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ORGANIZAÇÃO E EXTENSIBILIDADE DA CROMATINA EM HEPATÓCITOS DE CAMUNDONGO SOB CONDIÇÕES DE JEJUM E REALIMENTAÇÃO

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Co-Orientador: Prof. Dr. Benedicto de Campos Vidal

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“Quando um ideal de perfeição, impele a ser melhor, esse culto dos próprios méritos consolida, nos homens, a dignidade; quando o afã de parecer arrasta a todos os rebaixamentos, o culto da sombra inflama a vaidade.”

José Ingenieros

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Sumário

Resumo

O efeito de jejum por 48 h e de realimentação por 48 h após jejum na organização e extensibilidade da cromatina foi estudado em hepatócitos de camundongos adultos. A metodologia utilizada envolveu ensaios topoquímicos, análise de imagem, ação da gravidade e microscopia de polarização. O jejum aumentou os estados de empacotamento da cromatina, principalmente em áreas de cromatina não condensada, e houve uma drástica diminuição no nível de glicoproteínas de matriz nuclear e de freqüência de extensibilidade cromatínica sob a ação da gravidade. As mudanças no empacotamento da cromatina não foram acompanhadas por alterações na quantidade de DNA. O grau de desempacotamento da cromatina, mas não da extensibilidade cromatínica, em camundongos em jejum que foram realimentados foi maior que o dos controles bem alimentados. A realimentação também induziu aumento na quantidade de DNA. Contudo, a duração da realimentação foi, provavelmente, insuficiente para restabelecer o estereoarranjo do complexo cromatina-matriz nuclear e restaurar a fluidez da cromatina aos níveis vistos em camundongos bem alimentados. Dessa forma, as mudanças associadas com o jejum e a realimentação em núcleos de hepatócitos de camundongos adultos envolveu a perda, reestruturação e recuperação de proteínas nucleares, especialmente componentes da matriz nuclear, relacionados com a organização e extensibilidade da cromatina. Estas mudanças poderiam favorecer o silenciamento e reativação de atividades transcripcionais, dependendo do estado nutricional do organismo.

Abstract

The effect of a 48 h starvation and of 48 h of refeeding subsequent to starvation on chromatin organization and extensibility was studied in hepatocytes from adult mice. The methods used involved topochemical assays, image analysis, gravity action, and polarization microscopy. Starvation increased the chromatin packing states, especially in areas of non-condensed chromatin, and there was a drastic decrease in the level of nuclear matrix glycoproteins and in the frequency of chromatin extensibility under gravity. The changes in chromatin packing were not accompanied by alterations in the amount of DNA. The extent of chromatin unpackaging, but not of chromatin extensibility, in starved mice that were refed was greater than in well-fed controls. Refeeding also increased the amount of DNA. However, the duration of refeeding was probably insufficient to re-establish the stereoarrangement of the chromatin-nuclear matrix and to restore the chromatin fluidity to the level seen in well-fed mice. Thus, the changes associated with starvation and refeeding in the liver cell nuclei of adult mice involved the loss, restructure and regain of nuclear proteins, especially nuclear matrix components, related to chromatin organization and extensibility. These changes could favor the silencing and reactivation of transcriptional activities, depending on the organism's nutritional state.

Introdução

Organização nuclear

O DNA nos núcleos interfásicos acha-se organizado segundo uma hierarquia estrutural, alcançando níveis de compactação necessários para abrigar, por exemplo, 2 m de sua estrutura em um núcleo com diâmetros de 5 a 10 μm. As compactações do DNA na cromatina e da cromatina no núcleo são extremamente ordenadas (Cremer *et al.* 1993, 2000). As características mais comuns dessa organização são importantes para a atividade transcricional do genoma.

A estrutura primária de compactação do DNA ocorre nos nucleossomos. Estes, adicionalmente, se acham arranjados em cadeias polinucleossômicas (semelhantes a colares de conta), que se organizam em fibras de 30 nm (solenóides), de acordo com o modelo mais aceito atualmente para a organização da cromatina (Alberts *et al.* 2002). Cada nucleossomo consiste de 146 pb de DNA enrolados em um octâmero de histonas arranjado sob a forma de um tetrâmero $(H3\text{-}H4)_2$ e de dois dímeros H2A/H2B, posicionados em cada face do tetrâmero (nucleóide), além de uma porção de DNA de ligação ("DNA-linker"), ligando os nucleóides adjacentes, e perfazendo um total de 200 pb em cada nucleossomo. As histonas do núcleo octamérico possuem estruturas similares, com um domínio amino-terminal, um domínio globular e um domínio carboxi-terminal cada uma (Ramakrishnam 1994). Os domínios globulares dessas histonas estão envolvidos nos contatos histona-histona e histona-DNA que são responsáveis pela manutenção da integridade estrutural do nucleossomo. Esses contatos são essencialmente não-específicos e as interações entre DNA e histonas, por

exemplo, se dão apenas entre os grupos fosfato negativamente carregados do DNA e cadeias laterais positivamente carregadas dos resíduos de aminoácidos das histonas (Saecker & Record Jr. 2002), o que torna essa ligações sensíveis a mudanças na força iônica ou no pH do meio. A compactação dos nucleossomos em solenóides acontece graças à participação da histona H1 e das caudas das histonas dos nucleóides (Mello 2001 – revisão).

A estrutura solenoidal da fibra de cromatina está organizada em domínios em alça ("loop domains") com um comprimento médio de 86 kb cada domínio (Gerdes *et al.* 1994). Admite-se que na base de cada alça haja seqüências, ricas em pares de bases A-T, denominadas MARs ("matrix attachment regions"), ou SARs ("scaffold associated regions") que se liguem a proteínas da matriz nuclear (Davie 1995).

Especula-se que, juntamente com a cromatina, a matriz nuclear define a forma nuclear, fornecendo suporte estrutural para vários processos do metabolismo do núcleo interfásico, incluindo-se replicação do DNA (Pardoll *et al.* 1980), processamento de hnRNA (Zeitlin *et al.* 1989) e ação de hormônios esteróides (Rennie *et al.* 1983), além de controle da expressão gênica, transporte de RNAs e fosforilação ao nível de substrato (Taboga 2001 – revisão).

A matriz nuclear (Fig. 1) pode ser observada depois que a cromatina e o RNA são extraídos tratando-se o núcleo com tampões com alta força iônica, associados ou não com agentes quelantes (EDTA), detergentes não iônicos (como o Triton X-100) e nucleases (Berezney e Coffey 1977; Taboga 2001 – revisão).

