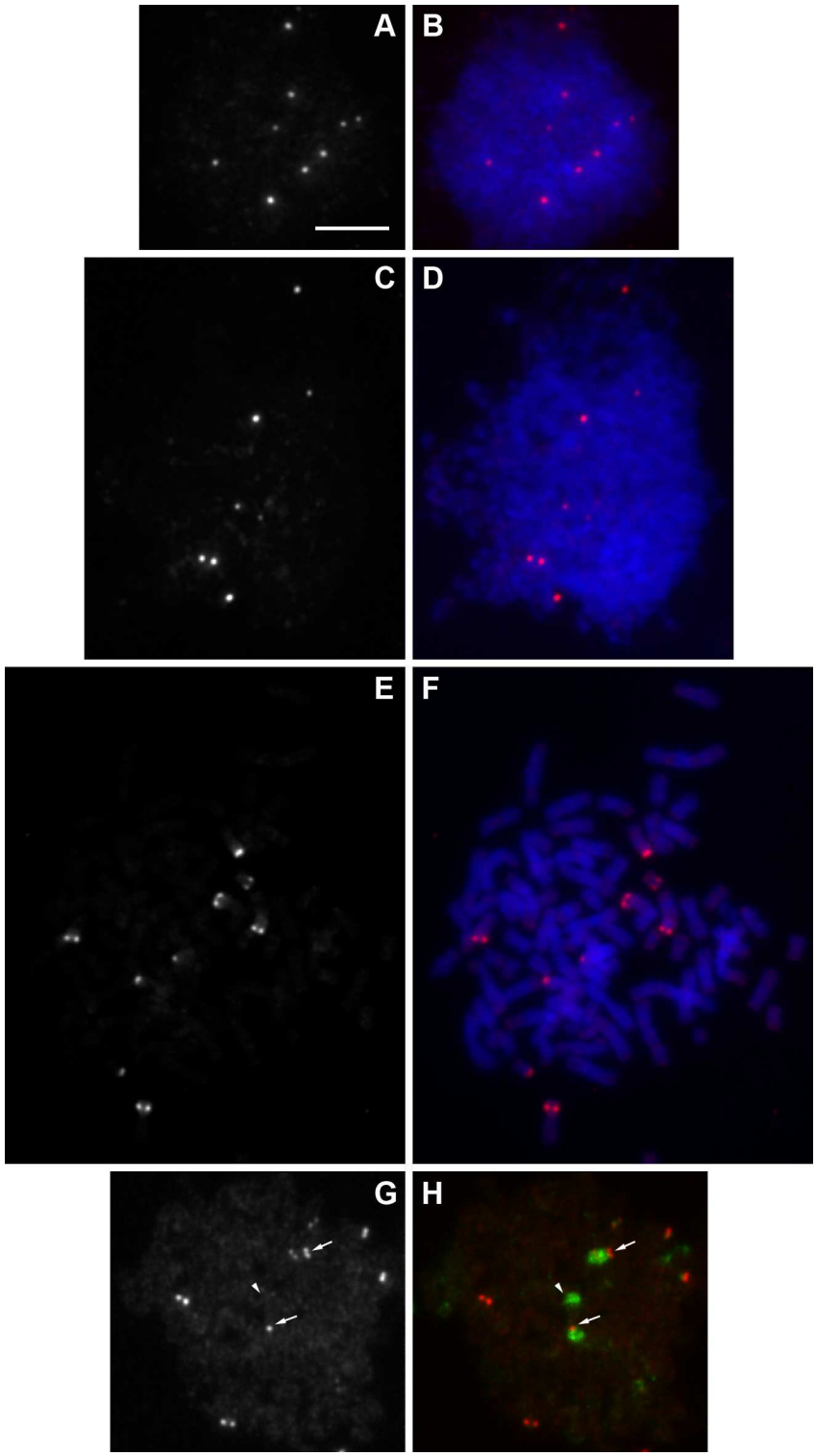


Fig. 6. A-F. UBF-positive NORs (**A-E**, red in **B-F**) in the interphase HeLa cells after calyculin A treatment.

A, **B**: G1 phase; **C**, **D**: S phase; **E**, **F**: G2 phase. DAPI counterstaining is in blue. 9 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. 3 F,G).

Chapter 6. Positioning of NORs and NOR-bearing chromosomes in relation to nucleoli

6.1. Introduction

During interphase, chromosomes exist in the form of well discernible, though highly porous, territories (Cremer et al., 1993; Verschure et al., 1999; Visser et al., 2000; Cremer and Cremer, 2001; Parada and Misteli, 2002; Pederson, 2004; Foster and Bridger, 2005; Cremer and Cremr, 2006). It is widely accepted that these chromosome domains occupy more or less fixed positions in the mammalian interphase nucleus, depending on the gene density, replication timing and the size of chromosome territory (Zink et al., 1998; Manders et al., 1999; Sun et al., 2000; Vazquez et al., 2001; Parada et al., 2003; Walter et al., 2003). Results of several studies indicate that the location of chromosomes is related to the activity of their genes (Mahy et al., 2002; Gilbert and Ramsahoye, 2005). The gene-rich chromosomes are preferentially located at the nuclear center, though the preference disappeared after inhibition of transcription, suggesting that the chromosome positioning may depend on the transcription activity. Other data argue that particular chromosome positioning places genes into special neighborhood favorable for their expression or silencing (Parada and Misteli, 2002). The situation is complicated by recent data indicating that chromosome territories partially intermingle in human cell nuclei (Branco and Pombo, 2007) and some genes can be found far beyond the area defined as chromosome territory (Baxter et al., 2002; Mahy et al., 2002; Chubb and Bickmore, 2003; Kioussis, 2005; Wegel and Shaw, 2005; Morey et al., 2007; Bártová et al., 2008). Thus, the large-scale order of chromosome positioning and its relation to gene activity remain unclear.

Human ribosomal genes represent a convenient model to address specific aspects of this problem. These genes form clusters, named nucleolus organizer regions or NORs, in each of the acrocentric chromosomes 13, 14, 15, 21 and 22 (Henderson et al., 1972; Long and Dawid, 1980; Puvion-Dutilleul et al., 1991). Essential components of the RNA polymerase I transcription machinery, including Upstream Binding Factor (UBF), can be detected by immunocytochemistry or silver staining on some NORs, termed

‘‘transcriptionally competent’’ or ‘‘competent’’, during mitosis. It is generally accepted that competent NORs are transcriptionally active during previous interphase (Weisenberger and Scheer, 1995; Roussel et al., 1996; Gebrane-Younes et al., 1997). Nucleoli reform after mitosis around transcriptionally active, and therefore necessarily competent, NORs (Ochs et al., 1985; Benavente et al., 1987; Jimenez-Garcia et al., 1994), and the integrity of nucleoli depends on expression of ribosomal genes (Melese and Xue, 1995; Scheer and Hock, 1999; Dousset et al., 2000). However, the position of the non-competent NORs that exhibit a condensed chromatin structure (O’Sullivan et al., 2002), and chromosomes carrying the non-competent NORs, with respect to nucleoli remains unclear.

Importantly, we have already shown regularities in distribution of competent as well as non-competent NORs among the specific chromosomes in two human-derived cell lines, transformed HeLa and primary LEP cells (Smirnov et al., 2006), and established that all HeLa cells, and more than 95% LEP cells, contain at least one non-competent NOR (Smirnov, unpublished observations). More specifically, we showed that in the aneuploid HeLa cells the NORs belong to acrocentric chromosomes 13, 14, 15, 21, 22 and a metacentric one painted with probe for chromosome 15. The signals of transcriptional competence (silver or UBF signals) were usually present on one chromosome 13, one chromosome 14, all chromosomes 15 (three acrocentrics and one metacentric), two chromosomes 21, and two chromosomes 22. In LEP cells, 10 NORs were regularly observed on mitotic chromosome spreads. In these cells, both copies of the chromosome 15, but only one of the chromosomes 13, regularly contained transcriptionally competent NORs. Both chromosomes 14 contained competent NORs in 85% cases. Small acrocentrics 21 and 22 displayed less regularity: one or both homologues carried competent NORs with comparable frequencies (Smirnov et al., 2006).

In the present study, we expand the results of Smirnov et al. (2006) and analyze nuclear positions of competent and non-competent NORs, as well as chromosomes bearing NORs, with respect to nucleoli in HeLa and LEP interphase cells.

6.2. Material and Methods

6.2.1. Cell culture

HeLa, aneuploid cell line, that have stable karyotype without considerable variations (Macville et al., 1999; Smirnov et al., 2006), and primary LEP cells were cultivated in flasks or on coverslips at 37°C in Dulbecco modified Eagle's medium (DMEM, Sigma, USA) containing 10% fetal calf serum, 1% glutamine, 0.1% gentamycin, and 0.85g/l $NaHCO_3$ in atmosphere supplemented with 5% CO_2.

6.3. Antibodies and DNA probes

Commercial Cy3- and FITC- labeled whole chromosome painting probes for human chromosomes 13, 14, 15, 21 and 22, supplied ready to use in hybridization mixture (Appligene Oncor, USA), and pA and pB rDNA probes, prepared from a pA and pB plasmid constructs (Erickson et al., 1981), kindly donated by James Sylvester (Nemours Children's Clinic Research, Orlando, FL) were used. The pA probe contains the 3` end of 18S rDNA, the 5.8S rDNA, both internal transcribed spacers, and most of the 28S rDNA. The pB probe contains the promoter, the external transcribed spacer, and the 5` end of the 18S subfragment. The probes were labeled by biotin using nick-translation kit BIONICK Labeling System (GIBCO-BRL, Invitrogen) according to the manufacturer`s instructions. The rDNA probes were stored in hybridization mixture containing 25 ng of probe, 0.5 mg/ml sonicated salmon sperm DNA, 50% deionized formamide, 2x SSC and 10% dextran sulfate at -20°C. Both rDNA probes exhibited the same pattern of FISH-labeling. Therefore, only the results obtained with pB probe were used for statistical analysis.

Primary monoclonal antibody against mouse fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA), was used for immunovisualization of nucleoli. Biotinylated rDNA probe was labeled after FISH with monoclonal rabbit anti-biotin antibodies (Enzo, Roche). Secondary anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) were conjugated with Cy3 or FITC.

6.3.1. Immunofluorescence

Cells growing on coverslips were washed in phosphate-buffered saline (PBS), fixed in methanol at -20°C for 30 min and air-dried. Following three washes in PBS, the cells were incubated with anti-fibrillarin antibody, washed in PBS and incubated with secondary antibodies conjugated with either FITC or Cy3.

6.3.2. Immuno-FISH and FISH

The combined detection of fibrillarin and *in situ* hybridization (immuno-FISH) was performed after Pliss et al., 2005. After fibrillarin imunolabeling, as described above, the cells were postfixed with methanol/acetic acid (3:1) overnight at -20°C, then the regular FISH procedure followed (Pliss et al., 2005), except the post hybridization washing. Namely, the cells were washed in 50% formamide in 2xSSC, pH 7, for 15 min at 43°C, in 0.1% Tween-20 /2xSSC for 8 min at 43°C; in 0.1% Igepal (ICN Biomedicals, Inc) / 4xSSC for 3 x 4 min at 37°C, in PBS 3 x 3 min at RT (Harničarová et al., 2006). After FISH, biotinylated rDNA probes were detected using respective primary and secondary antibodies.

For the combined detection of fibrillarin and double-FISH (i.e. triple-labeling), the fibrillarin immunolabeled cells were first photographed and their position on the slide marked before methanol-acetic acid postfixation. Then the FISH with rDNA and chromosome probes was performed, and the same cells were photographed again. This method was used to achieve the best visualization of nucleoli.

To ensure the detection of all extranucleolar rDNA foci, we employed an alternative approach avoiding fibrillarin labeling. Accordingly, the cells were fixed in methanol/acetic acid (3:1) for 30 min at -20°C. After air-drying the cells were processed for FISH as described above, and nucleoli were visualized by phase contrast and as DAPI negative areas. Although the nucleolar areas could not be identified as precisely as after fibrillarin immuno-labeling, the numbers of the extranucleolar rDNA foci matched well with the results obtained by the immuno-FISH. Thus we observed in HeLa cells no extranucleolar foci in 68% cells, one focus in 20% cells, two foci in 5% cells, three foci in 4% cells and four foci in 1% cells (compare with Fig. 4B).

