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A possible role of cholesterol-sphingomyelin/phosphatidylcholine in nuclear matrix during rat liver regeneration

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***Background/Aims*: Phospholipids and cholesterol in chromatin have been previously demonstrated. The lipid fraction changes during cell proliferation in relation to activation of enzymes of phospholipid metabolism. The aim of the present work is to clarify if chromatin lipids may derive or not from nuclear matrix and if they have different roles.**

***Methods*: The subnuclear fractions were isolated from rat hepatocyte nuclei and the lipid fraction was extracted and analysed by chromatography in normal and regenerating liver. The phosphatidylcholine-sphingomyelin metabolism enzymes activity was assayed, by using radioactive substrates.**

***Results*: In nuclear matrix, cholesterol and sphingomyelin are respectively five and three times higher than those present in chromatin; the amount of phosphatidylcholine, which it is enriched in saturated fatty acids, is lower, thus indicating a less fluid structure. The lower content in phosphatidylcholine may be justified by the phosphatidylcholine-dependent phospholipase C activity, which increases during liver regeneration, reaching a peak at the beginning of S-phase, when also cholesterol and sphingomyelin increase.**

***Conclusions*: The nuclear matrix lipids are independent from chromatin lipids; the ratio cholesterol-sphingomyelin/phosphatidylcholine is higher and, as a consequence, nuclear matrix is less fluid in relation to DNA synthesis, suggesting a specific role of nuclear matrix as a structure involved in DNA duplication.**

***Keywords*: Nuclear matrix; Cholesterol; Phosphatidylcholine; Sphingomyelin; Phosphatidylcholine-dependent phospholipase C**

1. Introduction

It has been demonstrated that a phospholipid (PLs) cellular fraction is associated with chromatin [1,2]. The chromatin phospholipid fraction (CPF), which can be considered a chromatin 'minor component', differs from that of microsomes and nuclear membranes as regard composition [1] and turnover [2]; it changes also in relation to cell function [3]. The main modifications regard principally sphingomyelin (SM) and phosphatidylcholine (PC). Many enzymes like sphingomyelinase (SMase) [4], and phosphatidylcholine-dependent phospholipase C (PC-PLC) [5], sphingomyelin-synthase (SM-synthase) [6] are also present in chromatin and show different physico-chemical characteristics with respect to those present in the nuclear membranes. Their activities change in relation to many physiological functions, such as cell proliferation [4,5,7]. Recently, in chromatin, the presence of SM-linked cholesterol (CHO) and its changes in relation to cell proliferation have been demonstrated [7]. The presence of PLs [8,9], the phospholipase A2 (PLase-A2) and the SMase activities [9] have been also demonstrated in nuclear matrix.

The nuclear matrix is the scaffolding of the cell nucleus that confers nuclear shape, organises the nuclear chromatin, and regulates many important intra-nuclear biochemical events [10]. Many nuclear functions, including DNA replication, relaxation of the superhelical strain in DNA, processing of hnRNA and snRNP, and RNA transport, depend on the presence of a nuclear matrix [11]. Various structural and functional components of the nuclear matrix represent potential targets for anticancer agents [11]. Many transcription factors have been shown to be associated with the nuclear matrix, including steroid hormone receptors, that

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represent the first transcription regulatory proteins localised in this compartment [12]. Recently, advances in microscopic techniques and the availability of new molecular probes have made it possible to localise functional domains within the nuclear matrix and demonstrate dynamic interactions between both soluble and insoluble components involved in the control of multiple nuclear transactions [13]. Current evidences suggest that histone acetyltransferase, histone deacetylase, transcription factors, and the transcription machinery mediate the transient attachments between nuclear matrix and active chromatin [14]. Waitz and Loidl showed that the nuclear localisation of c-myc protein changes during cell cycle, being transiently but specifically bound to the periphery of the nuclear matrix structure during S-phase [15]. Nuclear matrix seems important also in neoplastic transformation and apoptotic process [10].

The problem now arises if the chromatin PLs are derived from the nuclear matrix or are independent. The solution of this question may help also in clarifying the role of the lipid fraction in chromatin and in the nuclear matrix. In the present work we try to analyse lipid composition of nuclear matrix and to compare the results with those obtained in nuclear membranes and chromatin. The behaviour of the nuclear matrix lipid fraction during hepatic regeneration is studied at the end to identify a possible independent role on chromatin lipid fraction.

