Age-related association of rDNA and telomeres with the nuclear matrix in mouse hepatocytes

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Abstract

Transcribed sequences have been suggested to be associated with the nuclear matrix, differing from non-transcribing sequences, which have been reported to be contained in DNA loops. However, although a dozen of genes have their expression level affected by aging, data on chromatin–nuclear matrix interactions under this physiological condition are still scarce. In the present study, liver imprints from young, adult and old mice were subjected to FISH (fluorescence in situ hybridization) for 45S rDNA and telomeric sequences, with or without a lysis treatment to produce extended chromatin fibres. There was an increased amount of 45S rDNA sequences located in DNA loops as the animals grow older, while telomeric sequences were always observed in DNA loops irrespective of the animal age. We assume that active rRNA genes associate with the nuclear matrix, while DNA loops contain silent sequences. Transcription of each 45S rRNA repeat unit is suggested to be dependent on its interaction with the nuclear matrix.

Keywords: aging; chromatin extensibility; development; nuclear matrix; rDNA; telomere

1. Introduction

A prevailing theory on aging states that it results from an accumulation of tissue damage, similar to rust. This presumes that aging results inevitably from accumulated damage to cells as promoted by toxins, free-radical molecules, DNA-damaging radiation, disease and stress (Harman, 1998). Nevertheless, there is increasing evidence that specific genetic instructions and structural modifications drive the process making it an active process rather than a passive one (Oberdoerffer and Sinclair, 2007).

Studies by several authors, conducted in yeast, worms, flies and mice, have confirmed substantial changes in gene expression with aging (Burzynski, 2005). Among most important changes reported is the silencing of tumour suppressors and other genes involved in the control of cell cycle, apoptosis, detoxification and cholesterol metabolism (Cao et al., 2001).

The activity of rRNA genes in mouse hepatocytes gradually increases after birth, reaches a peak at 14 days of postnatal age and then decreases drastically as the animals grow older (Ma and Nagata, 1990). These changes are accompanied by an increase in protein synthesis at about 1 month postnatally, followed by a decrease in this synthesis along the adult life and low synthesis levels in aged animals (Ma and Nagata, 1990).

The rRNA genes, which in most eukaryotic species are repeated ~100–5000 times per haploid genome, are located at one or a few chromosomal sites called NORs (nucleolar organizer regions) (Gaubatz et al., 1976; Long and Dawid, 1980). The rRNA coding regions are organized as head-to-tail repeats, with the transcribed regions separated by segments of non-transcribed spacers (Fedoroff, 1979; Pan and Zhang, 2008). In rat hepatocytes, these sequences appear enriched in the purified nuclear matrices after DNase I digestion, thus indicating that the high transcription rates of rRNA genes and nuclear matrix interaction are interconnected (Pardoll and Vogelstein, 1980).

The nuclear matrix is an operationally defined nuclear skeletal structure assumed to be involved in many nuclear functions, including DNA replication, transcription and repair and pre-mRNA processing/transport. It is also a passenger concourse for proteins going in and out of the nucleus through the nuclear pore complex (Tsutsui et al., 2005). The nuclear matrix comprises a protein framework formed by the nuclear pore/nuclear lamina complex, a residual nucleolus and a residual internal fibrillar–globular mesh (Berezney and Coffey, 1974; Gasser and Laemmli, 1986; Berezney, 1991). Until relatively recently, the nuclear matrix was considered a rigid and static structure, but new theories now consider it as a dynamic structure (Tsutsui et al., 2005; Barboro et al., 2009).

The isolation of the nuclear matrix requires nuclease and high-salt treatments that remove chromatin and other soluble nuclear proteins from the cell nuclei (Nickerson, 2001). An ordinary method for nuclear matrix isolation involves the extraction of nuclei with highly concentrated solutions of monovalent salts such as 2 M NaCl (Berezney and Coffey, 1977). After that, the nuclei adopt a so-called nuclear halo configuration, in which nuclear DNA sticks out from the residual structure (nuclear matrix) as negatively supercoiled loops attached at their base by MARs (matrix attachment regions). If

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; e, erythroblast; ECF, extended chromatin fibre; FISH, fluorescence in situ hybridization; MAR, matrix attachment region; n, nucleoli.
nuclei are treated vertically, under the action of gravity, formation of ECFs (extended chromatin fibres) may occur, as they are strongly affected by nuclear matrix proteins, thus contributing to the higher-order chromatin structure (Gerdes et al., 1994; Haaf and Ward, 1994; Davie, 1995; Cremer et al., 2000; Vidal, 2000). An increased number of interactions between chromatin and nuclear matrix can render or even prevent the ECF formation, since there are salt-resistant interactions of MARs with the nuclear matrix (Moraes et al., 2005, 2007). After this extraction, the nuclear DNA can be fractionated into loop DNA and matrix-associated DNA; the latter accounts for 1% of total nuclear DNA and is enriched in rRNA sequences (Pardoll and Vogelstein, 1980). When this treatment is followed by FISH (fluorescence in situ hybridization) assay, transcriptionally inactive sequences produce long strings of signal extending out on to the DNA halo or loop, whereas active sequences remain tightly condensed as single spots within the residual nucleus, thus suggesting that the association of genes with the nuclear matrix may be a factor responsible for transcription activation (Gerdes et al., 1994).