The results of all single labeling (fibrillarin immuno-labeling and FISH), double labeling (fibrillarin immuno-labeling combined with FISH and double-FISH) and triple labeling experiments (fibrillarin immuno-labeling and double FISH) were compatible.

Coverslips were mounted in Mowiol supplemented with DABCO and viewed using Olympus AX70 Provis equipped with the Photometrics CCD camera or Leica TCS NT confocal microscope.

All statistical evaluations were obtained by analysis of 100 HeLa and LEP cells.

6.4. Mathematical 2D random model system

We chose 2D analysis, because it allows statistical evaluation of large numbers of images. 2D-analysis has been used for the study of nuclear positioning of DNA loci and chromosome territories in cells that are grown on glass surface and have flattened nuclei (see e.g. Volpi et al., 2000; Mahy et al., 2002; Parada et al., 2004b; Taslerová et al., 2006), and similar results with respect to the mutual orientation of these objects were obtained by 2D and 3D-analysis (Mahy et al., 2002; Morey et al., 2007).

We used a model in which polygonal chromosomes were randomly positioned within elliptic nucleus containing randomly positioned round nucleoli. The parameters: area of the nucleus, its major axis length, total area occupied by nucleoli and chromosomes, the number of nucleoli and chromosomes were obtained as mean values of measurements and counts on the cells after hybridization. The geometric parameters were measured by means of the Soft Imaging System (Analysis program).

6.5. Results

6.5.1. Nucleolar association of the interphase NOR-bearing chromosomes correlates with transcriptional competence of their NORs in HeLa and LEP cells

We analyzed nucleolar association of the NOR-bearing chromosomes in HeLa (containing in average 4.03 ± 0.12 nucleoli; mean value and standard error are indicated) and LEP cells (containing in average 2.04 ± 0.10 nucleoli), bearing in mind the established occurrence of competent and non-competent NORs in homologous NOR-bearing chromosomes (Smirnov et al., 2006). Fluorescent *in situ* hybridization with probes for chromosomes 13, 14, 15, 21 and 22 was performed on interphase cells (Fig. 1). Some of the studied chromosomes had no significant contact with nucleolus revealed by fibrillarin immunolabeling that is commonly used for the visualization of nucleoli. Such "extranucleolar" chromosomes were frequently distanced from nucleoli by more than one micrometer. On the other hand, the majority of NOR-bearing chromosome territories were associated with nucleoli. Different forms of the nucleolar association were observed for different chromosome homologues (Fig. 1). In the case of chromosomes 13 and 14 in both HeLa and LEP cells, the painted part of chromosome was typically straight, more or less elongated and entering nucleolus at one point. Second form is represented by the chromosomes 15 which, in both HeLa and LEP cells, often penetrated to the centre of nucleolus or even traversed its area. Other chromosomes (especially the chromosomes 21 and 22 in HeLa cells) appeared as semilunar structures embracing nucleolus. All these cases were considered here as an association of chromosome with nucleolus. Sometimes, we observed long thin filaments connecting the extranucleolar chromosome territory with nucleolus (Fig. 2). These cases were not considered as nucleolar associations

The percentage of the chromosomes associated with nucleoli in HeLa and LEP cells is shown in Fig. 3. In this Figure we also compared the experimental data with the results provided by a mathematical 2D random model system in which chromosomes and nucleoli were randomly scattered within an elliptic nucleus (see Material and methods). In this model nucleolar association depends on the chromosomal size which was determined by hybridization signal. Thus, the difference between the observed data and those predicted by the model reflected the affinity of a certain chromosome type towards

nucleoli and this affinity was not influenced by the size of chromosomes. In all studied cases this difference significantly exceeded the level of measurement error, and was proportional to the value (percentage) of the nucleolar association for each type of chromosome. It should be emphasized that the majority of NOR-bearing chromosomes were asscociated with nucleoli and the number of nucleoli-associated chromosomes generally exceeded the number of competent NORs (Fig. 3A; Smirnov et al., 2006).

In HeLa cells, chromosome 15, being the main contributor of competent NORs (Smirnov et al., 2006), was at the same time the most frequently associated with nucleoli (Fig. 3). Chromosomes 13 and 14, regularly containing respectively one and three NORs, but only one competent NOR (Smirnov et al., 2006), contacted nucleoli with a low frequency. Chromosomes 21 and 22 possessing an intermediate number of competent NORs (Smirnov et al., 2006) showed also an intermediate level of nucleolar association.

To assess correlation between the number of transcriptionally competent NORs in particular chromosomes and their association with nucleolus in LEP cells, we chose to compare chromosome 15 and 13, because they showed the most regular pattern and represented correspondingly the maximum and minimum number of competent NORs (Smirnov et al., 2006). We found that chromosomes 15 associated with nucleoli by far more frequently than chromosomes 13 (Fig. 3). This indicated that in the nuclei of diploid LEP cells, chromosomes carrying competent NORs had a tendency to be associated with nucleoli similarly as in the transformed HeLa cells.

We thus conclude that the tendency of rDNA-bearing chromosome homologues to associate with nucleoli correlates with the number of transcriptionally competent NORs in these homologues.

6.5.2. The majority of the transcriptionally non-competent NORs in HeLa and LEP cells are situated within the nucleoli

A simple explanation of the observed regularity in the nucleolar associations of the NOR-bearing chromosomes can be provided by supposing that only transcriptionally active or competent NORs participate in the formation of nucleoli. To check this hypothesis, we combined *in situ* hybridization with rDNA probe and fibrillarin immunolabeling (Fig. 4A). Although for obvious reasons we could not count individual

NORs within the nucleoli, this procedure enabled us to count rDNA foci localized outside the fibrillarin-positive nucleoli (Fig. 4B). Of course, such extranucleolar foci could include two or more coalesced rDNA clusters. Despite this, we found that the extranucleolar rDNA foci were absent in about 73% of HeLa cells and about 87% of LEP cells (Fig. 4B). We can, therefore, conclude that most NORs, both competent and non-competent, should be situated in nucleoli.

Since some chromosomes are quite frequently not associated with nucleoli (Figs. 1 and 3A), and most of non-competent NORs are found in nucleoli, our results also imply that some NORs may be distanced from the respective chromosome territories. To confirm this, we used the fact that all three homologues 14 in HeLa cells carry NORs (Smirnov et al., 2006), and not all of these chromosomes are found in association with nucleoli (Figs. 2, 3A). Performing FISH experiment with probes for chromosome 14 and for rDNA, in combination with fibrillarin immunolabeling, we regularly found that some extranucleolar chromosome territories were not co-localized with any rDNA (Fig. 5). Therefore, some NORs, or at least their parts containing rDNA coding regions, should be located within nucleoli, and connected to the respective chromosome territories via thin filamentous protrusions. Indeed, we sometimes identified a weak FISH signal connecting the chromosome 14 territory with the nucleolus (Fig. 2). Since the nucleolar association of NOR-bearing homologues correlates with the number of transcriptionally competent NORs in these homologues, we speculate that the NORs distanced from their chromosome territories are non-competent.

We thus conclude that most of the transcriptionally non-competent NORs are situated in the nucleoli, and some NOR-bearing chromosomes are positioned in such manner that their NORs are located in the nucleolus, while the bulk of the chromosome territory is distanced from the nucleolus.

6.6. Discussion

Results of the present work expand our knowledge of the intricate spatial relationship among nucleoli, NOR-bearing chromosomes and both competent and non-competent NORs in the interphase nucleus of human-derived Hela and LEP cells.

We found that the higher is the number of competent NORs in the given NOR-bearing homologues, the higher is the frequency of close nucleolar associations of these homologues (Fig. 3). It should be mentioned, however, that the given human homologues may carry predominantly either competent or non-competent NORs depending on the cell type. For instance, chromosomes 15 carry maximal number of competent NORs in both HeLa and LEP cells (Smirnov et al., 2006), whereas in human lymphocytes these chromosomes carry lower number of competent NORs than the other type of NOR-bearing homologues (Heliot et al., 2000).

The correlation between the close nucleolar associations of the NOR-bearing chromosomes and the transcription competence of their NORs, as observed in this study, can be explained straightforwardly by the effect of the rDNA transcription activity on the chromosome positioning. We have to mention in this respect that the total number of the competent NORs does not change significantly during the cell cycle (Smirnov et al., 2006), and rDNA transcription starts at the end of mitosis, when NORs still exist as individual entitities. Both nucleoli and chromosome territories are formed later on in G1 phase (Weisenberger and Scheer, 1995; Roussel et al., 1996; Gebrane-Younes et al., 1997; Raška et al., 2006; Smirnov et al., 2006). On the other hand, we observe in the present study that the number of chromosomes closely associated with nucleoli generally exceeds the number of competent NORs (Fig. 3A, Smirnov et al., 2006). For instance, in the case of HeLa chromosomes 14, only one of three NORs is usually competent, while in average more than one chromosome were found in close association with nucleoli (Figs. 2 and 5). Thus, nucleolar association of the NOR-bearing chromosome is not sufficient for the activity of its NOR. These data, therefore, suggest that transcription competence of NORs represent one factor that can cause the respective chromosome association with the nucleoli, but other players are apparently involved as well. Beside the role of the transcribed sequences of ribosomal genes, of course, together with associated protein and nucleoprotein complexes (Raška et al., 2006)**,** we cannot exclude a role of other sequences

including also the non-transcribed spacers and flanking sequences that can be found within, or at the periphery, of nucleoli (Kaplan et al., 1993; Santoro, 2005).

Our data enable us to draw an interesting conclusion about the localization of non-competent NORs. On the one hand, most of the HeLa and LEP cells do not possess any extranucleolar rDNA. On the other hand, all HeLa cells and more than 95% of LEP cells contain at least one non-competent NOR during mitosis (Smirnov, unpublished observations). Moreover, after treatment with calyculin A, that allows visualization and quantification of individual NORs in interphase, we find the same number of non-competent NORs as in mitosis (Smirnov et al., 2006). Therefore we conclude that a vast majority of NORs must be localized in nucleoli

Nucleolar location of the non-competent or transcriptionally silent NORs has been reported in several other studies. For instance, in a mouse/human hybrid system, where mouse cells contained one human acrocentric chromosome (Sullivan et al., 2001), the NOR belonging to this chromosome was localized in nucleolus, but remained transcriptionally silent. Clusters of inactive, methylated rDNA repeats were found in the nucleoli of normal mouse neurons (Akhmanova et al., 2000), although it was not demonstrated that these clusters consisted of silent NORs. Importantly, here we present the first focused documentation testifying for the accommodation of the majority of non-competent, and so presumably silent, NORs in the nucleoli of human cells. It is not yet clear why non-competent NORs are situated in the nucleolus, and we are unable to further discuss the causes for this phenomenon on the basis of results of the present study. Our data, however, indicate that the localization of NORs in the nucleolus is to some extent independent from their transcription activity.

Using triple labelings of the nucleoli, rDNA and chromosome 14 territory (Fig. 5), we have shown that some NORs (supposedly non-competent) are situated as far as several micrometers away from the areas occupied by the corresponding chromosome territory. In such cases we sometimes observe extended protrusions of chromatin connecting extranucleolar chromosome territories with the nucleoli. An analogous situation has been also described for a number of non-ribosomal genes that are found to be located on the long loops extending from the chromosome territories (Baxter et al., 2002; Mahy et al., 2002; Chubb and Bickmore, 2003; Kioussis, 2005; Morey et al., 2007; Bártová et al., 2008).

In summary, the large-scale positioning of the NOR-bearing chromosomes, through their associations with nucleoli, is closely linked to the transcription activity of rDNA. The tendency of rDNA-bearing chromosomes to associate with nucleoli correlates with the number of transcriptionally competent NORs in the respective chromosome homologues. Importantly, not only competent, but also most of non-competent NORs, are included in the nucleoli. Some intranucleolar NORs are situated on elongated chromatin protrusions connecting nucleoli with respective chromosome territories distanced from nucleoli.