2. Materials and methods

Radioactive PC (L-3-Phosphatidyl *N*-methyl-^{3}H choline 1,2 dipalmitoyl, 81.0 Ci/mmol) and radioactive SM (choline-metyl ^{14}C, 54.5 Ci/mol) were obtained from Amersham Pharmacia Biotech (Rainham, Essex, UK); Ecoscint A was obtained from National Diagnostic (Atlanta, GA, USA); PC, SM, phosphatidylinositol (PI), phosphatidylethanolamine (PE), phospatidylserine (PS), CHO were purchased from Sigma Chemical Co. (St. Louis, MO, USA)

2.1. Animals

Thirty-day-old Sprague–Dawley rats of either sex were used. They were kept in a normal light-dark period and had free access to pelleted food and water prior to killing between 09:00 and 10:00 h. Hepatectomy was performed, after anaesthesia, between 08:00 and 10:00 h according to Higgins and Handerson [16]. Sham operated animals were used as control. The animals were killed 6, 12, 18, 24 h after hepatectomy.

2.2. Preparation of hepatocyte nuclei, chromatin and nuclear membranes from rat liver

The nuclei were separated according to the method of Bresnick [17]. The absence of contamination by endoplasmic reticulum was monitored by electrophoretic analysis of RNA extracted from this preparation [18]. The nuclear pellet in 1 M sucrose still contained some rRNA which disappeared completely after two washings in Barnes et al. solution [19]. The nuclei were checked for possible cytoplasmic contamination also by evaluating the activity of a microsomal marker (NADH-cytochrome C-reductase) [20]. The chromatin was extracted according to the method of Shaw and Huang [21] modified by Viola Magni et al. [1]. Nuclear membranes were isolated according to the procedure of Kay and Johnston [22].

2.3. Nuclear matrix purification

After hepatocyte nuclei washing, the nuclei-depleted membrane were obtained by treatment with 0.1% Triton X-100 for 5 min followed by centrifugation. Previous experiments have shown that this Triton treatment removes all external contamination without any loss of lipids from internal membrane or chromatin [1]. The pellet was washed in Barnes et al. solution [19] for three times. The nuclear matrix purification was obtained after DNAse I digestion according to Cocco et al. [8]. The nuclear pellet was resuspended in 0.25 M sucrose, 10 mM Tris–HCl, 5 mM $MgCl_2$ pH 7.4 and digested with 3 μg/ml DNase I at 37°C for 15 min. Chromatin was removed by adding $(NH4)_2SO_4$ to a final concentration of 0.25 M under gentle shaking, as reported by Alberti et al. [23]. The sample was centrifuged at $1800 \times g$ 15 min. The nuclear matrix was extracted by 2 M NaCl, 10 mM Tris pH 7.4 at 4°C for 15 min. This treatment was repeated tree times.

2.4. Biochemical determinations

Protein, DNA and RNA contents were determined according to Lowry et al. [24], Burton [25] and Schneider [26], respectively. The total amount of PLs was determined by measuring inorganic phosphorus [27].

2.5. Lipid analysis

Lipids were extracted according to Folch et al. [28]. The organic phase was separated, for PLs analysis, on thin layer silica gel chromatography (TLC), in a bidimensional system as previously reported [1]. The lipids were detected with iodine vapour and scraped into test tubes for inorganic phosphorous determination [27]. CHO was separated by TLC using hexane/diethyl ether/acetic acid, 70:30:1 by vol, as solvent [29]. The spot of CHO was quantified according to Rudel et al. [30].

2.6. Phosphatidylcholine fatty acids composition in nuclear membrane, chromatin and nuclear matrix

For fatty acid composition, the PC was separated on TLC and analysed according to Leray et al. [31].

2.7. SMase, PC-PLC and SM-synthase activity assays

The SMase, PC-PLC and SM-synthase activities were measured in nuclear membranes and chromatin as previously reported [4–6]. The incubation conditions for nuclear matrix were the same as those reported for nuclear membranes [4–6].

2.8. Effect of CHO and SM on SMase, PC-PLC and SM-synthase in nuclear matrix

In order to establish if the increase of CHO-SM content in nuclear matrix, demonstrated during liver regeneration, may be correlated specifically with the increase of PC-PLC activity, nuclear matrix has been incubated, as previously reported [4–6] for the SMase, PC-PLC, SM-synthase activity assay, in the presence of 0.1 mM CHO or SM 0.1 mM to a final volume of 0.1 ml.