Taking into account that differences have previously been reported for the changes in the activity of rRNA genes with advancing age, in the present work, we aimed at comparing FISH signals of active and silenced 45S rDNA and repetitive silenced telomeric sequences in relation to their attachment to nuclear matrix after ECF formation was induced in young, adult and old mice hepatocytes.

2. Experimental procedures

2.1. Animals

Male mice from the inbred strain A/Uni, obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas were reared under normal conditions and fed extruded chow (Purina®) ad libitum. The animals used were 1–2 (young), 15–20 (adult) and 61–100 (aged) weeks old. At least three animals of each group were used. Animals were killed by decapitation. Their livers were then immediately removed and placed in cold 0.9% NaCl solution. Liver slices were imprinted on histological slides. All protocols involving animal care and use were approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP – 378-1).

2.2. Treatments

Freshly prepared imprints were fixed in a mixture of absolute ethanol and acetic acid (3:1, v/v, respectively) for 1 min and then rinsed in 70% ethanol for 5 min. The preparations were positioned vertically using a protocol previously described for ECF investigation (Moraes et al., 2005). Briefly, the lysis solution consisted of 2 M NaCl plus 1% Triton X-100 in Tris/HCl buffer (25 mM, pH 7.4). Treatment lasted for 5 h at 25°C, after which, the volume of solution was completed with absolute ethanol to a final concentration of 50%, and treatment was prolonged for another 10 min. The slides were then carefully removed from the lysis solution and transferred to 70% ethanol for 30 min. Fixed preparations not subjected to the lysis protocol were used as controls.

2.3. FISH

The plasmid HM 456, which contains part of the 18S and 28S rDNA of Xenopus laevis (Meunier-Rotival et al., 1979) was kindly provided by Dr A.M. Vianna-Morgante (IB-USP, São Paulo, Brazil), and the telomere-like oligonucleotide DNA sequence was synthesized by Invitrogen. Probes were labelled by nick translation with biotin-16dUTP (Bionick Labeling Kit, Life Technologies, Inc.). The protocol used was previously described by Mondin et al. (2007). Briefly, the preparations were treated with RNase A (Sigma Chemical Co.), followed by treatment with 0.01 M HCl for 2 min, washed in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), treated with 5 µg/ml pepsin for 10 min at 37°C and fixed in 4% paraformaldehyde for 5 min, followed by denaturation with 2 × SSC/50% formamide for 10 min at 80°C. Heat-denatured probes were hybridized at 37°C for 20 h. After hybridization and washes with 2 × SSC/50% formamide and 2 × SSC at 42°C, probe detection used a two-step procedure for amplification. Biotin was revealed using mouse anti-biotin and rabbit anti-mouse TRITC conjugated antibodies. Coverslips were mounted in Vectashield (Vector Laboratories) containing 0.5 µg/ml DAPI (4’,6-diamidino-2-phenylindole).

2.4. Microscopy

DNA FISH preparations were analysed under a Zeiss Axiophot 2 epifluorescence microscope equipped with an HBO-100 W stabilized mercury lamp as the light source and appropriate filters. Zeiss Plan ×25/0.50, ×40/0.75 and ×100/1.4 oil-immersion objectives were used. Digital images were obtained using a Sony CCD-IRIS/RGB Hyper HAD colour video camera and ISIS (MetaSystems, Altlussheim) software. Selected images were processed using CorelDraw®12 software.

2.5. Image processing

Images of DAPI-stained nuclei were segmented using supervised threshold to mark the nuclear region. Areas with average intensities above this threshold were set to 1 (nuclear region), and all other areas were set to 0. The resulting images were called DAPI mask.

FISH images for rDNA were converted into greyscale format and were segmented using supervised threshold to determine the region positive for this marker. The sum of pixel values of this region was calculated and was called IGL. The sum of pixel values with overlapping DAPI mask of value 1 was calculated, and its relative IGL percentage was called %IGLin and corresponds to the percentage of integrated grey levels of FISH signals inside nuclear remnants. The sum of pixel values with overlapping DAPI mask with value 0 was called %IGLout and corresponds to the percentual of integrated grey levels of FISH signals outside nuclear remnants (extended chromatin fibres and nuclear halos).
2.6. Statistics

All calculations and statistical analyses were done with Minitab 14 (State College, PA). The Mann–Whitney test was used.