6.7. Tables and figures

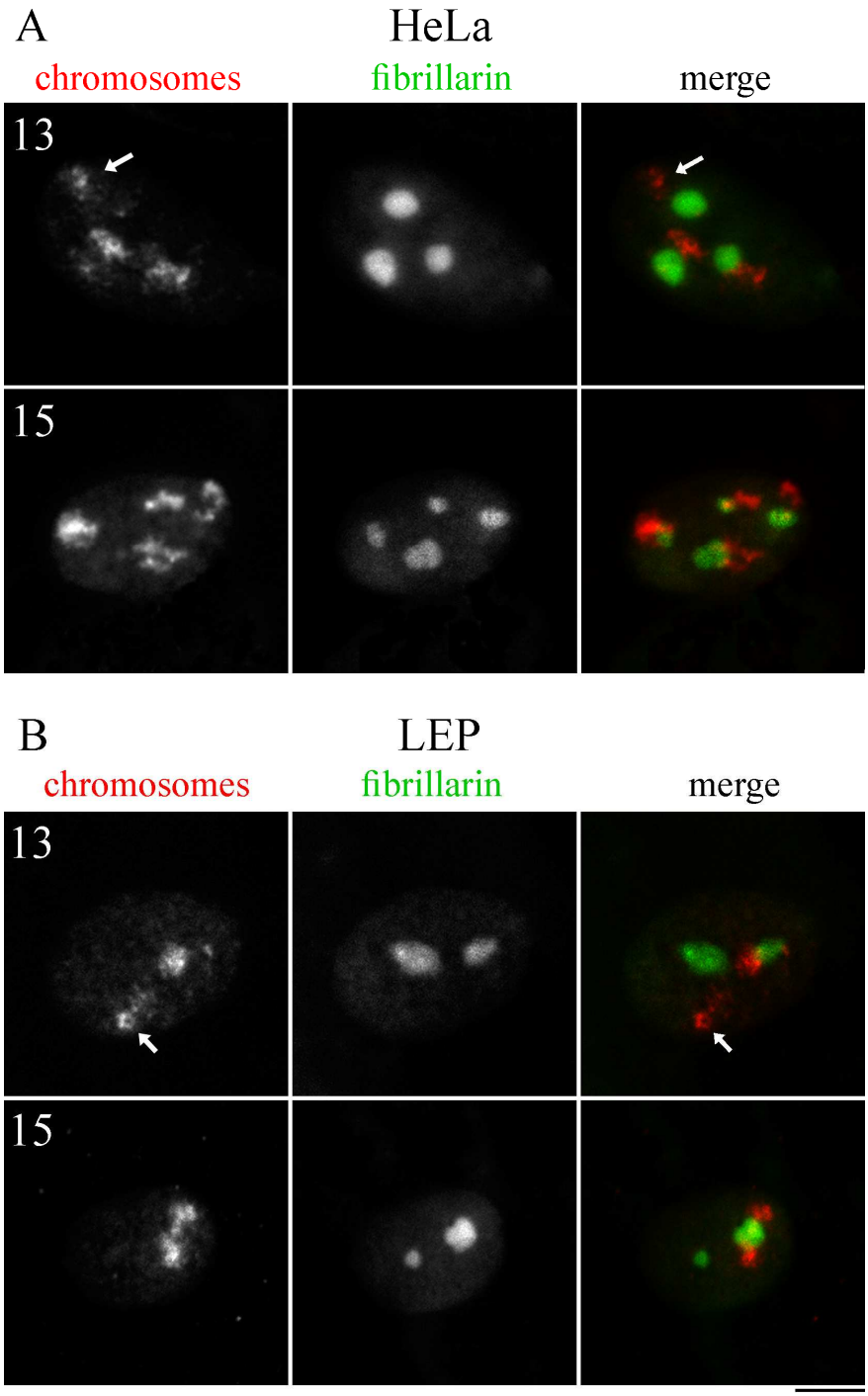


Fig. 1 . Association of the chromosomes 13 and 15 with nucleoli in interphase HeLa (A) and LEP (B) cells. FISH signal with the specific chromosome probes (in red) was observed in interphase cells. Immunocytochemistry with fibrillarin was used to visualize nucleoli (in green). The transformed HeLa cells contain three homologues of the chromosomes 13 and four homologues of the chromosome 15. The primary LEP cells have normal karyotype with two chromosomes 13 and 15. In both HeLa and LEP cells, the chromosome homologues 15 appeared usually in close association with nucleoli, while one of the homologues 13 was often distanced from the nucleoli (arrow). Bar: 10 μm

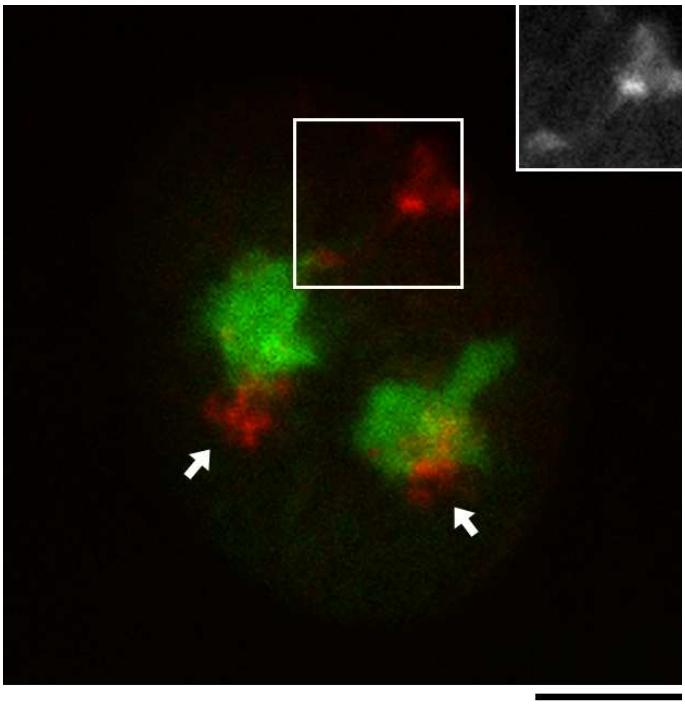


Fig. 2. A thin protrusion between the extranucleolar chromosome territory and nucleolus. Two chromosome homologues 14 (in red; arrows) in the HeLa cell are closely associated with fibrillarin-positive nucleoli (in green). The third chromosome homolog 14 (in red) is distanced from nucleolus and connected to it via a long chromatin protrusion that ends with a thickening at the contact point with the nucleolus. Since the corresponding chromatin signal is weak in the merge image, this protrusion is shown in the one channel grey scale insert. Bar: 10 μm

A

Cell line	Chromosomes	Proportion of chromosomes carrying competent NORs (%)	Observed proportion of chromosomes associated with nucleoli (%)	Proportion of associated chromosomes predicted by random model (%)	Difference between observed and predicted values (%)
HeLa	13	**30 ± 2**	60 ± 2	50	**10**
	14	**30 ± 2**	59 ± 2	47	**12**
	15	**99 ± 1**	98 ± 1	37	**61**
	21	**63 ± 3**	78 ± 2	35	**43**
	22	**67 ± 5**	69 ± 2	35	**34**
LEP	13	**50 ± 5**	58 ± 2	43	**15**
	15	**97 ± 4**	88 ± 1	38	**50**

B

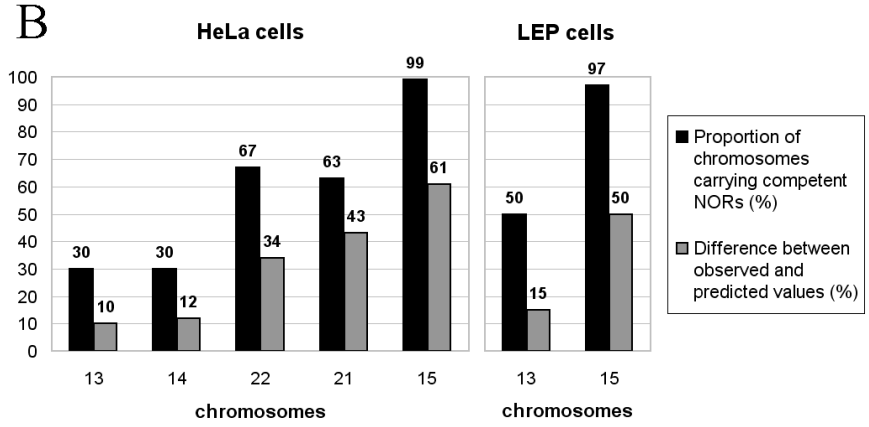
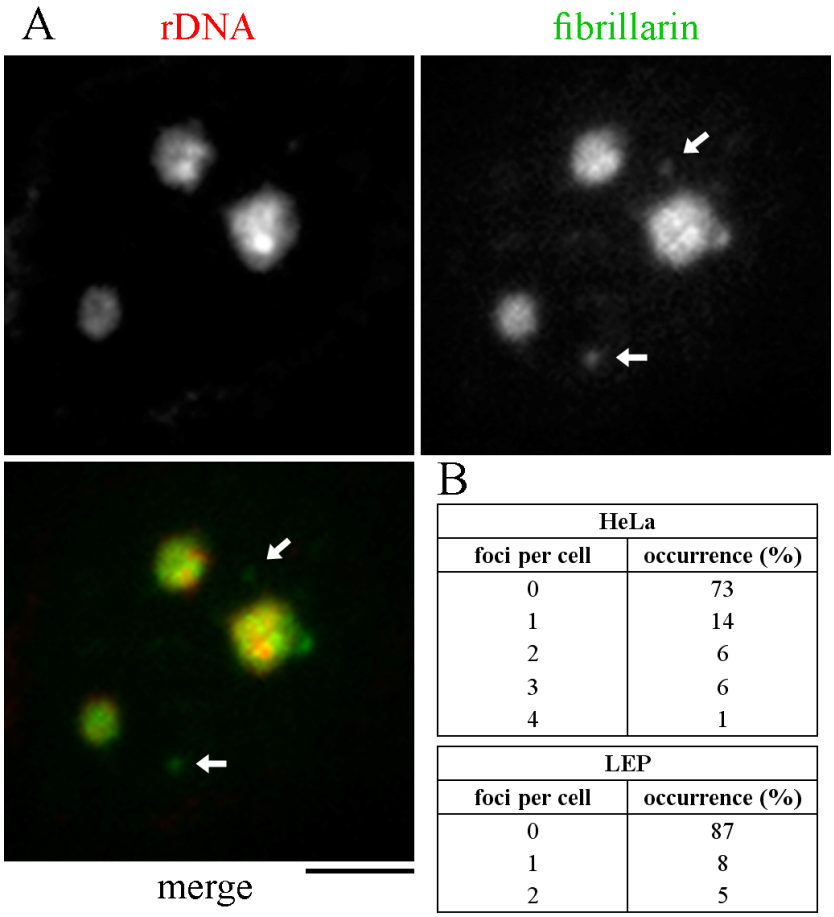


Fig. 3. Transcription competence of the NORs belonging to different chromosomes correlates with nucleolar associations of these chromosomes. (A) The percentage of the nucleoli closely associated chromosomes was counted in HeLa and LEP cells. Respective numbers of the competent NORs in different NOR-bearing chromosomes in these cells have been obtained from colocalization experiments (Smirnov et al.,2006). The results are compared with the data provided by mathematical random 2D model where the percentage of the association depends on the size of chromosomes detected by chromosomal probe. In all studied cases the differences between the observed data and those predicted by the model significantly exceed the level of measurement error and reflect the affinity of certain NOR-bearing chromosome homologues towards nucleoli. Mean values and standard errors are indicated. (B) The relevant bar diagrams are shown to clearly document the observed positive correlation between the proportion of chromosomes carrying competent NORs (black bars corresponding to the third column in Fig. 3A) and the close nucleolar association of the chromosomes (gray bars corresponding to the last column in Fig. 3A). Accordingly, the chromosomes in HeLa cells are, in contrast to Fig. 3A, arranged upon the increasing value of their nucleolar association.