3. Results

3.1. Biochemical composition

The nuclear matrix was characterised by a high content in protein, lipids and only a small amount of RNA and DNA. During the purification steps of the nuclear matrix, in the supernatant, we have 93.8% (min 89.5–max 98.9) of DNA, 63.9% (min 55.9–max 66.5) of RNA and PLs 9.2% (min

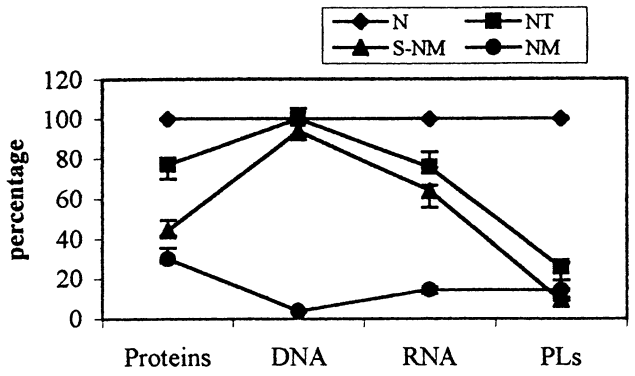

Fig. 1. Loss of proteins, DNA, RNA and phospholipids during the purification steps of nuclear matrix. The data are expressed as percentage relative to hepatocyte nuclei and indicate the median and range of three experiments N = nuclei, NT = nuclei treated whit Triton X-100, S-NMx = supernatant after nuclear matrix purification, NMx = nuclear matrix.

8.9–max 10.5) of the entire hepatocyte nuclei (N, Fig. 1). A small part of RNA together with proteins was lost during Triton X-100 nuclei treatment (NT). The comparison between nuclear membranes, chromatin and nuclear matrix showed that chromatin contained, as expected, almost all DNA and 78.8 % (min 76.9–max 79.1) of RNA whereas the nuclear matrix contained 22.3% (min 21.5–max 23.1) of nuclear proteins and only 5.1% (min 4.9–max 5.1) of RNA. A greater amount of RNA (17.2%, min 16.8–max 17.2) was present in the nuclear membranes.

As regards the lipid composition, the nuclear matrix showed a content of CHO and PLs lower than the nuclear membranes, but 4.7 and 2.6 times (respectively) higher than those present in the chromatin (Table 1). Therefore, the nuclear matrix is enriched in CHO content with respect to PLs content. TLC analysis of single PLs extracted from nuclear matrix showed an increase of approximately two times of all PLs with the exception of SM which is 3.2 times that present in the chromatin (Table 1). The data, expressed as percentage of total PLs, showed that the SM value is higher and PC value lower than those present in chromatin and nuclear membranes. The nuclear matrix CHO/SM ratio was similar to that found in nuclear membranes, but nuclear matrix CHO/PC was higher than that of nuclear membranes (Table 1). The fatty acid analysis showed that PC contains more saturated fatty acids than the nuclear membrane and chromatin, suggesting less fluidity of nuclear matrix with respect to nuclear membranes (Fig. 2).

3.2. SMase, PC-PLC and SM-synthase activity

In nuclear matrix, the PC-PLC showed higher activity, whereas SM-synthase and SMase were less active with respect to chromatin (Table 2).

3.3. Lipid modification in nuclear matrix and chromatin during liver regeneration

The liver regeneration was characterised by a strong increase of CHO and a decrease of PC in the nuclear matrix. The CHO in the liver, examined after anhaestesia, shows a modest increase from 2.54 (min 2.32–max 2.68) μg/mg protein to 3.05 (min 2.95–max 3.90). A value which comes back to normal in sham operated animals. In hepatectomised animals instead the CHO increased from the value 3.05 (min 2.95–max 3.90) μg/mg protein to 24.90 (min 24.10–max 27.30) during the first 18 h after hepatectomy, followed by a decrease at 24 h. Similar behaviour was shown by SM (Fig. 3a). A reduction to half value was observed for the PC (Fig. 3a), which may be justified by the increase in PC-PLC activity from 669 (min 657–max 684) pmol/mg protein/min to 3599 (min 3484–max 3848) pmol/mg protein/min (Fig. 3b).