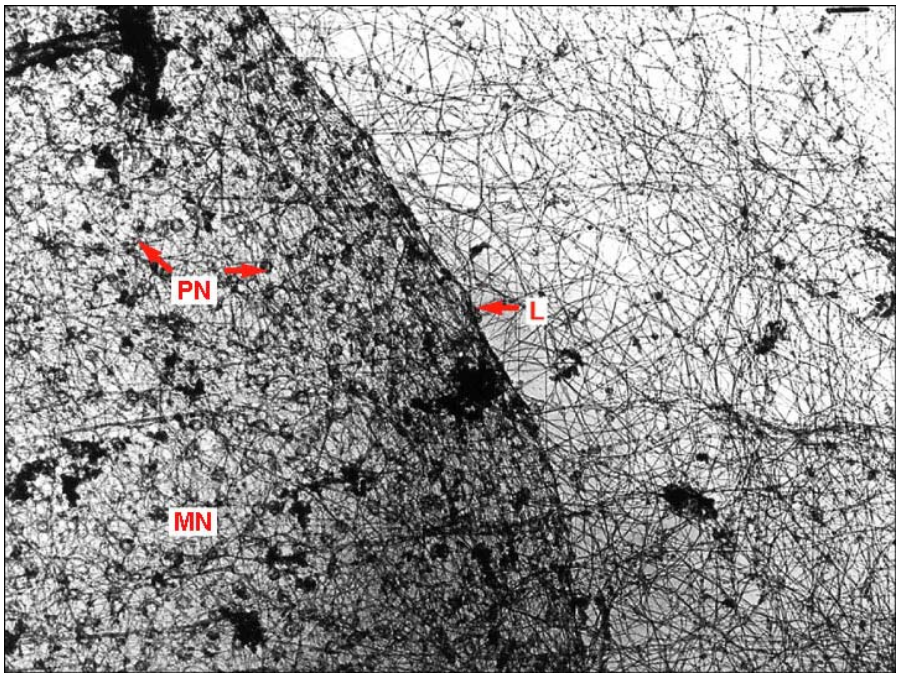

Figura 1. Micrografia eletrônica da matriz nuclear de uma célula epitelial de bexiga após extração salina, mostrando a matriz nuclear residual (MN) e os filamentos intermediários do citoesqueleto. A lâmina nuclear (L) e os poros nucleares (PN) também podem ser claramente observados (adaptado de http://www.rnweb.com/be_core/content/journals/u/data/1996/0700/u7a015.html).

A matriz nuclear é uma estrutura essencialmente protéica (98% do conteúdo é constituído de proteínas, 1,2% de RNA, 0,5% de fosfolipídios e 0,1% de DNA) (Taboga 2001 – revisão), podendo manter relações com a lâmina nuclear, os complexos de poro e as proteínas do citoesqueleto, além de conter também elementos fibrogranulares mais internos ao núcleo. É composta por 10-25% de conteúdo protéico total do núcleo e encontra-se firmemente ligada ao DNA e a alguns tipos de RNA (Taboga 2001 – revisão). Esse conteúdo protéico possui um perfil heterogêneo de proteínas não-histônicas, incluindo-se metaloproteínas, glicoproteínas, enzimas do metabolismo cromatínico, assim como proteínas denominadas de matrinas nucleares, que são as principais e maiores proteínas da matriz nuclear (Berezney *et al.* 1995; Taboga 2001 – revisão). Os cromossomos

ocupam posições fixas no núcleo porque eles estão ligados a vários componentes da matriz (Comings and Okada 1976; Nickerson *et al.* 1989; Cremer *et al.* 1993, 2000).

Vários autores têm estudado a presença de glicoproteínas no núcleo. As mais estudadas são as O-glicosiladas, localizadas nos poros nucleares e no nucleoplasma, mas também há a presença de glicoproteínas N-glicosiladas, localizadas nas lâminas nucleares e na eucromatina, associadas ao DNA. Várias dessas glicoproteínas podem ser reconhecidas no núcleo pela lectina concanavalina A e lectinas de propriedades similares, além de diversas outras lectinas (Hart *et al.* 1989).

As glicoproteínas da matriz nuclear respondem por 2% do total de proteínas da matriz interna e 6% do total de proteínas da matriz periférica (em íntima associação com a lâmina nuclear). Pouco se sabe a respeito da função dessas proteínas, mas vários estudos sugerem que essas glicoproteínas possam ter um papel chave na estruturação da matriz nuclear, sendo que a formação dessa estrutura pode depender, dentre outros fatores, das interações carboidrato-proteínas nela existentes (Ferraro *et al.* 1994). Além disso, pode haver uma correlação entre glicosilação nuclear e atividade transcricional. Estudos comparando o conteúdo glicoprotéico nuclear de células de fígado normais e de carcinomas hepáticos revelou indícios de mudanças no conteúdo glicoprotéico associadas aos diferentes padrões de expressão gênica associados ao tumor (Glass *et al.* 1981).

Deve-se também mencionar que, em termos dos níveis hierarquicamente superiores de organização da cromatina, observáveis à microscopia, diferentes estados de empacotamento podem ser descritos em núcleos interfásicos. Algumas compactações se relacionam a uma certa inatividade gênica por conta de diferenciação celular (ex: linfócitos humanos, eritrócitos nucleados, espermatozóides), morte celular (apoptose) ou ainda estados patológicos, como o avanço de alguns processos de tumorigênese (Mello and Russo 1990; Mello *et al.* 1995; Vidal *et al.* 1998).

Há, no entanto, regiões cromatínicas que permanecem condensadas durante toda a interfase denominadas heterocromatina, em contraposição às áreas de cromatina distendida denominadas eucromatina (Heitz 1928; 1934). A heterocromatina apresenta replicação de DNA mais lenta do que a eucromatina e certa inatividade gênica. A heterocromatina de regiões centroméricas, teloméricas e algumas vezes, intercalares de regiões homólogas de cromossomos homólogos, geralmente rica em DNA repetitivo ou satélite, é predominantemente não codificadora e denominada constitutiva (Mello 1978; 2001 – revisões) (nas definições de Heitz (1934), α-heterocromatina). Quando porções heterocromáticas constitutivas se associam constituindo corpos maiores, estes se denominam cromocentros. São particularmente identificáveis em núcleos de hepatócitos de camundongo e de células somáticas do hemíptero *Triatoma infestans* (Mello 2001 – revisão).

Diferindo da heterocromatina constitutiva, a heterocromatina facultativa (na definição de Heitz (1934), β-heterocromatina) não apresenta diferenças em tipo de

DNA, em comparação à eucromatina e seria mais um estado reprimido de atividade, podendo reverter ao estado de eucromatina. Como exemplo, pode ser citada a heterocromatina de um dos cromossomos X dos mamíferos placentários XX (Mello 1978 – revisão).

A heterocromatina exerce um papel funcional importante na célula, contribuindo para o controle da transcrição de genes que estejam dentro de seus domínios. A formação da heterocromatina telomérica é determinada pelas modificações covalentes das caudas das histonas (Luger and Richmond 1998; Cheung *et al.* 2000; Rice and Allis 2001; Alberts *et al.* 2002), que por sua vez são reconhecidas por proteínas denominadas Sir ("silent information regulator"). Essas proteínas tanto reconhecem regiões de cromatina cujas caudas não estejam acetiladas, como atuam como desacetilases, cuja atividade depende do cofator NAD^+, fornecendo o arcabouço para a ligação de outras proteínas Sir (Brachman *et al.* 1995; Guarente 1999, 2000; Alberts *et al.* 2002; Rosenberg and Parkhust 2002; Grewal and Moazed 2003). Durante o jejum prolongado, por exemplo, a concentração de NAD^+ aumenta na célula, ativando as proteínas Sir e contribuindo para a propagação da heterocromatina telomérica para regiões adjacentes aos telômeros. Possivelmente este seja um mecanismo de silenciamento de determinados genes através da compactação da cromatina, cuja atividade não é necessária para a célula durante a privação de alimento (Alberts *et al.* 2002).

Cromatinas condensadas, heterocromáticas ou não, podem apresentar diferenças em propriedades químicas e/ou citoquímicas, na dependência de seus componentes protéicos (Vidal 1972*a*, 1972*b*). Como exemplo, temos a cromatina

de espermatozóides, que possuem propriedades anisotrópicas e citoquímicas diferentes de acordo com o tipo de proteína responsável pela compactação do DNA nessas células (Mello & Vidal 1973; Taboga *et al.* 1996).