3. Results

3.1. rDNA sequences detach from the nuclear matrix with development and aging

The variable number of FISH signals observed when nuclei from animals of the three ages were compared with each other is a consequence of the well-known hepatic polyploidy (Gupta, 2000). In nuclei from young mice, the DNA content of which is mainly contained in the 2C class, only two rDNA spots were clearly observed (Figure 1A), whereas in nuclei from adult and old mice, more rDNA spots were visible according to the higher ploidy levels observed in these animals (most nuclei with DNA content 4C and 8C, respectively) (Figures 1B, 1C). The rDNA FISH signals observed in young mice hepatocytes were always condensed (Figure 1A). In contrast, the 45S rDNA sites observed in hepatocytes from adult (Figure 1B) and old (Figure 1C) mice were partially decondensed and appeared as expanded clouds; they were always seen in the periphery of the negative images of the n (nucleoli), and in association with DAPI-stained dense clusters (chromocentres) (Figures 1B, 1C). These variations can give us a clue of a different chromatin organization of rDNA sequences when different ages were compared and are probably a consequence either of a different packaging of rDNA sequences at different ages or a result of the different ploidy levels in young, adult and old mice. In the case of adult and old animals, amplification of the signal could be the result of increased numbers of rDNA copies in the polyploid genome.

In fixed and lysed hepatocytes from young mice, which do not properly form ECFs but uncommon nuclear halos instead, the small dots of 45S rDNA detected by FISH remained inside the nuclei after the lysis treatment (Figure 2A), whereas in fixed and lysed preparations from adult and old mice, the FISH signals spread throughout and outside the nuclei, such that many small spots could be seen in the nuclear halos and ECFs (Figures 2B, 2C). The 45S rDNA signal intensity outside the nuclei was higher in old mice when compared with adult mice (Figures 2B, 2C) (Table 1). Moreover, the intensity of FISH signals inside the nuclei was higher in adult hepatocytes than in old hepatocytes (Table 1), thus arguing in favour of a higher number of rDNA sequences attached to the nuclear matrix in nuclei from adult mice, when compared with nuclei from old mice. These results were observed in all the examined nuclei that formed ECFs.

Figure 1 DNA FISH of 45S rDNA on fixed hepatocyte nuclei from young (A), adult (B) and old (C) mice. Nuclei were counterstained with DAPI. FISH signals are seen around the nucleoli (n). Scale bars, 12 µm.
3.2. Telomeric sequences are always located in chromatin loops

The behaviour of telomeric repetitive motifs, known to be heterochromatic and non-genic was also observed, in order to test the hypothesis that only transcribed sequences remain attached to the nuclear matrix, after the lysis treatment here employed.

We observed that, in mouse hepatocyte nuclei, telomeres were also detected as small dots (young and adult mice) (Figures 3A, 3C). In adult (Figure 3C) and old mice (not shown), telomeric sequences were seen spread in all the analysed nuclei; due to the increased extent of ploidy, several blocks could be seen, most of them near the bright DAPI-stained chromocentres but not associated with them. In young mice, these sequences were

Table 1 Image analysis of rDNA FISH signals after lysis treatment

| Model | Specimen | %IGLin | %IGLout | In/out | M
|-------|----------|--------|---------|--------|---
| Adult | 1        | 31.60  | 68.40   | 0.46   | 0.46* |
|       | 2        | 40.57  | 59.43   | 0.68   |      |
|       | 3        | 33.17  | 66.83   | 0.50   |      |
|       | 4        | 9.93   | 90.17   | 0.11   |      |
|       | 5        | 21.13  | 78.87   | 0.27   |      |
| Old   | 1        | 7.04   | 92.96   | 0.08   | 0.10* |
|       | 2        | 9.40   | 90.60   | 0.10   |      |
|       | 3        | 5.77   | 94.23   | 0.06   |      |
|       | 4        | 3.72   | 96.28   | 0.04   |      |
|       | 5        | 11.68  | 88.32   | 0.13   |      |
|       | 6        | 9.09   | 90.91   | 0.10   |      |
|       | 7        | 18.43  | 81.57   | 0.23   |      |
|       | 8        | 8.89   | 91.31   | 0.10   |      |

* Statistical difference with $P<0.0104$. 

Figure 2 45S rDNA differently associates with the nuclear matrix in hepatocytes of different ages

rDNA FISH signals on fixed and vertically lysed hepatocyte nuclei from young (A), adult (B) and old (C) mice. Nuclei were counterstained with DAPI. Arrows indicate direction of gravity and of ECFs. Scale bars, 30 μm.
detected only in nuclei of erythroblasts (Figure 3A). In these cells, most of the signals appeared positioned as organized features, concentrated in one part of the cell.