In chromatin instead, the SM value of 0.75 μg/mg protein

Table 1
Comparison of lipid composition in nuclei, nuclear membrane, chromatin and nuclear matrix[a]

	Nuclei	Nuclear membranes	Chromatin	Nuclear matrix
CHO	2.41 (1.68–2.91)	8.10 (7.54–8.55)	0.52 (0.35–0.72)	2.48 (2.30–2.77)
PLs	35.22 (21.39–45.95)	114.80 (94.06–139.20)	5.62 (5.64–7.11)	14.74 (13.58–14.88)
PS	2.00 (1.32–2.19)	2.05 (1.82–2.22)	0.36 (0.27–0.58)	0.78 (0.43–1.19)
	5.31 (4.48–6.35)	2.05 (1.71–2.11)	5.99 (3.60–8.48)	6.76 (3.35–7.00)
PI	3.33 (2.54–3.92)	10.97 (7.80–16.49)	0.68 (0.44–1.00)	1.92 (1.80–2.42)
	9.81 (7.52–11.6)	10.44 (7.70–15.80)	11.02 (8.05–14.32)	15.20 (12.04–16.11)
SM	0.92 (0.49–1.37)	4.70 (3.37–5.66)	0.48 (0.48–0.65)	1.53 (1.33–1.53)
	2.80 (1.40–3.95)	3.98 (3.54–5.70)	7.79 (6.83–9.25)	11.50 (11.02–12.89)
PC	18.80 (13.9–24.7)	60.33 (45.85–69.15)	3.70 (3.12–4.09)	6.20 (6.13–6.70)
	58.71 (39.00–71.12)	59.01 (43.85–65.55)	54.09 (47.30–62.21)	44.92 (43.05–47.50)
PE	8.34 (7.90–9.70)	27.13 (26.64–28.06)	1.38 (1.27–1.40)	3.44 (3.24–3.50)
	26.90 (22.40–27.35)	26.31 (25.40–26.88)	20.50 (18.98–21.41)	3.35 (3.28–3.56)
CHO/SM	2.61	1.72	1.10	1.62
CHO/PC	0.13	0.13	0.14	0.40

[a] The values are expressed as μg/mg protein (in the second line the percentage of total phospholipids) and represent the median and range (in parenthesis) of three independent experiments. CHO, cholesterol; PLs, phospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine. The content of CHO, PLs and SM is higher in nuclear matrix with respect to chromatin.

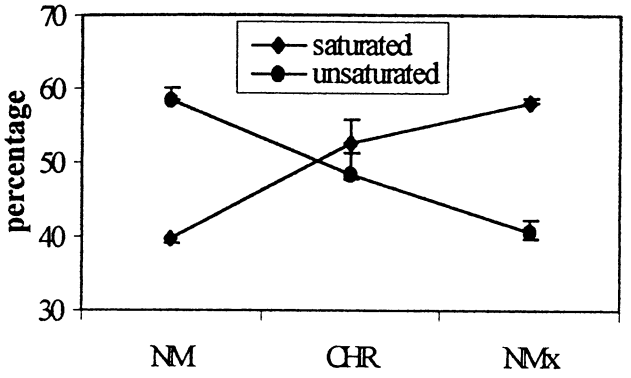

Fig. 2. Percentage of saturated and unsaturated fatty acids of phosphatidylcholine in nuclear membrane, chromatin and nuclear matrix. The data are expressed as percentage of fatty acid distribution and indicate the median and range of three experiments. NM = nuclear membrane, CHR = chromatin, NMx = nuclear matrix. The content of saturated fatty acids is higher in nuclear matrix with respect to nuclear membrane, suggesting a less fluidity.

(min 0.65–max 0.83) at 6 h after hepatectomy increased by three times at 12 h (2.30 μg/mg protein, min 2.04–max 2.40), then decreased at 18 h by 60% (0.94 μg/mg protein, min 0.56–max 1.23) and at 24 h the value was 1.17 μg/mg protein (min 1.12–max 1.20). The PC value of 5.73 μg/mg protein at 6 h (min 5.62–max 5.79) remained constant up to 12 h, increased 2-fold at 18 h (12.86 μg/mg protein, min 11.72–max 13,80) and decreased by 27% at 24 h (9.36 μg/mg protein, min 8.61–max 10.15).