Fibras estendidas de cromatina

As propriedades físicas dos cromossomos ou da cromatina em núcleos interfásicos são importantes para entender suas mudanças estruturais em nível molecular e supramolecular associadas às suas respectivas alterações funcionais (Perkins *et al.* 1997; Poirier *et al.* 2000). Dentre tais propriedades físicas, padrões de extensibilidade cromatínica podem fornecer informações relevantes sobre alterações estruturais e de supraorganização da cromatina sob condições fisiológicas particulares. Admite-se que estas possam diferir nos núcleos interfásicos com o desenvolvimento, a diferenciação celular, a morte celular e o envelhecimento.

Fibras estendidas de cromatina (extended chromatin fibers – ECFs) são estruturas fibrosas de cromatina, que podem fluir do núcleo quando se utilizam tratamentos específicos, incluindo-se tratamento com m-AMSA (N-[4-(9-acridinilamino)-3-metoxifenil]metanosulfonamida) (Heng *et al.* 1992), soluções alcalinas ou desnaturantes (Fidlerová *et al.* 1994), ou extração em soluções contendo alta concentração salina e detergentes (Haaf and Ward 1994, 1995; Vidal 2000). Os tratamentos citados são responsáveis pela lise celular e nuclear, mas a extensibilidade da cromatina depende também de forças mecânicas atuando nas células, como a ação da gravidade (Haaf and Ward 1994, 1995; Vidal 2000), ou o estiramento manual dos núcleos e conseqüente liberação e extensão

da cromatina (Fidlerová *et al.* 1994). Soluções concentradas de NaCl removem histonas, o que contribui para aumentar a mobilidade da cromatina (Bekhor *et al.* 1969; Mello e Cordeiro 1985).

A extensibilidade cromatínica irá depender de propriedades reológicas do DNA e dos complexos DNA-proteínas nela presentes (Steger and Workman 1996; Strick *et al.* 1996) além da interação do DNA com a matriz nuclear. Através de procedimentos que possam revelar fibras estendidas de cromatina é possível detectar-se uma distribuição ordenada de marcadores cromossômicos, sejam eles seqüências de fibras cromatínicas de diferentes espessuras, supraorganização e orientação macromolecular (Vidal 2000), sejam determinados *loci* gênicos identificáveis pela técnica de hibridação *in situ* fluorescente (FISH) (Heng *et al.* 1992; Gerdes *et al.* 1994; Haaf and Ward 1995) ou ainda determinadas famílias de proteínas identificáveis por anticorpos (Hirano and Mitchison 1994) e citoquímica (Vidal 2000).

Assim, é de se esperar que a extensibilidade cromatínica mostre diferentes padrões em núcleos com diferentes quantidades de heterocromatina, como em hepatócitos de camundongo sob a ação do jejum, uma vez que nessas circunstâncias podem ocorrer diferentes graus de complexação do DNA à proteínas nucleares (Castro *et al.* 1986; Amaral and Mello 1989).

Mudanças na estrutura cromatínica causadas por mudanças no estado nutricional foram relatadas por vários autores. Em hepatócitos de camundongos, o jejum ou a subnutrição induz modificações no conteúdo de histona H1 e também de histonas do núcleo octamérico, assim como produz hipometilação de DNA e

mudanças na atividade de topoisomerases e de enzimas que desestabilizam a estrutura da dupla-hélice (Castro *et al.* 1986). Um aumento generalizado na condensação da cromatina após um período de jejum de 52 h foi observado em hepatócitos de ratos e camundongos através de ensaios moleculares e topoquímicos (Castro e Sevall 1980; Castro *et al.* 1986; Amaral e Mello 1989).

Em hepatócitos de codornas japonesas, a privação de alimento por cinco dias provoca mudanças na quantidade de algumas proteínas nucleoplásmicas não histônicas (Palyga *et. al.* 1991). A estrutura cromatínica de regiões promotoras de genes de certas enzimas hepáticas de frangos é afetada durante condições de jejum (Ma and Goodridge 1992). Uma dieta pobre em proteínas durante cinco dias causa redução no conteúdo protéico total nuclear em 35%, o qual se torna recuperado rapidamente com a realimentação (exceto pelas histonas, cuja concentração retorna aos níveis normais mais lentamente) (Cassia *et al.* 1991).

Em células de insetos hematófagos, áreas de heterocromatina descondensam em resposta ao jejum, possivelmente ativando genes que estejam normalmente silentes (Mello 1989).

Além do reconhecimento das caudas desacetiladas das histonas pelas proteínas Sir, a metilação das caudas das histonas favorece o reconhecimento destas por outro tipo de proteína envolvida na formação de heterocromatina: a proteína HP1, identificada em *Drosophila*, e que possui outras isoformas de mesma função em outros organismos (Eissenberg *et al.* 1992). Nos macronúcleos de *Tetrahymena thermophila*, uma proteína similar à HP1 foi relatada como

estando envolvida na expressão de certos genes ligados a respostas induzidas por jejum (Huang *et al.* 1999).

Como descrito até aqui, desde que a estrutura e supraorganização da cromatina são afetadas pelo jejum ou subnutrição, é de se esperar que a viscoelasticidade da cromatina e a formação de ECFs poderiam estar alteradas nessas condições. Adicionalmente, a avaliação do conteúdo de glicoproteínas nucleares forneceria informações relevantes quanto ao papel de tais proteínas nas eventuais modificações estruturais da cromatina frente à situação de jejum.

Objetivos

Os objetivos do presente trabalho consistiram em:

- Estudar, através de análise de imagem, mudanças na organização cromatínica em núcleos de hepatócitos de camundongos adultos bem alimentados, em jejum e realimentados, corados pela reação de Feulgen .
- Quantificar, através de microespectrofotometria de varredura, a reatividade de glicoproteínas de núcleos de hepatócitos de camundongos adultos bem alimentados, em jejum e realimentados, à lectina Con-Br.
- Testar dois protocolos para obtenção de fibras estendidas de cromatina, buscando entender seu mecanismo de formação e os fatores envolvidos.
- Estudar, através de microscopia de polarização, as propriedades anisotrópicas das fibras estendidas de cromatina, além da reatividade dessas fibras à reação de Feulgen e à lectina Con-Br, além de comparar o padrão de extensibilidade cromatínica em núcleos de hepatócitos de camundongos adultos bem alimentados, em jejum e realimentados

Capítulo 1

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Chromatin organization and extensibility in mouse hepatocytes following starvation and refeeding

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Running title: Chromatin extensibility following starvation

Abstract

The effect of a 48 h starvation and of 48 h of refeeding subsequent to starvation on chromatin organization and extensibility was studied in hepatocytes from adult mice. The methods used involved topochemical assays, image analysis, gravity action, and polarization microscopy. Starvation increased the chromatin packing states, especially in areas of non-condensed chromatin, and there was a drastic decrease in the level of nuclear matrix glycoproteins and in the frequency of chromatin extensibility under gravity. The changes in chromatin packing were not accompanied by alterations in the amount of DNA. The extent of chromatin unpackaging, but not of chromatin extensibility, in starved mice that were refed was greater than in well-fed controls. Refeeding also increased the amount of DNA. However, the duration of refeeding was probably insufficient to re-establish the stereoarrangement of the chromatin-nuclear matrix and to restore the chromatin fluidity to the level seen in well-fed mice. Thus, the changes associated with starvation and refeeding in the liver cell nuclei of adult mice involved the loss, restructure and regain of nuclear proteins, especially nuclear matrix components, related to chromatin organization and extensibility. These changes could favor the silencing and reactivation of transcriptional activities, depending on the organism's nutritional state.