After lysis treatment, the telomeric sequences extended outside the nuclei at all the ages studied, but with less intensity in nuclei from young mice (Figure 3B), where they appeared at the nuclear halos. In this case, a DNA mass distributed homogenously out and inside the nuclei could be clearly observed. A similar pattern of telomeric sequences inside the nuclei and spreading out of them was observed in adult mice (Figure 3D). However, the intensity of the signals in the halo and in the ECFs of the adult mice was significantly brighter than those observed in the nuclei of young mice. Moreover, some dense blocks were clearly seen in the halo, a phenomenon not observed in nuclei of young mice. The telomeric sequences that remained in the nuclei were seen also on the bases of the DNA loop structures located at the nuclear periphery, which is in accordance with the notion that telomeres are often associated with the nuclear envelope (Pandita et al., 2007).

4. Discussion

The 45S rDNA repeats, which are responsible for the production of a large amount of rRNAs (Wellauer et al., 1974; Perry, 1976; Kominami et al., 1978), were expected to differently bind to the nuclear matrix with development and aging, since decreased transcription levels have been reported under these conditions (Ma and Nagata, 1990). Indeed, the present results indicate that, as the animal ages, there is an increase in the amount of 45S rDNA
repeats non-associated with the nuclear matrix and located in the nuclear halos or ECFs, thus suggesting that the attachment of these sequences to the nuclear matrix is related to gene activation.

According to the model proposed by Heng et al. (2004), there are two types of MAR sequences with ability to bind to the nuclear matrix: (i) the constitutive MARs, which are found in the bases of the DNA loops and outside transcribed sequences, presenting a structural role; (ii) facultative MARs, which are located inside the genes, being responsible for their transcriptional activity. Since the MARs found until now on rDNA sequences are located only in flanking sequences, it seems that they are of the constitutive type (Chen et al., 1997). In fact, there is no description of facultative MARs on rDNA genes.

In this regard, our present results confirm the existence of MARs in rRNA genes, since many rDNA copies remained attached to the nuclear matrix even after the lysis treatment. The association of MARs with the nuclear matrix is salt-resistant (Cook et al., 1976; Moraes et al., 2007).

Additionally, the results regarding the 45S rDNA genes suggest that, in multicopy genes, there is a nuclear matrix-dependent control of gene expression different from that of single copy genes. In single copy genes, the adhesion to the nuclear matrix determines their transcriptional level (Allen et al., 2000; Whitelaw et al., 2000), while in the case of 45S rDNA, this interaction may determine if each gene copy will be transcribed or not. Thus, the amount of transcripts produced could be regulated by controlling the production average of the thousands of gene copies rather than by controlling the level of activity of each single repeating unit.

In relation to the telomeric sequences, the results clearly show that the association of the chromatin with the nuclear matrix is a feature common to transcribing sequences. After the lysis treatment, most telomeric sequences exit the nuclei, remaining attached only to the nuclear envelope, and it is independent of the animal developmental state or age. Since telomeric repetitive motifs are devoid of genes, it is reasonable to conclude that non-transcribing sequences do not associate with the nuclear matrix. However, a question still remains on whether other heterochromatic sequences such as pericentric repeats have a similar behaviour.

It is probable that the telomeric sequences in nuclei from young mice are so packed that they were unavailable to telomeric probes in fixed nuclei or that, in adult and old mice, there is unpackaging of the telomeric structure, either as a natural physiological event or as a by-product of the genomic instability that is characteristic of the aging process. Nevertheless, after lysis, which is responsible for solubilizing several histone and non-histone proteins, the telomeres became accessible to the telomeric probes, thus binding them and revealing the sequences located at the nuclear halos. However, it is not to be overlooked that the telomeric repetitive motifs were seen in control nuclei only of polyplid adult and old hepatocytes and not in diploid nuclei from young mice, where there are less telomeric repeats present and, due to the sensitivity of the method, these sequences were detectable only when stretched with the lysis treatment.

In the present study, we showed that, when 45S rDNA genes are being highly transcribed, as in the case of young mice (Ma and Nagata, 1990), all of these genes were found completely attached to the nuclear matrix, while with development and aging, their copies are progressively switched off and consequently detached from the nuclear matrix. At the same time, the non-coding telomeric sequences were found detached from the nuclear matrix irrespective of the age or developmental state of the animal, but associated with the nuclear envelope, thus supporting the idea that non-coding regions probably do not associate with the nuclear matrix.

Author contribution

Alberto Moraes carried out FISH experiments, microscopy, image processing, statistics and drafted the manuscript. Mateus Mondin and Margarida Aguiar-Perecin carried out FISH experiments and drafted the manuscript. Marcelo Beletti carried out image processing and image analysis and drafted the manuscript. Ana Guaraldo provided animal facilities and carried out bioterism services. Maria Luiza Mello co-ordinated the study and drafted the manuscript.

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