3.4. Effect of cholesterol and sphingomyelin on nuclear matrix phosphatidylcholine-dependent phospholipase C

To establish if the increase of CHO-SM, demonstrated in rat liver regeneration, at 18 h after hepatectomy influences the PC-PLC activity, NMx was incubated in the presence of CHO or SM. The results showed that incubation with CHO and SM increased the PC-PLC activity by 2.02 and 1.35 times, respectively, whereas no variations were found in SM-synthase and SMase activity (Fig. 4).

Table 2
Enzymatic activities of PL-PLC, SM-synthase, and SMase in nuclear membranes, chromatin and nuclear matrix[a]

	PC-PLC	SM-synthase	SMase
Nuclear membranes	1672 (1669–1723)	758 (751–787)	649 (642–670)
Chromatin	209 (199–216)	270 (267–297)	20 (18–24)
Nuclear matrix	683 (670–688)	83 (79–88)	11 (10–15)

[a] The values are expressed as pmol/mg prot./min and represent the median and range (in parenthesis) of three independent experiments. PC-PLC, phosphatidylcholine-dependent phospholipase C; SM-synthase, sphingomyelin-synthase; SMase, sphingomyelinase. In nuclear matrix, PC-PLC activity is higher and SM-synthase lower with respect to chromatin.

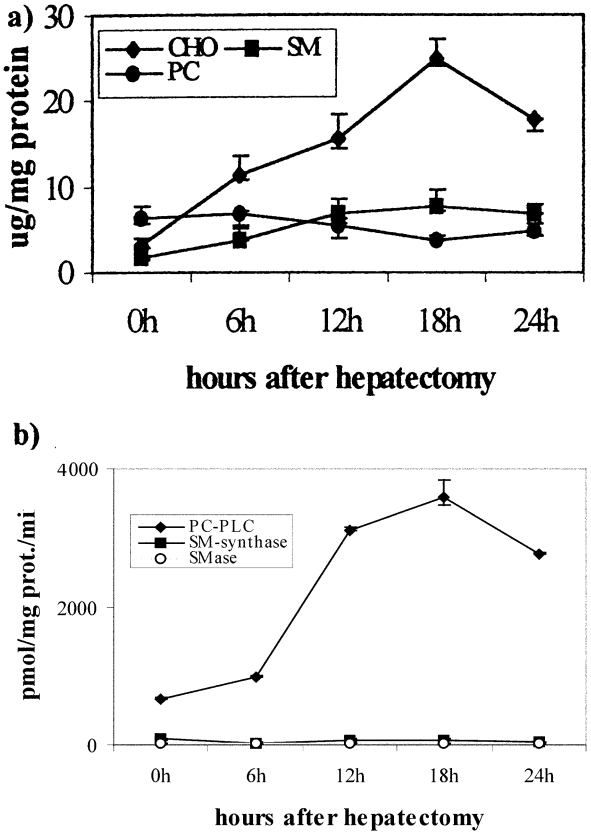

Fig. 3. Modifications in nuclear matrix during rat liver regeneration of (a) cholesterol, sphingomyelin and phosphatidylcholine content. The data are expressed as μg/mg protein and represent the median and range of three single experiments; (b) phosphatidylcholine-dependent phospholipase C, sphingomyelin-synthase and sphingomyelinase activity. The data are expressed as pmol/mg prot./min and represent the median and range of three single experiments. The content of CHO increases and PC decreases, with respect to 0 h up to 18 h after hepatectomy.

4. Discussion

The presence of nuclear matrix has been the object of many discussions about its real existence. Recent data are in favour not only of its existence as scaffolding of nuclear structure, but as a structure which has many roles in cell proliferation and in gene transcription regulation [11]. In fact, many transcription factors are associated to the matrix as well as to some receptor proteins [12].