Keywords: chromatin organization, extended chromatin fibers, starvation, refeeding, nuclear matrix.

Introduction

Chromatin and DNA flow from cell nuclei following treatment with concentrated saline and detergent solutions and, under the action of gravity or manual mechanical stretching, can form extended chromatin fibers (ECF) (Heng et al. 1992; Haaf and Ward 1994, 1995; Fidlerova et al. 1994; Poirier et al. 2000; Vidal 2000). ECF formation depends on the rheological properties of DNA (Strick et al. 1996; Perkins et al. 1997), but is also affected by other nuclear molecules. The molecules that play a part in chromatin composition, organization and metabolism are histones, histone variants, and non-histone proteins, including enzymes that modify histones and factors that bind to histones (ex.: Sir family proteins) (Brachmann et al. 1995; Steger et al. 1996; Guarente 1999, 2000; Alberts et al. 2002; Bannister et al 2002; Rosenberg and Parkhurst 2002; Grewal and Moazed 2003). Some of these proteins may affect ECF formation, e.g. nuclear matrix proteins which can influence chromatin extensibility through their contribution to higher-order chromatin structure (Davie 1995; Cremer et al. 2000).

Changes in chromatin structure caused by nutritional status have been reported. In rodent liver cells, starvation or malnutrition modifies histone H1 and histones of the nucleosome core, and can promote DNA hypomethylation as well as changes in the enzymatic activity of topoisomerases and helix-destabilizing proteins (Castro et al. 1986). A generalized increase in chromatin higher-order structure (condensation) after up to 52 h of starvation has been shown using molecular biology and topochemical assays (Castro and Sevall 1980; Castro et al. 1986, Amaral and Mello 1989).

Food deprivation for five days causes changes in the non-histone nucleoplasmic proteins of Japanese quail liver cells (an increase in the amount of 40 and 125 kDa proteins and a decrease in a 63 kDa protein) (Palyga et al. 1991). The chromatin structure around the genes of certain hepatic enzymes in the chicken is affected by starvation and there is a decrease in the hypersensitivity to DNase I that is simultaneous with the onset of nucleosome formation in these areas (Ma and Goodridge 1992). Protein depletion for five days causes a 35% decrease in the total nuclear protein in mouse liver. A fast recovery of lost proteins, except for histones, which are restored at a lower rate, is observed when depleted mice are refed a normal diet (Cassia et al. 1991).

In cells of blood-sucking insects, areas of heterochromatin decondense in response to starvation, possibly in an attempt to activate usually silent genes (Mello 1989; Mello et al. 2001). In the macronuclei of *Tetrahymena thermophila,* a protein similar to the heterochromatin-associated protein HP1 of *Drosophila melanogaster* has been reported to function in the establishment and/or maintenance of a specialized condensed chromatin environment that facilitates the expression of certain genes linked to a starvation-induced response (Huang et al. 1999).

Since the structure and supraorganization of chromatin are affected by starvation or malnutrition, the viscoelasticity of chromatin and ECF formation would also be expected to be altered under these conditions. In the present study, the chromatin organization and ECF formation in hepatocyte nuclei from well-nourished, starved and refed mice were compared using topochemical methods,

image analysis and polarization microscopy. Two protocols that are assumed to preserve nuclear proteins to different extents were used.

Materials and methods

Male mice of the inbred strain A/CEMIB, obtained from the Multidisciplinary Center for Biological Investigation of the State University of Campinas (CEMIB/UNICAMP) were reared under normal conditions and fed standard extruded chow (Purina®) *ad libitum* until 15 weeks old. A group of these mice was subsequently deprived of food for 48 h but received water *ad libitum*. Some of the starved mice were then refed for 48 h. Fully-nourished (4), starved (5), and refed (5) mice were killed by decapitation and their livers immediately removed and placed in cold physiological solution (0.9 % NaCl in distilled water). Liver slices were used to prepare imprints on glass slides. All of the protocols involving animal care and use were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas (CEEA/IB) and met the guidelines of the Canadian Council on Animal Care.

Treatments

Freshly prepared imprints were fixed in an absolute ethanol – glacial acetic acid mixture (3:1, v/v) for 1 min and then rinsed in 70% ethanol for 5 min. The slides were positioned vertically and horizontally and the imprints were immediately lysed in 2 M NaCl plus 1% Triton X-100 in Tris-HCl buffer (25 mM, pH 7.4) for 5 h at 25ºC to obtain ECFs.

Some unfixed preparations were subjected to the same lysis protocol but for a reduced period of time (10 min), after which the volume of solution was completed with absolute ethanol to a final concentration of 50% for 10 min. Following this step, the slides were carefully removed from the lysis solution and transferred to 70% ethanol for 30 min (Heng et al. 1992; Haaf and Ward 1994; Vidal 2000).

Fixed preparations not subjected to the lysis protocol were used as controls.

Topochemistry and optical anisotropy

Staining was carried out with a 0.025% toluidine blue (Merck, Darmstadt, Germany) solution in McIlvaine buffer at pH 4.0 for 15 min (Vidal 2000). The preparations were then rapidly (5 s) rinsed in distilled water, air dried, cleared in xylene and mounted in Canada balsam.

Birefringence and linear dichroism (selective absorption of polarized light) were investigated in the toluidine blue-stained nuclear materials under polarized light with a Zeiss polarizing microscope equipped with 16/0.32 Planachromatic objective and photographed in a Zeiss Axiophot 2 microscope equipped with 10/0.30, 20/0.50, 40/0.75, and 100/1.30 Pol-Neofluar objectives, optovar 1.25, 1.4 condenser. Kodak Gold ISO 100 film was used for photomicrographs.

The frequency of nuclei from fully-nourished, starved, and refed mice showing ECFs was estimated and compared in the toluidine blue-stained preparations examined under polarized light.

Staining was done with a 0.025% toluidine blue (Merck, Darmstadt, Germany) solution in McIlvaine buffer at pH 4.0 for 15 min (Vidal 2000). The preparations were then rapidly (5 s) rinsed in distilled water, air dried, cleared in xylene and mounted in Canada balsam.

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Some preparations were subjected to the Feulgen reaction (hydrolysis done in 4 M HCl at 25ºC for 60 min) to detect DNA. Imprints were also screened for the presence of histones and non-histone proteins in the ECFs and nuclear images using alkaline and acid fast green (Sigma, St. Louis, MO, U.S.A.) staining, respectively (Deitch 1966). The ability of ECF to bind to Con-Br (Con-A-like lectin extracted from *Canavalia brasiliensis* seeds according to Moreira and Cavada (1984)) was also investigated. Briefly, the slides were incubated at room temperature with the Con-Br solution for 10 min followed by a peroxidase solution for 10 min and by a peroxidase substrate solution containing 3,3'-diaminobenzidine

(Fluka Chemie, Switzerland), according to the protocols of Kiernan (1975) and Vidal et al. (1997).