B

HeLa	
foci per cell	**occurrence (%)**
0	73
1	14
2	6
3	6
4	1

LEP	
foci per cell	**occurrence (%)**
0	87
1	8
2	5

Fig. 4. Most HeLa and LEP cells contain no extranucleolar rDNA. (A) rDNA (red), fibrillarin (green) and merged image in a HeLa cell. No rDNA signals are present outside the fibrillarin-positive nucleoli. The arrows indicate Cajal bodies. Bar: 10 μm. (B) The percentage of extranucleolar rDNA foci in HeLa and LEP cells. The extranucleolar rDNA foci are absent in about 73% of HeLa cells and in about 87% of LEP cells.

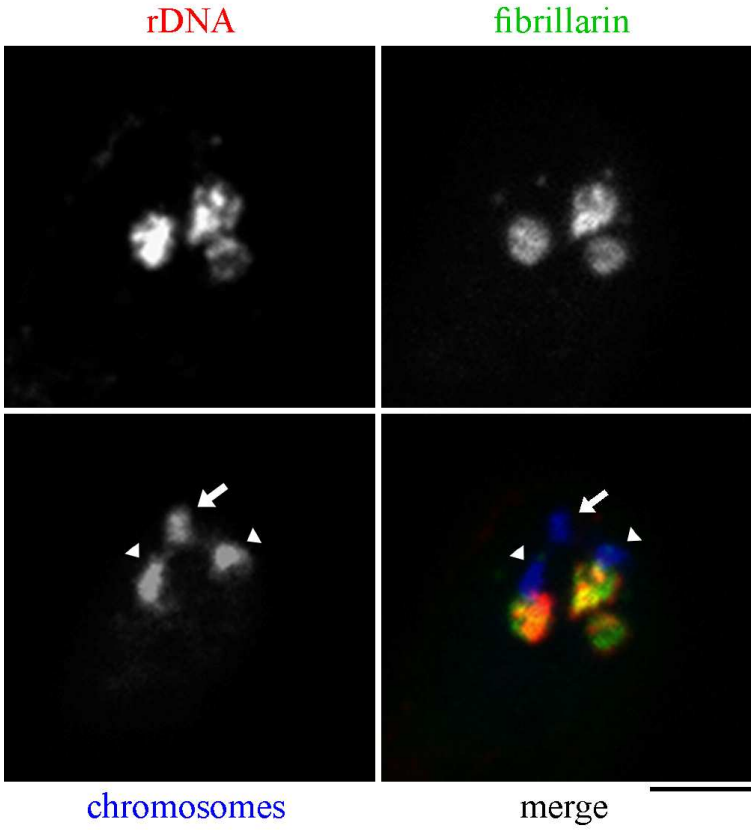


Fig. 5. Triple labeling of chromosomes 14, fibrillarin and rDNA in a HeLa cell. The chromosomes are shown in blue, fibrillarin in green, and rDNA in red. Two chromosome territories are closely associated with nucleoli (arrowheads). The third chromosome territory (arrow) is distanced from the nucleolus and is not colocalized with rDNA signal. Since no rDNA signals were found outside the nucleoli, the NOR belonging to this chromosome should be in the nucleolus. Bar: 10 μm

Chapter 7. Positioning of the NOR-bearing chromosomes in relation to nucleoli in daughter cells after mitosis

7.1. Introduction

Chromosomes are not randomly arranged in the vertebrate cell nucleus (Cremer and Cremer 2001; Parada and Misteli 2002; Pederson, 2004; Foster and Bridger 2005; Cremer and Cremer, 2006). But it is not clear to what extent their nuclear positions, together with their neighbourhoods, are conserved in daughter cells (Bickmore and Chubb, 2003). Using similar experimental approaches, the results of recent studies argued that the chromosomes were arranged similarly in maternal and daughter cells (Gerlich et al., 2003; Essers et al., 2005), or that positions of chromosomes in daughter nuclei were conserved only partly and in most cases largely differed from the positions seen in mother cell nuclei (Walter et al., 2003).

To address specific aspects of this problem, we used the model of the chromosomes carrying ribosomal genes. These genes are organized in clusters termed Nucleolus Organizer Regions (NORs) (McClintock, 1934; Bush and Smetana, 1970). Nucleoli disintegrate during mitosis, and at the beginning of the next G1 phase NORs from more than one chromosome cluster and participate in the formation of a given nucleolus (Raška, 2003; Raška et al., 2004). In the middle of G1 phase the position of chromosomes and the number of nucleoli in the nucleus are already stable and do not change significantly until the end of the interphase (Parada and Misteli, 2002; Walter et al., 2003; Foster and Bridger, 2005; Cremer and Cremer, 2006). We therefore selected for our study pairs of daughter cells in this period of the cell cycle, and compared the association of chosen NOR-bearing chromosomes (NOR-chromosomes) with nucleoli in the pairs of daughter cells from the human derived HeLa cell line. We thus did not investigate the maternal cell with regard to the daughter cells, but focused on the similarity between the two daughter cells.

The aim of our study was to establish how frequently the daughter cells had equal numbers of the homologues of certain NOR-chromosomes associated with individual nucleoli. Since the inheritance of the chromosome positioning in relation to nucleoli

depends on the number of nucleoli per nucleus, we also compared the numbers of nucleoli in the two daughter cells. It should be mentioned that the approach used here did not allow us discriminate between the individual homologues of the chromosomes associated with each nucleolus.

7.2. Material and Methods

HeLa cells were cultivated in flasks at 37°C in Dulbecco modified Eagle's medium (DMEM, Sigma, USA) containing 10% fetal calf serum, 1% glutamine, 0.1% gentamycin, and 0.85g/l $NaHCO_3$ in atmosphere supplemented with 5% CO_2. The preparations of the couples of postmitotic cells were obtained by shaking and seeding mitotic cells on the glass coverslips. In such procedure we could get sufficient numbers of clearly distinguished pairs of the postmitotic daughter cells. *In vivo* time-lapse observations encompassing a period from mitosis to mid G1 showed that the cells of different pairs did not mix during this period (data not shown).

Commercial Cy3- and FITC- labeled whole chromosome painting probes for human chromosomes 13, 14, 15, 21 and 22, supplied ready to use in hybridization mixture (Appligene Oncor, USA). Primary monoclonal antibody against mouse fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA), was used for immunovisualization of nucleoli. Secondary anti-mouse antibodies (Jackson ImmunoResearch Laboratories) were conjugated with Cy3 or FITC.

The combined detection of fibrillarin and *in situ* hybridization (immuno-FISH) was performed after Pliss et al. (2005). After fibrillarin imunolabeling the cells were postfixed with methanol/acetic acid (3:1) overnight at -20°C, then the regular FISH procedure followed (Pliss et al., 2005), except the post hybridization washing. Namely, the cells were washed in 50% formamide in 2xSSC, pH 7, for 15 min at 43°C, in 0.1% Tween-20 /2xSSC for 8 min at 43°C; in 0.1% Igepal (ICN Biomedicals, Inc) / 4xSSC for 3 x 4 min at 37°C, in PBS 3 x 3 min at RT (Harničarová et al., 2006). Coverslips were mounted in Mowiol supplemented with DABCO and viewed using Olympus AX70 Provis equipped with the Photometrics CCD camera.

7.3. Results

Nuclei of HeLa cells contained usually 2-5 nucleoli, with average number 4.03 ± 0.12 (Kalmárová et al., 2007). The number of nucleoli were most frequently different in the daughter cells (Fig. 1). Specifically, in 77% cases, the daughter cells contained different numbers of nucleoli. We additionally compared our data with a random model. In this model the appearance of the pairs of daughter cells with *i* and *j* nucleoli was calculated as product of the experimentally found frequencies of the cells with *i* and *j* nucleoli. Comparing the incidence of the nucleoli in 100 pairs of daughter cells, we found a close correspondence with the random model (Fig. 1).

Next we visualized chromosomes 14 and 15, performing hybridization with Cy3- and FITC- labeled probes, in combination with immuno labeling of nucleoli using antibody against fibrillarin. The HeLa cells typically possess four homologues of chromosome 15 and three homologues of chromosome 14. Different numbers of these chromosomes can be associated with each nucleolus (Kalmárová et al., 2007). Accordingly, different cells may have different combinations of the nucleolar association. In case of the chromosome 15, all four homologues are nucleoli-associated (Kalmárová et al., 2007; Smirnov et al., 2006). For instance, five combinations are possible in cells with four nucleoli (Fig. 2). In one extreme situation, all four chromosomes are associated with one nucleolus. In the other extreme sitiuation, there is one chromosome associated with each of the nucleoli (Fig. 3, A-C). In case of the chromosome 14, not all homologues are associated with nucleoli (Fig. 3, D-F) (Kalmárová et al., 2007; Smirnov et al., 2006), which increases the number of possible combinations to seven (Fig. 2).

Comparing these combinations in the daughter cells, we surprisingly found that in 50% of cell pairs, for both chromosome 14 and chromosome 15, the combinations were identical (Table). To evaluate these data, we used a random pairing model in which appearance of the pairs of daughter cells with combinations *i* and *j* was calculated as product of the experimentally found frequencies of the cells with the combinations *i* and *j*. The pairs with identical combinations appeared with significantly higher frequency in the experiment (50%) than in the random model (32% for chromosome 15 and 25% for chromosome 14) (Table).

Additionally, in the case of chromosome 14 we observed a significant symmetry in the distribution of the non-associated chromosomes after mitosis: in 62% cases the daughter cells had equal number of such chromosomes, while the random model predicted only 44%.

7.4. Discussion

In this study we observed that the daughter cells typically had different numbers of nucleoli (Fig. 1). Such an asymmetry, observed also by other authors (see e.g. Leung et al., 2004), is not entirely compatible with the claim that global chromosome positions are basically heritable through mitosis (Gerlich et al., 2003). In this respect, our results are in harmony with the work of Walter et al. (2003), according to which there is only a limited similarity in chromosome positioning between the daughter cells. However, we found that chromosomes 14 and 15, showed a similar pattern of nucleolar associations more frequently than predicted by the random pairing model (Fig. 3, Table). Surprisingly, this result was obtained even for such cases (chromosome 14, Fig. 2) where the total number of chromosomes associated with nucleoli was variable.

Taken together, our data indicate that the distribution of the NOR-bearing chromosomes among the nucleoli is partly conserved through mitosis.

7.5. Tables and figures

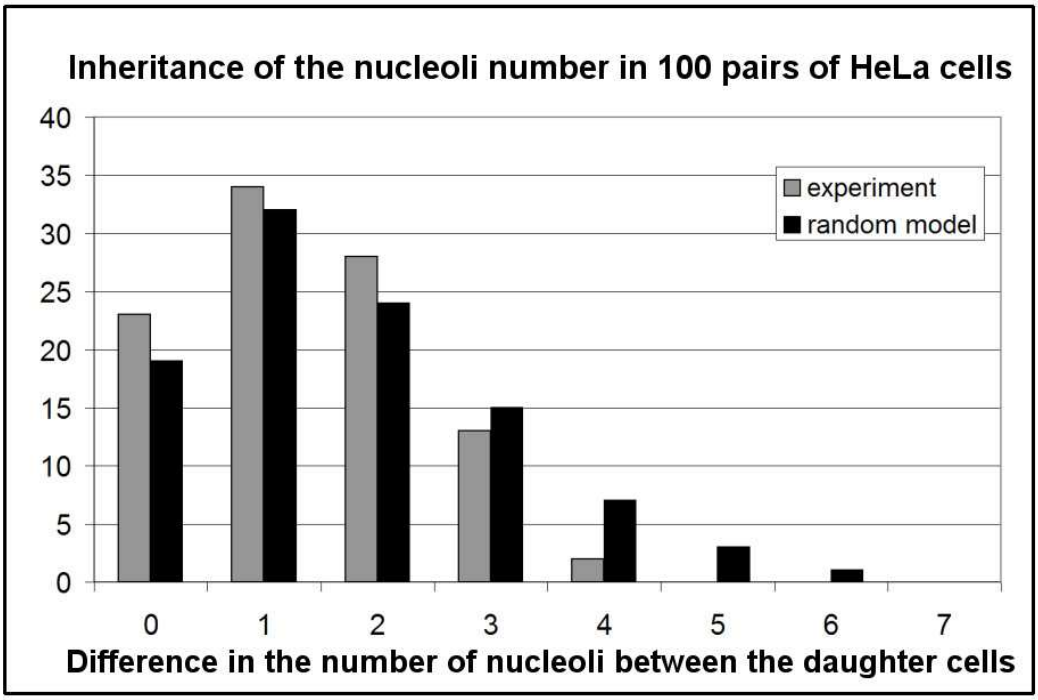


Fig. 1. Differences in the nucleoli number between the daughter HeLa cells. The number of nucleoli in the daughter cells most frequently differed by one (grey bars). Only in 23% of cell pairs, the number of nucleoli was identical. Observed differences in the number of nucleoli between the daughter cells closely corresponded to those in the random model (black bars).