Since previous descriptions of this structure have shown the existence of a lipid fraction, it cannot be excluded that the lipids present in the chromatin may be due to the presence of nuclear matrix in our chromatin preparation. In the previous work great attention was devoted to exclude that the chromatin lipids fraction may be due to nuclear membrane contamination [33], but nothing has been done regarding nuclear matrix. In the present work we have separated nuclear matrix from hepatocytes following the standard procedure and have analysed its composition with

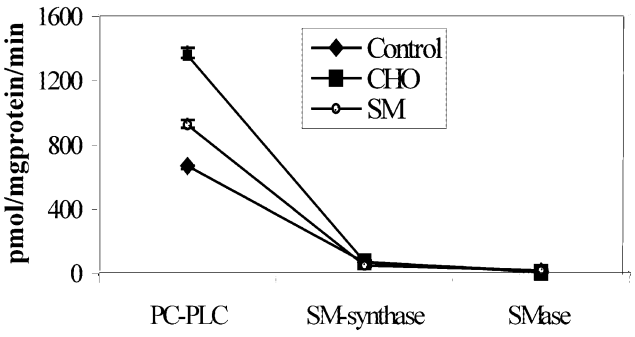

Fig. 4. Effect of cholesterol and sphingomyelin on nuclear matrix phosphatidylcholine-dependent phospholipase C, sphingomyelin-synthase and sphingomyelinase activity. The data are expressed as pmol/mg prot./min and represent the median and range of three single experiments. CHO, cholesterol; SM, sphingomyelin.

particular attention to the lipids fraction and some enzymes related to PLs metabolism. The results showed that the nuclear matrix, which contains a great amount of proteins with the presence of low percentages of DNA, RNA, has a lipid composition different with respect to nuclear membranes and chromatin. The presence of CHO is very high in the nuclear matrix and the ratio CHO/PC is three times that observed in the other nuclear structures. This is due to the fact that PC value is lower, whereas that of CHO is particularly high. The fatty acid composition evidentiates an enrichment of PC saturated fatty acids with respect to the chromatin. Also SM content is higher in nuclear membrane than in chromatin. In conclusion these results exclude the fact that the lipids found in chromatin are similar to those of the nuclear matrix, which also differ from those of the nuclear membranes. On the basis of lipids composition the nuclear matrix seems more rigid than the nuclear membranes. These considerations are confirmed by the analysis of the behaviour during hepatic regeneration. The nuclear matrix CHO increases by 8.16. times during the first 18 h after hepatectomy, followed by a decrease at 24 h. In nuclear membrane no significant variations of CHO are observed [32], whereas in chromatin a 5-fold increase in CHO content, during the first 18 h after hepatectomy, is observed [7]; the value is nevertheless less than that found in nuclear matrix (7.01 times). In nuclear membrane, the SM content increases by 1.18 times between 12 and 18 h [32] whereas the PC is unchanged up to 24 h [34]. In the chromatin, during the first hours after hepatectomy, a SM decrease, following to an increase in SMase activity, which reaches the peak 18 h after hepatectomy [4], is observed; thereafter SM increases due to the activation of SM-synthase [7]. In the nuclear matrix instead the enzyme, which increases its activity reaching a maximum at 18 h, is PC-PLC thus causing a decrease by 40% of PC. In the nuclear membranes the PC-PLC activity is strongly reduced after hepatectomy and increases slowly during the following hours reaching the 50% of the normal value after 24 h [6]. In the chromatin, this enzyme activity is also less than normal during the first 6 h after hepatectomy and shows an increase at 12 h, in coincidence with the beginning of S-phase [6]. The behaviour of chromatin PC-PLC is in agreement with the increase of PC. Previous experiments on cell in culture have found an increase in PC content in nuclei during the S-phase [35] which coincides with the chromatin PC behaviour. The nuclear matrix PC instead shows a decrease accompanied by an increase of CHO and SM which also reach the peak at the same time after hepatectomy of PC-PLC enzyme activity. The CHO and SM stimulate specifically the nuclear matrix PC-PLC activity. It is possible that the increase of nuclear matrix CHO and SM can stimulate the PC-PLC activity with a decrease of PC during rat liver regeneration; as a consequence, the increase of CHO-SM/PC ratio may reduce the fluidity. It is clear therefore that the rigidity of nuclear matrix changes in relation to cell proliferation reaching the maximum during DNA duplication.

Then, the following conclusions may be made: the lipids described in chromatin are not the results of contamination either from that of the nuclear membranes, or from that of nuclear matrix on the basis not only of their composition or metabolism, but also on the basis of their different behaviour during the first hours after hepatectomy. The data are also in favour of the existence of nuclear matrix which may have a role in cell duplication. It has been shown that this structure may favour DNA relaxation of superhelical strain [13]. It is possible that change in rigidity may have the function to avoid that during duplication the DNA may be more dispersed inside the nucleus, thus making a correct rearrangement of chromosome more difficult.

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