Video image analysis

Carl Zeiss/Kontron equipment and Kontron KS400 software (Oberkochen/ Munich, Germany) were used. The microscope images were obtained with a Zeiss Axiophot 2 microscope equipped with a Neofluar 40/0.75 objective, optovar factor 2, 0.90 condenser and λ = 546 nm. The images to be processed were fed from the microscope into a Pentium computer through a Sony CCD – IRIS/RGB Hyper HAD color video camera. Under these conditions, 1 μm corresponded to 5.77 pixels. The software provided quantitative information on nuclear area (μm^2), optical density (OD = absorbances), integrated optical density (IOD = Feulgen-DNA content), SDtd (standard deviation of the total densitometric values per nucleus or absorbance variability per nucleus, which reflects the variability in the degree of chromatin packing per nucleus), and entropy (number of bits needed to store the densitometric values per nucleus image, or extent of pixel variability in a nucleus) (Mello et al. 2003). Slides from three mice were used for each nutritional condition. Seventy nuclei per mouse were chosen at random for image analysis. The total number of nuclei for each nutritional condition was 210.

Scanning microspectrophotometry

Con-Br stained areas (in μm^2) were determined for the cell nuclei with a Zeiss automatic scanning microspectrophotometer interfaced to a personal

computer. The software program and the interface hardware were developed by Mr. Linus Vidal. The operating conditions and microscope settings were: a predominantly monodirectional scanning motion, a Planapo 100/1.25 objective, optovar 2, a measuring diaphragm diameter of 0.16 mm, a field diaphragm diameter of 0.2 mm, an LD-Epiplan 16/0.30 condenser, a scanning spot size of 0.5 μm X 0.5 μm, λ = 560 nm obtained with a Schott monochromator filter ruler, an R-928 photomultiplier and a Pentium II microcomputer. Grid points showing absorbances $\leq$0.020 were considered to be background and were automatically removed from the nuclear image. Cut-off points of 0.200 and 0.300 were selected to evaluate the Con-Br positive areas based on a preliminary test using nuclei from fully-nourished mice.

Statistical analysis

All calculations and statistical analyses were done using the Minitab 12 software (State College, PA). The applied tests consisted of one-way ANOVA for unstacked data and Mann-Whitney's test with a 95 % confidence interval both.

Results

In fixed control preparations not subjected to the lysis treatment, the hepatocyte nuclei stained metachromatically and showed birefringence after toluidine blue staining (Fig. 1A-D) and also stained with the alkaline and acid fast green solutions. The fixed preparations stained positively in the Feulgen reaction (Fig. 1E-G) and image analysis yielded the values shown in Tables 1 and 2. These

data showed that the nuclear areas and Feulgen-DNA amounts did not differ significantly whereas the absorbances increased, and entropy and SDtd decreased, with starvation. Refeeding increased the nuclear areas and Feulgen–DNA amounts compared to the fully nourished controls; the absorbance values returned to those shown by the fully-nourished controls, and the entropy and SDtd decreased significantly.

Only nuclei from fully-nourished mice responded strongly to the Con-Br reaction (Fig. 2A-D). Practically no response was seen in nuclei from starved mice (Fig. 2B). In hepatocyte nuclei from refed mice, there was considerable variability in the Con-Br response; some nuclei exhibited this response in the form of small granules only at the nuclear periphery (Fig. 2C and D). The use of a cut-off point of 0.200 allowed the identification of Con-Br positive areas in fully-nourished and refed mice. The cut-off point of 0.300 identified more densely packed points reactive in the Con-Br test (Table 3). The areas identified with the cut-off point of 0.200 coincided with the Con-Br positive visual images.

The microspectrophotometric evaluation of the data for the Con-Br-treated preparations indicated that Con-Br reactive binding sites were no longer present in the hepatocyte nuclei after starvation. Refeeding partially restored these sites (Table 3).

The chromatin of fixed and unfixed hepatocyte nuclei from well-fed and starved mice, and from refed mice treated with the lysis solution and subsequently with the toluidine blue solution, stained metachromatically and showed intense birefringence. Only in slides positioned vertically did chromatin flow from the cell

nuclei, provided the nuclei were treated with the lysis solution (Fig. 3A-D). In vertically-positioned preparations, the lysis protocols used were capable of inducing ECF formation. However, in unfixed preparations subjected to short lysis treatment, all of the nuclei produced ECFs (Figs. 3B,D and 4), whereas in the fixed preparations, only some of the cell nuclei showed these fibers (Figs. 5 and 6). The ECFs for the unfixed nuclei were extremely long (Fig. 4) whereas those for the fixed nuclei were shorter and slender (Fig. 5). Chromatin areas extending from chromocenters were occasionally identified.

In all cases, the ECFs stained metachromatically and showed optical anisotropy (interference colors of birefringence (Fig. 4A) and linear dichroism (Fig. 4B,C)) when treated with the toluidine blue solution, responded positively to the Feulgen reaction (Fig. 4D,E), and appeared unstained after treatment with alkaline fast green solution. In unfixed preparations, the ECF stained lightly with the acid fast green solution (not shown) and showed some response to the Con-Br reaction (Fig. 4F,G). The linear dichroism was negative (i.e., the absorbances of fibers positioned perpendicular to the plane of polarized light were larger than those of the same fibers positioned parallel to this plane), as is typical of DNA molecules (Fig. 4B,C). Some hepatocyte nuclei from starved mice produced ECFs with a few points showing a positive response to the Con-Br test (Fig. 4G).

Since variation in ECF formation was detected only in fixed preparations, with many nuclei not producing evident ECF (Figs. 5 and 6), the frequencies of responsive cells in toluidine blue-stained preparations from fully nourished, starved, and refed mice were determined and compared (Table 4). The values

were significantly different for fully nourished vs. starved mice ($p<0.05$, Mann-Whitney test). Refeeding did not change the values obtained after starvation, as shown by the same statistical test.

In lysed, fixed preparations from starved and fully-nourished mice, positive staining with acid fast green was seen in nuclei which did not develop ECF formation (Fig. 6C). A strong positive Con-Br response was still evident in hepatocytes from well-fed mice, inclusively in nuclei with only slight ECF formation. In this case, the Con-Br reactive points were located mostly within the nuclei, although a few points were also evident on ECFs (Fig. 6D). Compared to non-lysed preparations from starved mice, lysed preparations from these mice contained some nuclei with a few Con-Br reactive points (Fig. 6E). In hepatocytes from refed mice, the Con-Br positive response after fixation and treatment with the lysis protocol was more evident than that observed in nuclei from starved mice, but less intense and more peripherally distributed than that in nuclei from fully nourished mice (Fig. 6F).

Discussion

The present results show that starvation for 48 h changes the chromatin organization and extensibility of mouse hepatocyte nuclei, in agreement with other reports (Castro and Sevall 1980; Castro et al. 1986; Amaral and Mello 1989; Davie 1995; Cremer et al. 2000). A partial recovery in chromatin organization but not in chromatin extensibility was observed after refeeding for 48 h.

Starvation increased the optical densities (absorbances) while simultaneously decreasing the standard deviation of the total densitometric values per nucleus (SDtd) and the nuclear entropy, as shown by image analysis of Feulgen-stained preparations. Thus, food deprivation increased the degree of chromatin packing in areas of more loosely packed chromatin, thereby diminishing the contrast between highly and less densely packed states of chromatin. Indeed, under food deprivation, there is an increase in the cytosolic concentration of NAD^+, with the activation of several Sir proteins that spread the telomeric heterochromatin to the adjacent gene-containing DNA (Alberts et al. 2002). This situation favors gene silencing (Castro et al. 1986; Alberts et al. 2002).

The increase in the Feulgen-DNA absorbances associated with chromatin condensation during starvation led to a slight decrease in nuclear areas. However, this trend was not significant (Table 1) so there was no difference between starved mice and well-fed controls.