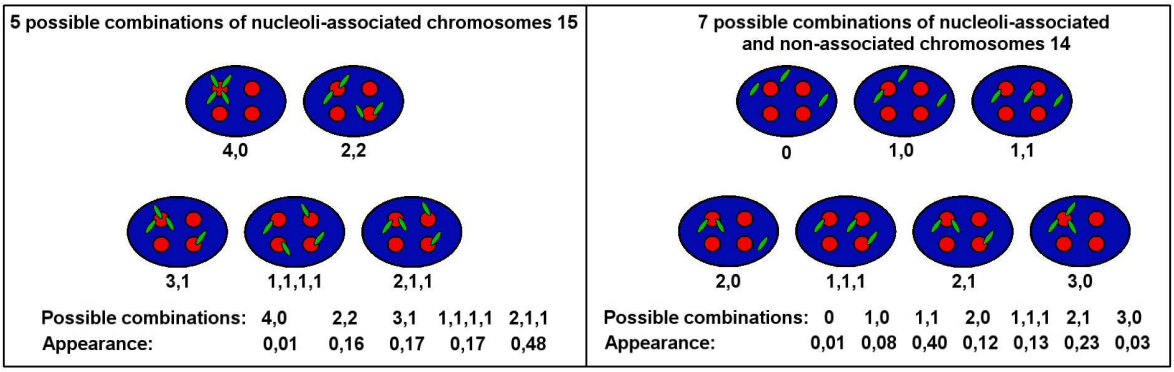


Fig. 2. Scheme depicting relations between nucleoli (red) and chromosomes 14 and 15 (green) in the cell nucleus (blue): all possible combinations of the nucleolar associations for the case of cells with four nucleoli are shown. All chromosomes 15 are associated with nucleoli, but some chromosomes 14 are not nucleoli-associated.

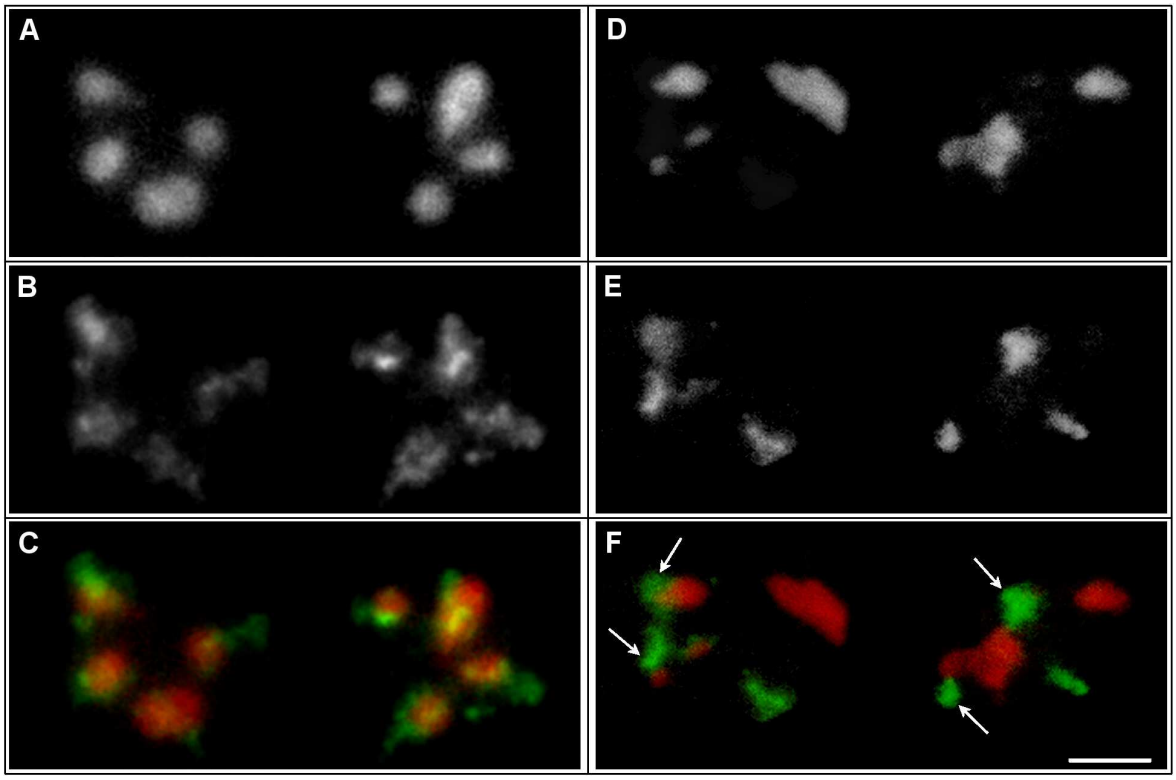


Fig. 3. Combinations of positions of chromosomes 14 and 15 in relation to nucleoli as compared in daughter HeLa cells. FISH signal with the specific probes for chromosomes 14 and 15 (B, E; green in C, F) in a couple of interphase daughter cells. Immunocytochemistry with fibrillarin was used to visualize nucleoli (A, D; red in C, F). In the chosen example of chromosome 15 (A, B, C), all four homologues are associated with nucleoli, deeply penetrating into them, which is typical for the chromosome 15 (Kalmárová et al. 2007). This case corresponds to the combination (1, 1, 1, 1) in Fig. 2. In the chosen example of chromosome 14 (D, E, F), two chromosome homologues are nucleoli associated (arrows in F), and one is separated from the nucleoli. This case corresponds to the combination (1, 1) in Fig. 2. Bar: 10 μm.

Table. Similarity of the position of the NOR-bearing chromosomes with respect to nucleoli in the daughter cells. In 50% of the cell pairs the combinations are identical for both chromosome 14 and chromosome 15, which significantly exceeds the values predicted by the random model

Chromosomes	**Frequency of symmetrical distribution**		
	Experiment	**Random model**	**n**
15	0.50	0.32	160
14	0.50	0.25	100

.

Chapter 8. General discussion and summary

In human cells ribosomal genes are organized as clusters called Nucleolus Organizer Regions (NORs) that are situated on the short arms of acrocentric chromosomes. It was found that essential components of the RNA polymerase I transcription machinery, including Upstream Binding Factor (UBF), can be detected on some NORs, termed "competent" NORs, during mitosis. The competent NORs are believed to be transcriptionally active during interphase. But since individual NORs cannot be observed in the cell nucleus, their interphase status remained 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, which 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.

Our results thus suggest that the competence of individual NORs is maintained throughout the cell cycle, since the average numbers of competent NORs does not significantly change. However, we have to emphasize that the maintenance of the average number does not exclude minor changes of the competence. Such as a change of the competence status of just one NOR (among all NORs) could be namely hidden within the statistical error of the experimental procedure. This change, however, could be detected using transfected cell lines expressing the recombinant protein UBF-GFP.

It is widely accepted that chromosomes occupy more or less fixed positions in mammalian interphase nucleus. However, relation between large-scale order of chromosome positioning and gene activity remained unclear. We approached this problem by studying the model of the human ribosomal genes. Employing FISH and

immunocytochemistry, we found that, in HeLa and LEP cells, the large-scale positioning of the NOR-bearing chromosomes (NOR-chromosomes) with regard to nucleoli is linked to the transcription activity of rDNA. Namely, the tendency of rDNA-bearing chromosomes to associate with nucleoli correlates with the number of transcriptionally competent NORs in the respective chromosome homologues. Regarding the position of NORs, we found that not only competent but also most of the non-competent NORs are included in the nucleoli. Some intranucleolar NORs (supposedly non-competent) are situated on elongated chromatin protrusions connecting nucleoli with respective chromosome territories spatially distanced from nucleoli. The cause of such an arrangement of the apparently non-competent NORs remains to be elucidated.

It is not clear to what extent nuclear positions of chromosomes, together with their neighbourhood, are conserved in daughter cells. We studied this problem by comparing the association of chosen NOR-chromosomes with nucleoli, as well as the numbers of nucleoli, in the pairs of daughter cells, and established how frequently the daughter cells had equal numbers of the homologues of certain NOR-chromosomes associated with individual nucleoli. The daughter cells typically had different numbers of nucleoli. As NOR-chromosomes associate with nucleoli, nucleoli have to play a crucial role in the arrangement of chromosomes in the cell nucleus, our data show that the position of chromosomes cannot be precisely maintained through mitosis. But at the same time, using immuno-FISH with probes for chromosomes 14 and 15 in HeLa cells, we found that the cell pairs with identical combinations of nucleolar associations of NOR-bearing homologues appeared significantly more frequently than predicted by the random model. Thus, although the total number of chromosomes associated with nucleoli is variable, our data indicate that the position of the NOR-bearing chromosomes in relation to nucleoli is partly maintained through mitosis.

Our data on position of NOR-bearing chromosomes are in harmony with the model of nuclear self-organization (Misteli, 2007). Its central idea is that the sum of all functional properties of a chromosome (i.e. the frequency and linear distribution of its active and inactive regions) determines its position, and chromosomes with functionally equivalent regions cluster in the nucleus. Importantly, this model does not exclude that position of the NOR-chromosome is conserved in one or even both daughter cells. However, this situation should occur not frequently as we observed large differences in

the numbers of nucleoli between the daughter cells. Indeed, as nucleoli are in nuclei usually separated by several micrometers, our result thus means that different sets of NOR-bearing chromosomes necessarily associate with a given nucleolus in the daughter cells. Nucleoli disintegrate during mitosis and chromosomes, including NOR-chromosomes, are inherited. Importantly, our results at the same time showed that the distribution of the NOR-bearing chromosomes among the nucleoli is partly conserved in the daughter cells. Although the number of nucleoli in mother and daughter, as well as in pairs of the two daughter cells, may differ, the functionally equivalent NOR regions from several NOR-bearing chromosomes, in agreement with the self-organization model, cluster within the nucleus and give rise to nucleoli. Taken together, our results obtained on NOR-chromosomes and nucleoli indicate that the position of NOR-chromosomes is largely affected by the interaction of functionally equivalent regions on different chromosomes and is conserved only partly.

Chapter 9. Conclusions

Concerning the behaviour of NORs during the cell cycle, several conclusions were reached:

- HeLa cells show an abnormal but stable pattern of rDNA distribution, however, not all acrocentric chromosomes carry ribosomal genes. In LEP cells all 10 acrocentric chromosomes are rDNA positive.
- Around 70 or 80% of the NORs, in HeLa and LEP cells respectively, are transcriptionally competent. In both HeLa and LEP cells, the transcription competence is non-randomly distributed among the NOR-bearing chromosomes.
- Stimulated transcription activity of the NORs exactly follows the pattern of transcription competence, which persists over the whole cell cycle, except probably for a short period in S phase.
- Employing premature chromosomal condensation to assess persistence of the transcription competence on the different NORs during the cell cycle, the pattern of transcription competence observed on mitotic chromosomal spreads persists throughout the interphase, except for a short period in S phase.