Refeeding probably stimulated polyploidy since, under this physiological condition, the Feulgen-DNA values and nuclear areas surpassed those of the fully-nourished controls. The decrease in SDtd and entropy compared to nuclei after 48 h of starvation indicated an even chromatin unpackaging (Mello et al. 2003), in agreement with the recovery of transcriptional activities after refeeding (Cassia et al. 1991).

The ECFs studied here were produced by the action of gravity, provided some of the chromatin, nuclear matrix and nuclear envelope components were disrupted and removed by lysis, which was more efficient in unfixed nuclei. Highly

concentrated salt solutions disrupt the interactions between DNA and nuclear proteins by solubilizing the proteins and extracting the RNA, thereby making the chromatin more fluid (Bekhor et al. 1969). Additionally, the use of a non-ionic detergent such as Triton X-100 can disrupt the nuclear envelope and allow the histone-depleted chromatin to flow from the nucleus under the force of gravity.

The ECFs obtained from the mouse hepatocyte nuclei consisted basically of DNA, as shown by the Feulgen staining and the optical anisotropy following toluidine blue binding. The anisotropic properties (linear dichroism and birefringence) of toluidine blue-stained ECFs revealed the highly ordered molecular arrangement of the DNA, especially in unfixed preparations, since these properties are typical of dye-binding molecules that are helically distributed around the DNA macromolecule. Occasional Con-Br positive points in the ECFs revealed glycoproteins, possibly from the nuclear matrix, in association with DNA and resistant to lysis.

The short slender ECFs seen in some of the fixed cell nuclei were most likely promoted by the fixative. Although the acetic acid-ethanol mixture used here has been reported to remove a significant part of the histones and non-histone proteins from cell nuclei (Dick and Johns 1968), ethanol may counteract the removal of histone by acetic acid (Petitte et al. 1994). Indeed, acid fast green staining and a positive Con-Br response were seen in fixed cell nuclei even after lysis. The decreased frequency of ECF production seen following starvation most certainly reflected the higher-order packing states associated with chromatin supra-organization, as mentioned above. However, although some chromatin

decondensation occurred with refeeding, the frequency of ECF migration apparently did not change. This could be because the duration of refeeding was too short for the nuclear matrix components to recover the architecture typical of the well-fed controls, with a consequent deleterious effect on chromatin extensibility.

The microscopic and microspectrophotometric data indicated that Con-Br mannose/glucose reactive residues, assumed to be nuclear matrix protein components that are stable to the acetic acid-ethanol fixation and treatment with lysis solution (Ferraro et al. 1994; Vidal et al. 1997; Vidal 2000), were no longer detectable in fixed hepatocyte nuclei after starvation. Con-A binding sites have already been demonstrated in the glycoproteins of nuclear matrix preparations and in nuclear sites with a predominance of transcriptionally active chromatin (Kan and da Silva 1986; Hart et al. 1989).

The response of hepatocyte nuclei to Con-Br treatment suggested that there was protein degradation during starvation (Cassia et al. 1991). However, a few Con-Br reactive granules were observed under the same physiological conditions in ECF flowing from unfixed, lysed nuclei or in fixed, lysed nuclei with no ECF formation. This suggested that some of the nuclear proteins reactive with Con-Br were retained after the 48 h starvation, although the remaining binding sites were unavailable to the lectin, perhaps because of steric hindrance related to the changes in chromatin supra-organization (increased chromatin packing) detected in Feulgen-stained preparations. The Con-Br reactive sites probably became exposed during treatment with the lysis solution, which removes RNA and most

nuclear proteins from unfixed or acetic acid-ethanol-fixed nuclei (Dick 1969; Mello and Cordeiro 1985), and possibly alters the stereoarrangement of the remaining nuclear matrix proteins. An adverse effect of steric hindrance on the response to Con-A has also been reported for collagen fibers (Pimentel and Vidal 1990).

Refeeding resulted in a partial recovery of the Con-Br positive response, as seen in fixed nuclei that were not lysed. This finding agrees with the restoration of protein synthesis and the migration of newly synthesized proteins to the cell nuclei, and with the decrease in protein breakdown seen after the cessation of food deprivation (Cassia et al. 1991). The variation seen in the localization of Con-Br positive granules in the hepatocyte nuclei, with most granules being distributed in the outer and inner areas of the nuclear border, probably indicated that newly synthesized protein components of the nuclear matrix were being imported into the nucleus.

In conclusion, the changes in the chromatin of hepatocytes from adult mice during starvation and refeeding involved the loss, restructure and regain of nuclear proteins, especially nuclear matrix components, associated with chromatin organization and extensibility. These changes could favor the silencing and reactivation of transcriptional activities, depending on the animal's nutritional state.

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Table 1. Image analysis data for fixed Feulgen-stained hepatocyte nuclei.

Parameters	Fully-nourished		Starved		Refed	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Nuclear area (μm^2)	129.45	63.77	118.78	48.56	156.15	72.44
OD	0.89	0.14	0.97	0.13	0.89	0.09
IOD	114.83	58.54	115.45	49.69	140.00	69.17
Entropy	4.94	0.28	4.84	0.23	4.66	0.28
SDtd	8.09	1.67	7.56	1.36	6.64	1.40

n = 210; IOD = integrated optical density (Feulgen-DNA content); OD = optical density (= absorbance); s = standard deviation; SDtd = standard deviation of total densitometric values per nucleus; $\bar{x}$ = arithmetic mean

Table 2. ANOVA comparison for image analysis parameters of Feulgen-stained nuclei from fully-nourished (N), starved (S) and refed (R) mice.

Parameters	Comparisons	F	P
Nuclear area (μm^2)	N X S X R	20.00	0.000
	N X S	3.73	0.054
	N X R	16.07	0.000
	S X R	38.56	0.000
OD	N X S X R	30.97	0.000
	N X S	36.96	0.000
	N X R	0.04	0.846
	S X R	55.29	0.000
IOD	N X S X R	12.15	0.784
	N X S	0.01	0.908
	N X R	16.20	0.000
	S X R	17.45	0.000
Entropy	N X S X R	58.76	0.000
	N X S	15.01	0.000
	N X R	102.39	0.000
	S X R	51.29	0.000
SDtd	N X S X R	51.28	0.000
	N X S	12.86	0.000
	N X R	92.60	0.000
	S X R	46.38	0.000

IOD = integrated optical density (Feulgen-DNA content); OD = optical density (= absorbance); SDtd = standard deviation of total densitometric values per nucleus

Table 3. Con-Br positive areas in mouse hepatocyte nuclei.

Nutritional status	Cut-off point : 0.200		Cut-off point : 0.300	
	$\bar{x}$	s	$\bar{x}$	s
Fully nourished				
A_T	143.30	77.10	143.30	77.10
A_C	41.43	40.67	24.75	28.03
A_C %	26.39	12.24	15.27	8.94
Starved				
A_T	101.66	35.47	101.66	35.47
A_C	-----	-----	-----	-----
A_C %	-----	-----	-----	-----
Refed				
A_T	170.60	81.50	170.60	81.50
A_C	21.39	2.96	6.77	0.98
A_C %	12.63	1.59	4.29	0.63

A_T – Total nuclear area (μm^2); A_C – area calculated after applying the indicated cut-off point (Con-Br area)(μm^2); A_C % - Con-Br area relative to A_T; s = standard deviation; $\bar{x}$ = arithmetic mean

Table 4. ECF frequency in fixed hepatocyte nuclei subjected to a lysis protocol and stained with toluidine blue.