Study analysing nuclear positions of NORs, and NOR-chromosomes with respect to nucleoli, showed that in HeLa and LEP interphase cells:

- There is a positive correlation between the close nucleolar associations of the NOR-bearing chromosomes and the transcription competence of their NORs.
- Most of the non-competent NORs are located in nucleoli.
- Some intranucleolar NORs are situated on elongated chromatin protrusions connecting nucleoli with respective chromosome territories that are distanced from nucleoli.

The study correlating position of NOR-bearing chromosomes and nucleoli in the two daughter cells showed that:

- The two daughter cells typically have different numbers of nucleoli.
- The distribution of the NOR-bearing chromosomes among the nucleoli is partly conserved in daughter cells.

Chapter 10. References

Abranches R, Beven AF, Aragón-Alcaide L, Shaw PJ, 1998. Transcription sites are not correlated with chromosome territories in wheat nuclei. J Cell Biol 143, 5-12.

Akhmanova A, Verkerk T, Langeveld A, Grosveld F, Galjart N, 2000. Characterisation of transcriptionally active and inactive chromatin domains in neurons. J Cell Sci 24, 4463-4474.

Babu KA, Verma RS, 1985. Structural and functional aspects of nucleolar organizer regions (NORs) of human chromosomes. Int Rev Cytol 94, 151-76.

Bártová E, Krejčí J, Harničarová A, Kozubek S, 2008. Differentiation of human embryonic stem cells induces condensation of chromosome territories and formation of heterochromatin protein 1 foci. Differentiation 76, 24-32.

Baxter J, Merkenschlager M, Fisher AG, 2002. Nuclear organisation and gene expression. Curr Opin Cell Biol 14, 372-376.

Bazett-Jones DP, Leblanc B, Herfort M, Moss T, 1994. Short-range DNA looping by the Xenopus HMG-box transcription factor, xUBF. Science 264, 1134-7.

Bednár J, Horowitz RA, Dubochet J, Woodcock CL, 1995. Chromatin conformation and salt-induced compaction: three-dimensional structural information from cryoelectron microscopy. J Cell Biol 131, 1365-76.

Belmont AS, Straight AF, 1998. In vivo visualization of chromosomes using lac operator-repressor binding. Trends Cell Biol 8, 121-4.

Benavente R, Rose KM, Reimer G, Hugle-Dorr B, Scheer U, 1987. Inhibition of nucleolar reformation after microinjection of antibodies to RNA polymerase I into mitotic cells. J Cell Biol 105,1483-1491.

Bezrookove V, Smits R, Moeslein G, Fodde R, Tanke HJ, Raap AK, Darroudi F, 2003. Premature chromosome condensation revisited: a novel chemical approach permits efficient cytogenetic analysis of cancers. Genes Chromosomes Cancer 38, 177-86.

Biberfeld P, 1971. Morphogenesis in blood lymphocytes stimulated with phytohaemagglutinin (PHA). A light and electron microscopic study. Acta Pathol Microbiol Scand Suppl 223, 1-70.

Bickmore WA, Chubb JR, 2003. Dispatch. Chromosome position: now, where was I? Curr Biol 13, 357-9.

Boisvert FM, van Koningsbruggen S, Navascués J, Lamond AI, 2007. The multifunctional nucleolus. Nat Rev Mol Cell Biol 8, 574-85.

Bolzer A, Kreth G, Solovei I, Koehler D, Saracoglu K, Fauth C, Müller S, Eils R, Cremer C, Speicher MR, Cremer T, 2005. Three-dimensional maps of all chromosomes in human male fibroblast nuclei and prometaphase rosettes. PLoS Biol 3, e157.

Borden J, Manuelidis L, 1988. Movement of the X chromosome in epilepsy. Science 242, 1687-91.

Boyle S, Gilchrist S, Bridger JM, Mahy NL, Ellis JA, Bickmore WA, 2001. The spatial organization of human chromosomes within the nuclei of normal and emerin-mutant cells. Hum Mol Genet 10, 211-9.

Branco MR, Pombo A, 2006. Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations. PLoS Biol 4, e138.

Branco MR, Pombo A, 2007. Chromosome organization: new facts, new models. Trends Cell Biol 17, 127–134.

Bridger JM, Bickmore WA, 1998. Putting the genome on the map. Trends Genet 14, 403 9.

Bridger JM, Boyle S, Kill IR, Bickmore WA, 2000. Re-modelling of nuclear architecture in quiescent and senescent human fibroblasts.Curr Biol 10, 149-52.

Bui HT, Yamaoka E, Miyano T, 2004. Involvement of histone H3 (Ser10) phosphorylation in chromosome condensation without Cdc2 kinase and mitogen-activated protein kinase activation in pig oocytes. Biol Reprod 70, 1843-51.

Busch H, Smetana K, 1970. The nucleolus. Academic Press, New-York.

Chen TR, 1988. Re-evaluation of HeLa, HeLa S3, and HEp-2 karyotypes. Cytogenet Cell Genet 48, 19-24.

Chen D, Dundr M, Wang C, Leung A, Lamond A, Misteli T, Huang S, 2005. Condensed mitotic chromatin is accessible to transcription factors and chromatin structural proteins. J Cell Biol 1, 41-54.

Chubb JR, Bickmore WA, 2003. Considering nuclear compartmentalization in the light of nuclear dynamics. Cell 112, 403-6.

Copenhaver GP, Putnam CD, Denton ML, Pikaard CS, 1994. The RNA polymerase I transcription factor UBF is a sequence-tolerant HMG-box protein that can recognize structured nucleic acids. Nucleic Acids Res 22, 2651-7.

Cremer C, Zorn C, Cremer T, 1974. An ultraviolet laser microbeam for 257 nm. Microsc Acta 75, 331-7.

Cremer T, Lichter P, Borden J, Ward DC, Manuelidis L, 1988. Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genet 80, 235-46.

Cremer T, Kurz A, Zirbel R, Dietzel S, Rinke B, Schrock E, Speicher MR, Mathieu U, Jauch A, Emmerich P, Scherthan H, Ried T, Cremer C, Lichter P, 1993. Role of chromosome territories in the functional compartmentalization of the cell nucleus. Cold Spring Harb Symp Quant Biol 58, 777–792.

Cremer T, Cremer C, 2001. Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat Rev Genet 2, 292-301.

Cremer M, Küpper K, Wagler B, Wizelman L, von Hase J, Weiland Y, Kreja L, Diebold J, Speicher MR, Cremer T, 2003. Inheritance of gene density-related higher order chromatin arrangements in normal and tumor cell nuclei. J Cell Biol 162, 809-20.

Cremer T, Cremer C, 2006. Rise, fall and resurrection of chromosome territories: a historical perspective Part II. Fall and resurrection of chromosome territories during the

1950s to 1980s. Part III. Chromosome territories and the functional nuclear architecture: experiments and models from the 1990s to the present. Eur J Histochem 50, 223-272.

de Capoa A, Felli MP, Baldini A, Rocchi M, Archidiacono N, Aleixandre C, Miller OJ, Miller DA, 1988. Relationship between the number and function of human ribosomal genes. Hum Genet 79, 301-4.

Dobigny G, Ozouf-Costaz C, Bonillo C, Volobouev V, 2002. "Ag-NORs" are not always true NORs: new evidence in mammals. Cytogenet Genome Res 98, 75-7.

Dousset T, Wang C, Verheggen C, Chen D, Hernandez-Verdun D, Huang S, 2000. Initiation of nucleolar assembly is independent of RNA polymerase I transcription. Mol Biol Cell 11, 2705-17.

Dragon F, Gallagher JE, Compagnone-Post PA, Mitchell BM, Porwancher KA, Wehner KA, Wormsley S, Settlage RE, Shabanowitz J, Osheim Y, Beyer AL, Hunt DF, Baserga SJ, 2002. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature 417, 967-70.

Dundr M, Misteli T, 2001. Functional architecture in the cell nucleus. Biochem J 356, 297-310.

Dundr, M, Hoffmann-Rohrer, U, Hu, Q, Grummt, I, Rothblum, LI, Phair, RD, Misteli, T, 2002. A kinetic framework for a mammalian RNA polymerase *in vivo*. Science 298, 1623-26

Edelmann P, Bornfleth H, Zink D, Cremer T, Cremer C, 2001. Morphology and dynamics of chromosome territories in living cells. Biochim Biophys Acta 1551, M29-39.

Erickson JM, Rushford CL, Dorney DJ, Wilson GN, Schmickel RD, 1981. Structure and variation of human ribosomal DNA: molecular analysis of cloned fragments. Gene 16, 1 9.

Essers J, van Cappellen WA, Theil AF, van Drunen E, Jaspers NG, Hoeijmakers JH, Wyman C, Vermeulen W, Kanaar R, 2005. Dynamics of relative chromosome position during the cell cycle. Mol Biol Cell 16, 769-75.

Fatica A, Tollervey D, 2002. Making ribosomes. Curr Opin Cell Biol 14, 313-8.

Ferraro M, Prantera G, 1988. Human NORs show correlation between transcriptional activity, DNase I sensitivity, and hypomethylation. Cytogenet Cell Genet 47, 58-61.

Foster HA, Abeydeera LR, Griffin DK, Bridger JM, 2005. Non-random chromosome positioning in mammalian sperm nuclei, with migration of the sex chromosomes during late spermatogenesis. J Cell Sci 118, 1811-20.

Foster HA, Bridger JM, 2005. The genome and the nucleus: a marriage made by evolution. Genome organisation and nuclear architecture. Chromosoma 114, 212-29.

Franke WW, Scheer U, Spring H, Trendelenburg M, Zentgraf H, 1979. Organization of nucleolar chromatin. In: Busch H (eds) The cell nucleus, Academic Press, New York, 7, 49-95.

French SL, Osheim YN, Cioci F, Nomura M, Beyer AL, 2003. In exponentially growing Saccharomyces cerevisiae cells, rRNA synthesis is determined by the summed RNA polymerase I loading rate rather than by the number of active genes. Mol Cell Biol 23, 1558-68.

Friedrich JK, Panov KI, Cabart P, Russell J, Zomerdijk JC, 2005. TBP-TAF complex SL1 directs RNA polymerase l pre-initiation complex formation and stabilizes UBF at the rDNA promoter. J Biol Chem 280, 29551-29558.

Gébrane-Younès J, Fomproix N, Hernandez-Verdun D, 1997. When rDNA transcription is arrested during mitosis, UBF is still associated with non-condensed rDNA. J Cell Sci 110, 2429-40.

Gerlich D, Beaudouin J, Kalbfuss B, Daigle N, Eils R, Ellenberg J, 2003. Global chromosome positions are transmitted through mitosis in mammalian cells. Cell 112, 751-64.

Ghosh S, Ghosh I, 1975. Variation of stemline karyotype in a HeLa cell line.Z Krebsforsch Klin Onkol Cancer Res Clin Oncol 84, 129-33.

Gilbert N, Gilchrist S, Bickmore WA, 2005. Chromatin organization in the mammalian nucleus.Int Rev Cytol 242, 283-336.

Gilbert N, Ramsahoye B, 2005. The relationship between chromatin structure and transcriptional activity in mammalian genomes. Brief Funct Genomic Proteomic 4, 129–142.

Gonda K, Fowler J, Katoku-Kikyo N, Haroldson J, Wudel J, Kikyo N, 2003. Reversible disassembly of somatic nucleoli by the germ cell proteins FRGY2a and FRGY2b. Nat Cell Biol 5, 205-10.

Goodpasture C, Bloom SE, 1975. Visualization of nucleolar organizer regions im mammalian chromosomes using silver staining. Chromosoma 53, 37-50.

Grummt I, 2007. Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes. Hum Mol Genet 16, R21-7.

Hadjiolov AA, 1985. The nucleolus and ribosome biogenesis. In: Beerman AM, Goldstein L, Portrer KR, Sitte P (eds) Cell biology monographs. Springer, Berlin Heidelberg New York, 1-263.

Hameister H, Sperling K, 1984. Description of a chromosome replication unit in individual prematurely condensed human S-phase chromosomes.Chromosoma 90, 389-93.

Harničarová A, Kozubek S, Pacherník J, Krejči J, Bártová E, 2006. Distinct nuclear arrangement of active and inactive c-myc genes in control and differentiated colon carcinoma cells. Exp Cell Res 312, 4019-4035.

Héliot L, Mongelard F, Klein C, O'Donohue MF, Chassery JM, Robert-Nicoud M, Usson Y, 2000. Nonrandom distribution of metaphase AgNOR staining patterns on human acrocentric chromosomes. J Histochem Cytochem 48, 13-20.

Henderson AS, Warburton D, Atwood KC, 1972. Location of ribosomal DNA in the human chromosome complement. Proc Natl Acad Sci U S A 69, 3394-8.

Howell WM, Black DA, 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method.Experientia 36, 1014-5.

Hu CH, McStay B, Jeong SW, Reeder RH, 1994. xUBF, an RNA polymerase I transcription factor, binds crossover DNA with low sequence specificity. Mol Cell Biol 14, 2871-82.

Jantzen HM, Admon A, Bell SP, Tjian R, 1990. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature 344, 830-836.

Jimenez-Garcia LF, Segura-Valdez ML, Ochs RL, Rothblum LI, Hannan R, Spector DL, 1994. Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. Mol Biol Cell 5, 955-966.

Jordan P, Mannervik M, Tora L, Carmo-Fonseca M, 1996. *In vivo* evidence that TATA-binding protein/SL1 colocalizes with UBF and RNA polymerase I when rRNA synthesis is either active or inactive. J Cell Biol 133, 225-34.

Kalmárová M, Smirnov E, Mašata M, Koberna K, Ligasová A, Popov A, Raška I, 2007. Positioning of NORs and NOR-bearing chromosomes in relation to nucleoli. J Struct Biol 160, 49-56.

Kaplan FS, Murray J, Sylvester JE, Gonzalez IL, O'Connor JP, Doering JL, Muenke M, Emanuel BS, Zasloff MA, 1993. The topographic organization of repetitive DNA in the human nucleolus. Genomics 15, 123-32.

Kim SH, McQueen PG, Lichtman MK, Shevach EM, Parada LA, Misteli T, 2004. Spatial genome organization during T-cell differentiation. Cytogenet Genome Res 105, 292-301.

Kioussis D, 2005. Gene regulation: kissing chromosomes. Nature 435, 579-80.

Koberna K, Staněk D, Malínský J, Eltsov M, Pliss A, Čtrnáctá V, Cermanová S, Raška I, 1999. Nuclear organization studied with the help of a hypotonic shift: its use permits hydrophilic molecules to enter into living cells. Chromosoma 108, 325-35.

Koberna K, Staněk D, Malínský J, Čtrnáctá V, Cermanová S, Novotná J, Kopský V, Raška I, 2000. *In situ* fluorescence visualization of bromouridine incorporated into newly transcribed nucleolar RNA. Acta Histochem 102, 15-20.

Koberna K, Malínský J, Pliss A, Mašata M, Večeřová J, Fialová M, Bednár J, Raška I, 2002. Ribosomal genes in focus: new transcripts label the dense fibrillar components and form clusters indicative of "Christmas trees" in situ. J Cell Biol 157, 743-8. Erratum in: J Cell Biol 2002 158, 369.

Kuroda M, Tanabe H, Yoshida K, Oikawa K, Saito A, Kiyuna T, Mizusawa H, Mukai K, 2004. Alteration of chromosome positioning during adipocyte differentiation. J Cell Sci 117, 5897-903.

Lam YW, Lamond AI, Mann M, Andersen JS, 2007. Analysis of nucleolar protein dynamics reveals the nuclear degradation of ribosomal proteins. Curr Biol 17, 749-60.

Lamond AI, Sleeman JE, 2003. Nuclear substructure and dynamics. Curr Biol 13, R825-8.

Learned, RM, Cordes, S, Tjian, R, 1985. Purification and characterization of a transcription factor that confers promoter specificity to human RNA polymerase I. Mol Cell Biol 5, 1358-1369.

Leitch AR, Mosgöller W, Shi M, Heslop-Harrison JS, 1992. Different patterns of rDNA organization at interphase in nuclei of wheat and rye. J Cell Sci 101, 751-757.

Leung AK, Gerlich D, Miller G, Lyon C, Lam YW, Lleres D, Daigle N, Zomerdijk J, Ellenberg J, Lamond AI, 2004. Quantitative kinetic analysis of nucleolar breakdown and reassembly during mitosis in live human cells. J Cell Biol 166, 787-800.

Lewis MS, Pikaard DJ, Nasrallah M, Doelling JH, Pikaard CS, 2007. Locus-specific ribosomal RNA gene silencing in nucleolar dominance. PLoS ONE 2, e815.

Lichter P, Cremer T, Tang CJ, Watkins PC, Manuelidis L, Ward DC, 1988. Rapid detection of human chromosome 21 aberrations by in situ hybridization. Proc Natl Acad Sci U S A. 85, 9664-8.

Long EO, Dawid IB, 1980. Repeated genes in eukaryotes. Annu Rev Biochem 49, 727-64.

Macville M, Schröck E, Padilla-Nash H, Keck C, Ghadimi BM, Zimonjic D, Popescu N, Ried T, 1999. Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. Cancer Res 59, 141-50.

Mahy NL, Perry PE, Bickmore WA, 2002. Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH. J Cell Biol 159, 753-63.

Mais C, Wright JE, Prieto JL, Raggett SL, McStay B, 2005. UBF-binding site arrays form pseudo-NORs and sequester the RNA polymerase I transcription machinery. Genes Dev 19, 50-64.

Manders EM, Kimura H, Cook PR, 1999. Direct imaging of DNA in living cells reveals the dynamics of chromosome formation. J Cell Biol 144, 813-21.

Manuelidis L, 1985. Individual interphase chromosome domains revealed by in situ hybridization.Hum Genet 71, 288-93.

Martin C, Beaujean N, Brochard V, Audouard C, Zink D, Debey P, 2006a. Genome restructuring in mouse embryos during reprogramming and early development. Dev Biol 292, 317-32.

Martin C, Brochard V, Migné C, Zink D, Debey P, Beaujean N, 2006b. Architectural reorganization of the nuclei upon transfer into oocytes accompanies genome reprogramming. Mol Reprod Dev 73, 1102-11.

Mayer R, Brero A, von Hase J, Schroeder T, Cremer T, Dietzel S, 2005. Common themes and cell type specific variations of higher order chromatin arrangements in the mouse. BMC Cell Biol 6, 44.

McClintock B, 1934. The relatioship of a particular chromosomal element to the development of the nucleoli in Zea Mays. Z Zellforsch Mikrosk Anat 21, 294–328.

McStay B, 2006. Nucleolar dominance: a model for rRNA gene silencing. Genes Dev 20, 1207-14.

Meaburn KJ, Misteli T, Soutoglou E, 2007a. Spatial genome organization in the formation of chromosomal translocations. Semin Cancer Biol 17, 80-90.

Meaburn KJ, Cabuy E, Bonne G, Levy N, Morris GE, Novelli G, Kill IR, Bridger JM, 2007b. Primary laminopathy fibroblasts display altered genome organization and apoptosis. Aging Cell 6, 139-53.

Mélèse T, Xue Z, 1995. The nucleolus: an organelle formed by the act of building a ribosome. Curr Opin Cell Biol 7, 319-24.

Mellink CH, Bosma AA, De Haan NA, 1994. Variation in size of Ag-NORs and fluorescent rDNA in situ hybridization signals in six breeds of domestic pig. Hereditas 120, 141-9.

Miller OL Jr, Beatty BR, 1969. Visualization of nucleolar genes. Science 164, 955-7.

Misteli T, 2007. Beyond the sequence: cellular organization of genome function. Cell 128, 787-800.

Morey C, Da Silva NR, Perry P, Bickmore WA, 2007. Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation. Dev 134, 909-919.

Mougey EB, O'Reilly M, Osheim Y, Miller OL Jr, Beyer A, Sollner-Webb B, 1993. The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes. Genes Dev 7, 1609-19.

Murmann AE, Gao J, Encinosa M, Gautier M, Peter ME, Eils R, Lichter P, Rowley JD, 2005. Local gene density predicts the spatial position of genetic loci in the interphase nucleus. Exp Cell Res 311, 14-26.

Nikiforova MN, Stringer JR, Blough R, Medvedovic M, Fagin JA, Nikiforov YE, 2000. Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. Science 290, 138-41.

Ochs RL, Lischwe MA, Shen E, Carroll RE, Busch H, 1985. Nucleologenesis: composition and fate of prenucleolar bodies. Chromosoma 92, 330-336.

Ochs RL, Smetana K, 1989. Fibrillar center distribution in nucleoli of PHA-stimulated human lymphocytes. Exp Cell Res 184, 552-7.

Ochs RL, Press RI, 1992. Centromere autoantigens are associated with the nucleolus. Exp Cell Res 200, 339-50.

Ogushi S, Palmieri C, Fulka H, Saitou M, Miyano T, Fulka J Jr., 2008. The maternal nucleolus is essential for early embryonic development in mammals. Science 319, 613-6.

Olson MO, Dundr M, Szebeni A, 2000. The nucleolus: an old factory with unexpected capabilities. Trends Cell Biol 10, 189-96.

Olson MO, Hingorani K, Szebeni A, 2002. Conventional and nonconventional roles of the nucleolus. Int Rev Cytol 219, 199-266.

Osheim YN, French SL, Keck KM, Champion EA, Spasov K, Dragon F, Baserga SJ, Beyer AL, 2004. Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to being cleaved from nascent transcripts in Saccharomyces cerevisiae. Mol Cell 16, 943-54.

O'Sullivan AC, Sullivan GJ, McStay B, 2002. UBF binding *in vivo* is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. Mol Cell Biol 22, 657-68.

Parada L, Misteli T, 2002. Chromosome positioning in the interphase nucleus. Trends Cell Biol 12, 425-432.

Parada LA, Roix JJ, Misteli T, 2003. An uncertainty principle in chromosome positioning. Trends Cell Biol 13, 393–396.

Parada LA, McQueen PG, Misteli T, 2004a. Tissue-specific spatial organization of genomes. Genome Biol 5, R44.

Parada LA, Sotiriou S, Misteli T, 2004b. Spatial genome organization. Exp Cell Res 296, 64-70.

Pederson T, 2004. The spatial organization of the genome in mammalian cells. Curr Opin Genet Dev 14, 203–209.

Pliss A, Koberna K, Večeřová J, Malínský J, Mašata M, Fialová M, Raška I, Berezney R, 2005. Spatio-temporal dynamics at rDNA foci: global switching between DNA replication and transcription. J Cell Biochem 94, 554-565.

Preuss S, Pikaard CS, 2007. rRNA gene silencing and nucleolar dominance: insights into a chromosome-scale epigenetic on/off switch. Biochim Biophys Acta 1769, 383-92.

Prieto JL, McStay B, 2005. Nucleolar biogenesis: the first small steps. Biochem Soc Trans 33, 1441-1443.

Puvion-Dutilleul F, Bachellerie JP, Bernadac A, Zalta JP, 1977. Transcription complexes in subnuclear fractions isolated from mammalian cells: ultrastructural study. C R Acad Sci Hebd Seances Acad Sci D 284, 663-666.

Puvion-Dutilleul F, Bachellerie JP, Puvion E, 1991. Nucleolar organization of HeLa cells as studied by in situ hybridization. Chromosoma 100, 395-409.

Raška I, Armbruster BL, Frey JR, Smetana K, 1983a. Analysis of ring-shaped nucleoli in serially sectioned human lymphocytes.Cell Tissue Res 234, 707-11.

Raška I, Rychter Z, Smetana K, 1983b. Fibrillar centres and condensed nucleolar chromatin in resting and stimulated human lymphocytes. Z Mikrosk Anat Forsch 97, 15-32.

Raška I, Ochs RL, Salamin-Michel L, 1990. Immunocytochemistry of the cell nucleus.Electron Microsc Rev 3, 301-53.

Raška I, 2003. Oldies but goldies: searching for Christmas trees within the nucleolar architecture. Trends Cell Biol 13, 517-25.

Raška I, Koberna K, Malínský J, Fidlerová H, Mašata M, 2004. The nucleolus and transcription of ribosomal genes. Biol Cell 96, 579-94.

Raška I, Shaw PJ, Cmarko D, 2006. New insights into nucleolar architecture and activity. Int Rev Cytol 255,177-235.

Robinett CC, Straight A, Li G, Willhelm C, Sudlow G, Murray A, Belmont AS, 1996. *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. J Cell Biol 135, 1685-700.

Roix JJ, McQueen PG, Munson PJ, Parada LA, Misteli T, 2003. Spatial proximity of translocation-prone gene loci in human lymphomas. Nat Genet 34, 287-91.

Roussel P, Hernandez-Verdun D, 1994. Identification of Ag-NOR proteins, markers of proliferation related to ribosomal gene activity. Exp Cell Res 214, 465-72.

Roussel P, André C, Comai L, Hernandez-Verdun D, 1996. The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. J Cell Biol 133, 235-46.

Rufas JS, Giménez-Martín G, Esponda P, 1982. Presence of a chromatid core in mitotic and meiotic chromosomes of grasshoppers. Cell Biol Int Rep 6, 261-7.

Santoro R, 2005. The silence of the ribosomal RNA genes. Cell Mol Life Sci 62, 2067-2079.

Santos AP, Abranches R, Stoger E, Beven A, Viegas W, Shaw PJ, 2002. The architecture of interphase chromosomes and gene positioning are altered by changes in DNA methylation and histone acetylation. J Cell Sci 115, 4597-605.

Savino TM, Gébrane-Younès J, De Mey J, Sibarita JB, Hernandez-Verdun D, 2001. Nucleolar assembly of the rRNA processing machinery in living cells. J Cell Biol 153, 1097-110.

Scheer U, Zentgraf H, Sauer HW, 1981. Different chromatin structures in Physarum polycephalum: a special form of transcriptionally active chromatin devoid of nucleosomal particles. Chromosoma 84, 279-90.

Scheer U, Benavente R, 1990. Functional and dynamic aspects of the mammalian nucleolus. Bioessays 12, 14-21.

Scheer U, Hock R, 1999. Structure and function of the nucleolus. Curr Opin Cell 11, 385-90.

Shaw PJ, Jordan EG, 1995. The nucleolus. Annu Rev Cell Dev Biol 11, 93-121.

Sirri V, Roussel P, Hernandez-Verdun D, 1999. The mitotically phosphorylated form of the transcription termination factor TTF-1 is associated with the repressed rDNA transcription machinery. J Cell Sci 112 , 3259-68.

Sirri V, Roussel P, Hernandez-Verdun D, 2000. The AgNOR proteins: qualitative and quantitative changes during the cell cycle. Micron 31, 121-6.

Smetana K, Lejnar J, Potměsil M, 1967. A note to the demonstration of DNA in nuclei of blood cells in smear preparations. Folia Haematol Int Mag Klin Morphol Blutforsch 88, 305-17.

Smetana K, Freireich EJ, Busch H, 1968. Chromatin structures in ring-shaped nucleoli of human lymphocytes. Exp Cell Res 52, 112-28.

Smirnov E, Kalmárová M, Koberna K, Zemanová Z, Malínský J, Mašata M, Cvačková Z, Michalová K, Raška I, 2006. NORs and their transcription competence during the cell cycle. Folia Biol 52, 59-70.

Spector DL, 2001. Nuclear domains. J Cell Sci 114, 2891-3.

Spector DL, 2003. The dynamics of chromosome organization and gene regulation. Annu Rev Biochem 72, 573-608.

Stadler S, Schnapp V, Mayer R, Stein S, Cremer C, Bonifer C, Cremer T, Dietzel S, 2004. The architecture of chicken chromosome territories changes during differentiation. BMC Cell Biol 5, 44.

Stahl A, Hartung M, Vagner-Capodano AM, Fouet C, 1976. Chromosomal constitution of nucleolus-associated chromatin in man. Hum Genet 35, 27-34.

Stefanovsky VY, Pelletier G, Bazett-Jones DP, Crane-Robinson C, Moss T, 2001. DNA looping in the RNA polymerase I enhancesome is the result of non-cooperative in-phase bending by two UBF molecules. Nucleic Acids Res 29, 3241-7.

Stefanovsky V, Moss T, 2006. Regulation of rRNA synthesis in human and mouse cells is not determined by changes in active gene count. Cell Cycle 5, 735-9.

Sullivan GJ, Bridger JM, Cuthbert AP, Newbold RF, Bickmore WA, McStay B, 2001. Human acrocentric chromosomes with transcriptionally silent nucleolar organizer regions associate with nucleoli. EMBO J 20, 2867-74.

Sun HB, Yokota H, 1999. Correlated positioning of homologous chromosomes in daughter fibroblast cells. Chromosome Res 7, 603-10.

Sun HB, Shen J, Yokota H, 2000. Size-dependent positioning of human chromosomes in interphase nuclei. Biophys J 79, 184-190.

Suzuki H, Wakana S, Yonekawa H, Moriwaki K, Sakurai S, Nevo E, 1996. Variations in ribosomal DNA and mitochondrial DNA among chromosomal species of subterranean mole rats. Mol Biol Evol 13, 85-92.

Taslerová R, Kozubek S, Bártová E, Gajdušková P, Kodet R, Kozubek M, 2006. Localization of genetic elements of intact and derivative chromosome 11 and 22 territories in nuclei of Ewing sarcoma cells. J Struct Biol 155, 493-504.

Tosuji H, Fusetani N, Seki Y, 2003. Calyculin A causes the activation of histone H1 kinase and condensation of chromosomes in unfertilized sea urchin eggs independently of the maturation-promoting factor. Comp Biochem Physiol C Toxicol Pharmacol 135, 415-24.

Trendelenburg MF, 1974. Morphology of ribosomal RNA cistrons in oocytes of the water beetle, Dytiscus marginalis L. Chromosoma 48, 119-35.

Vazquez J, Belmont AS, Sedat JW, 2001. Multiple regimes of constrained chromosome motion are regulated in the interphase Drosophila nucleus. Curr Biol 11, 1227–1239.

Verschure PJ, van Der Kraan I, Manders EM, van Driel R, 1999. Spatial relationship between transcription sites and chromosome territories. J Cell Biol 147, 13-24.

Visser AE, Eils R, Jauch A, Little G, Bakker PJ, Cremer T, Aten JA, 1998. Spatial distributions of early and late replicating chromatin in interphase chromosome territories. Exp Cell Res 243, 398-407.

Visser AE, Aten JA, 1999. Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. J Cell Sci 112, 3353-60.

Visser AE, Jaunin F, Fakan S, Aten JA, 2000. High resolution analysis of interphase chromosome domains. J Cell Sci 113, 2585-93.

Volpi EV, Chevret E, Jones T, Vatcheva R, Williamson J, Beck S, Campbell RD, Goldsworthy M, Powis SH, Ragoussis J, Trowsdale J, Sheer D, 2000. Large-scale chromatin organization of the major histocompatibility complex and other regions of

human chromosome 6 and its response to interferon in interphase nuclei. J Cell Sci 113, 1565-1576.

Wachtler F, Hopman AH, Wiegant J, Schwarzacher HG, 1986. On the position of nucleolus organizer regions (NORs) in interphase nuclei. Studies with a new, non-autoradiographic in situ hybridization method. Exp Cell Res 167, 227-40.

Walter J, Schermelleh L, Cremer M, Tashiro S, Cremer T, 2003. Chromosome order in HeLa cells changes during mitosis and early G1, but is stably maintained during subsequent interphase stages. J Cell Biol 160, 685-97.

Warburton D, Henderson AS, 1979. Sequential silver staining and hybridization in situ on nucleolus organizing regions in human cells. Cytogenet Cell Genet 24,168-75.

Wegel E, Shaw P, 2005. Gene activation and deactivation related changes in the three-dimensional structure of chromatin.Chromosoma 114, 331-7.

Weisenberger D, Scheer U, 1995. A possible mechanism for the inhibition of ribosomal RNA gene transcription during mitosis. J Cell Biol 129, 561-75.

Wiech T, Timme S, Riede F, Stein S, Schuricke M, Cremer C, Werner M, Hausmann M, Walch A, 2005. Human archival tissues provide a valuable source for the analysis of spatial genome organization. Histochem Cell Biol 123, 229-38.

Williams RR, Fisher AG, 2003. Chromosomes, positions please! Nat Cell Biol 5, 388-90.

Woodcock CL, Dimitrov S, 2001. Higher-order structure of chromatin and chromosomes. Curr Opin Genet Dev 11, 130-5.

Zink D, Cremer T, Saffrich R, Fischer R, Trendelenburg MF, Ansorge W, Stelzer EH, 1998. Structure and dynamics of human interphase chromosome territories in vivo. Hum Genet 102, 241-51.

Zink D, Fischer AH, Nickerson JA, 2004. Nuclear structure in cancer cells. Nat Rev Cancer 4, 677-87

Zurita F, Jiménez R, Burgos M, de la Guardia RD, 1998. Sequential silver staining and in situ hybridization reveal a direct association between rDNA levels and the expression of

homologous nucleolar organizing regions: a hypothesis for NOR structure and function. J Cell Sci 111,1433-9.

Zurita F, Jiménez R, Diaz de la Guardia R, Burgos M, 1999. The relative rDNA content of a NOR determines its level of expression and its probability of becoming active. A sequential silver staining and in-situ hybridization study. Chromosome Res 7, 563-70.

Publications of Markéta Kalmárová (Fialová)

Koberna K, Malínský J, Pliss A, Mašata M, Večeřová J, **Fialová M**, Bednár J, Raška I, 2002. Ribosomal genes in focus: new transcripts label the dense fibrillar components and form clusters indicative of „Christmas trees“ in situ. J Cell Biol 157, 743-748. IF 12,522.

Koberna K, Ligasová A, Malínský J, Pliss A, Siegel AJ,Cvačková Z, Fidlerová H, Mašata M, **Fialová M**, Raška I, Berezney R, 2005. Electron Microscopy of DNA Replication in 3-D: Evidence for Similar-Sized Replication Foci Throughout S-Phase, J Cell Biochem 94, 126-38. IF 2,664.

Pliss A, Koberna K,Večeřová J, Malínský J, Mašata M, **Fialová M**, Raška I, Berezney R, 2005. Spatio-temporal dynamics at rDNA foci: Global switching between DNA replication and transcription. J Cell Biochem 94, 554-65. IF 2,664.

Fidlerová H, Mašata M, Malínský J, **Fialová M**, Cvačková Z, Loužecká A, Koberna K, Berezney R, Raška I, 2005. Replication-Coupled Modulation of Early Replicating Chromatin Domains Detected by Anti-Actin Antibodies. J Cell Biochem 94, 899-916. IF 2,664.

Smirnov E, **Kalmárová M**, Koberna K, Zemanová Z, Malínský J, Mašata M, Cvačková Z, Michalová K, Raška I, 2006. NORs and Their Transcription Competence during the Cell Cycle. Folia Biol 52, 59-70. IF 0,719.

Kalmárová M, Smirnov E, Mašata M, Koberna K, Ligasová A, Popov A, Raška I, 2007. Positioning of NORs and NOR-bearing chromosomes in relation to nucleoli. J Struct Biol 160, 49-56. IF 3,496.

Kalmárová M, Smirnov E, Kováčik L, Popov A, Raška I, 2008. Positioning of the NOR-bearing chromosomes in relation to nucleoli in daughter cells after mitosis. Physiol Res 3. Article in press. IF 2,093.

Kalmárová M, Kováčik L, Popov A, Testillano PS, Smirnov E, 2008. Asymmetrical distribution of the transcriptionally competent NORs in mitosis. J Struct Biol (Accepted) IF 3,496