Nutritional conditions	Specimen code	No. of nuclei counted	Frequency of ECF formation (%)
Fully nourished	1	6704	22.0
	2	3363	13.6
	3	11357	13.3
	4	3791	9.7
Starved	5	6369	9.1
	6	9447	6.8
	7	3825	7.6
	8	4903	1.5
	9	6001	2.0
Refed	10	3393	8.2
	11	3405	6.3
	12	4539	2.3
	13	3538	11.1
	14	3459	7.2

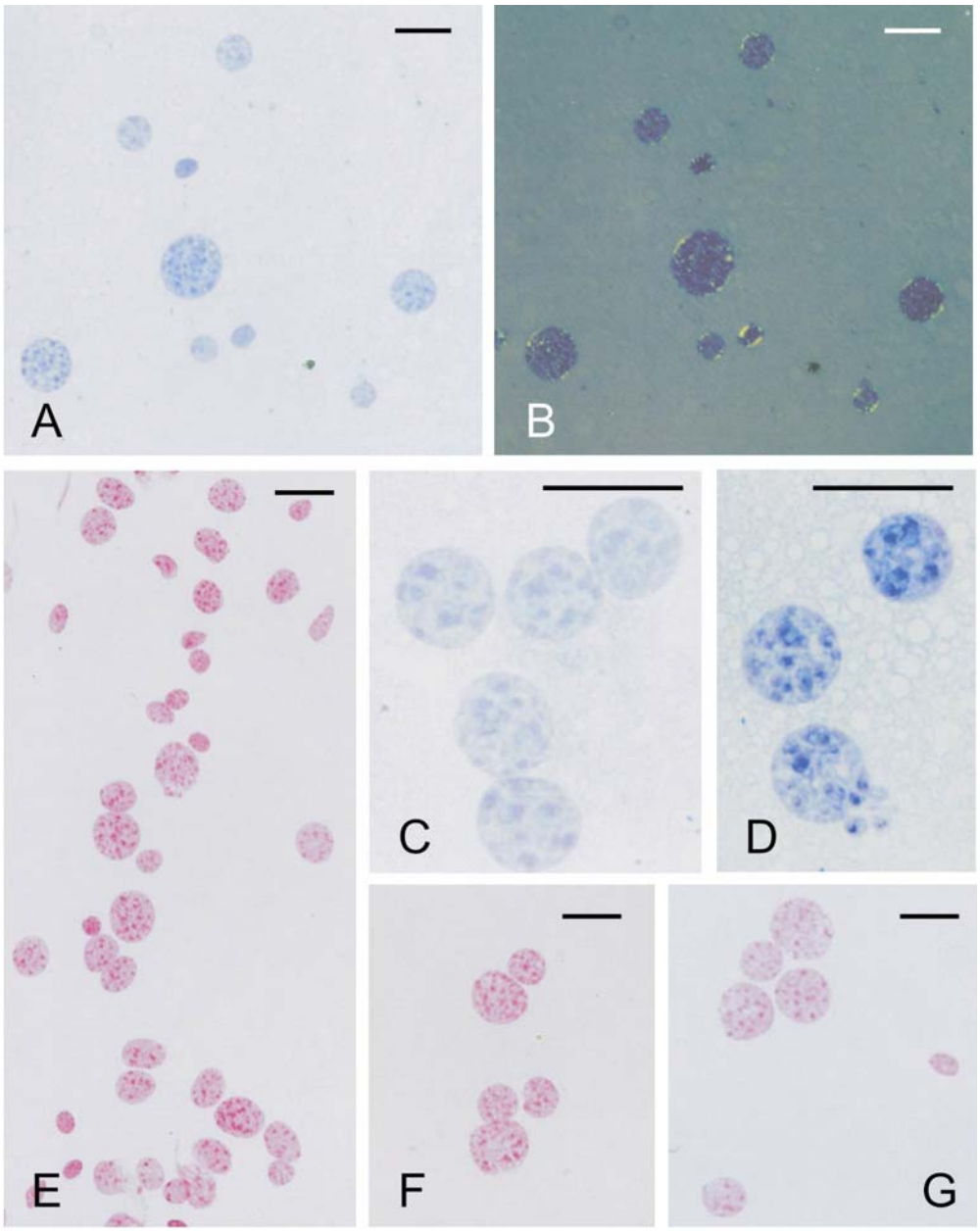

Figure 1

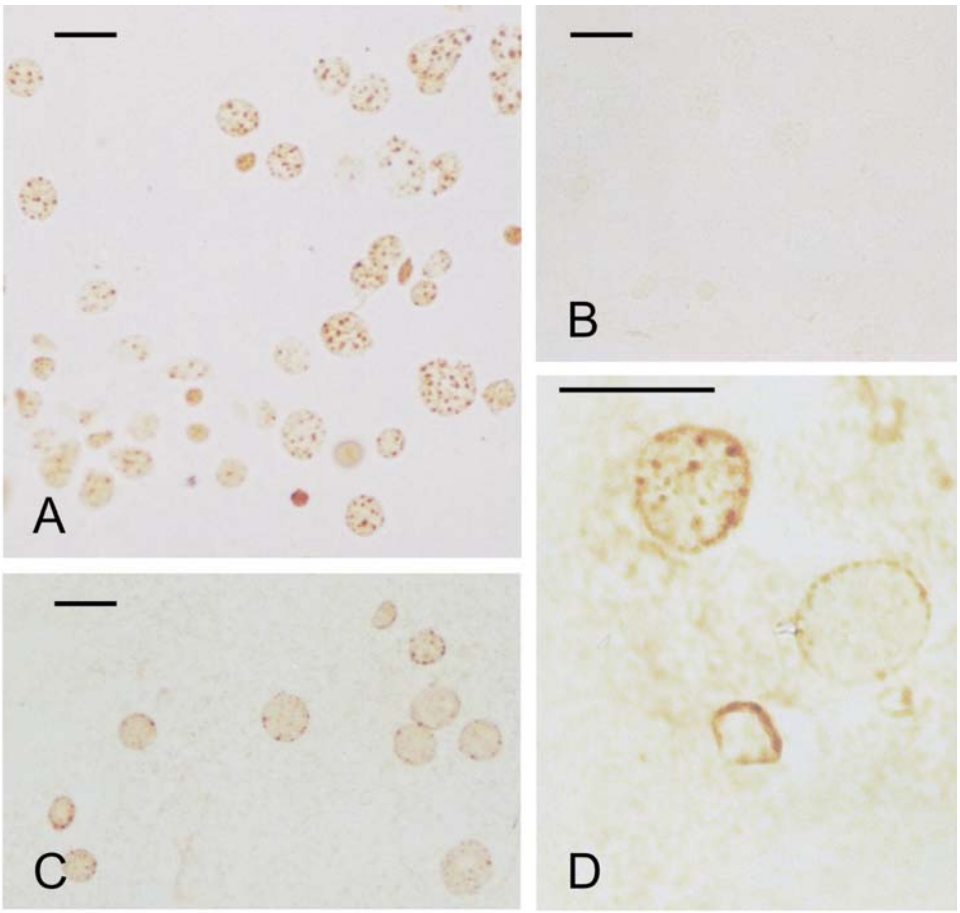

Figure 2

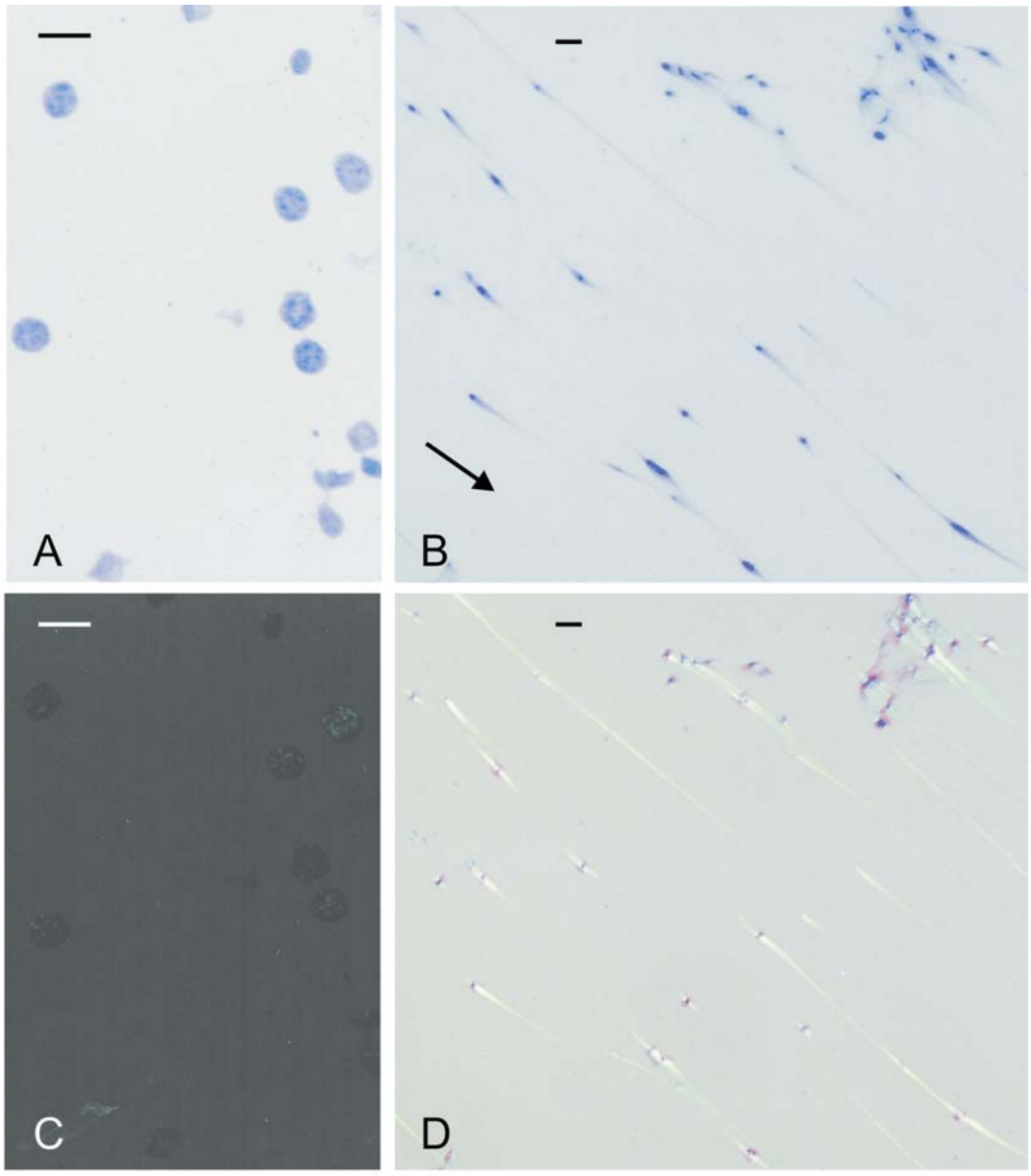

Figure 3

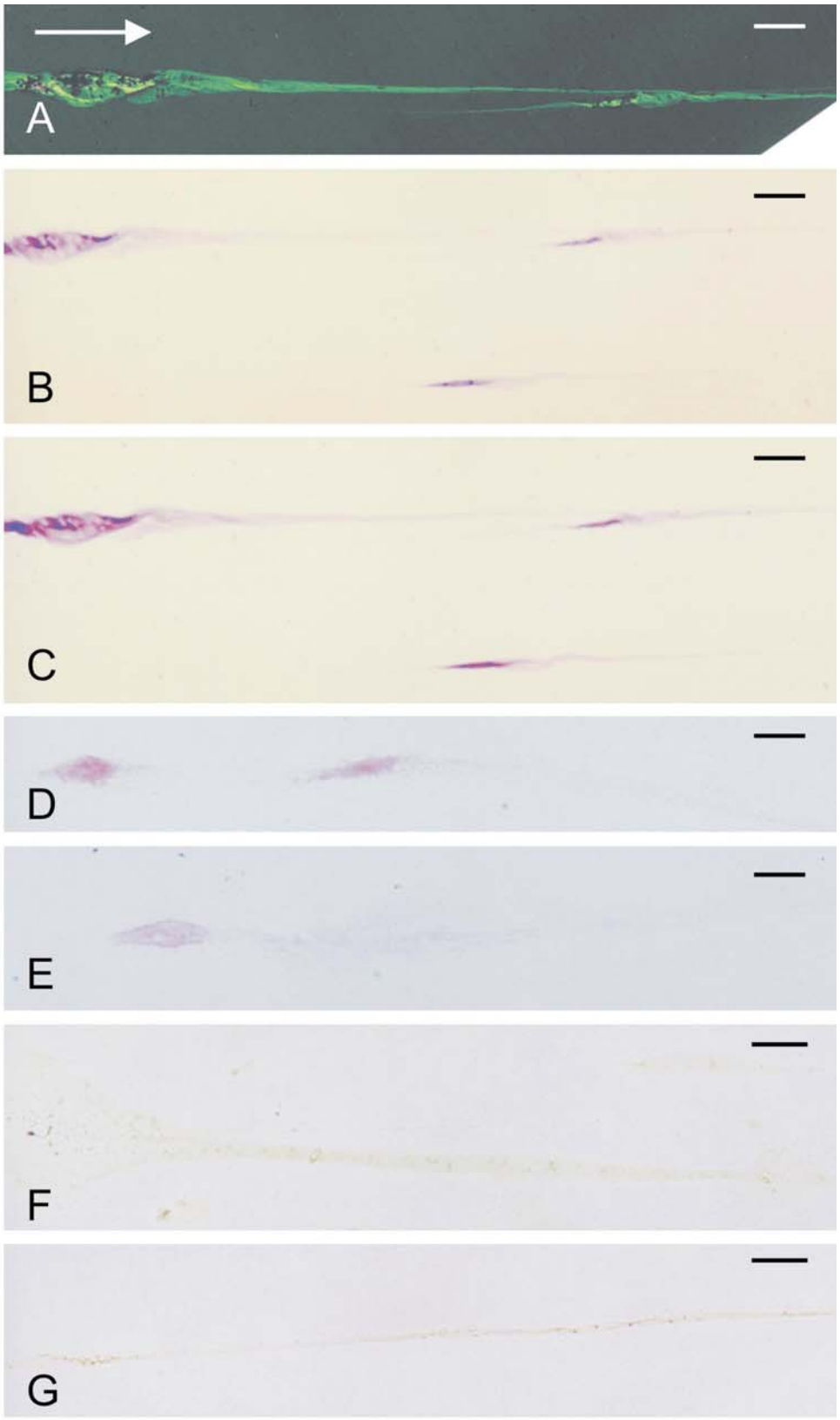

Figure 4

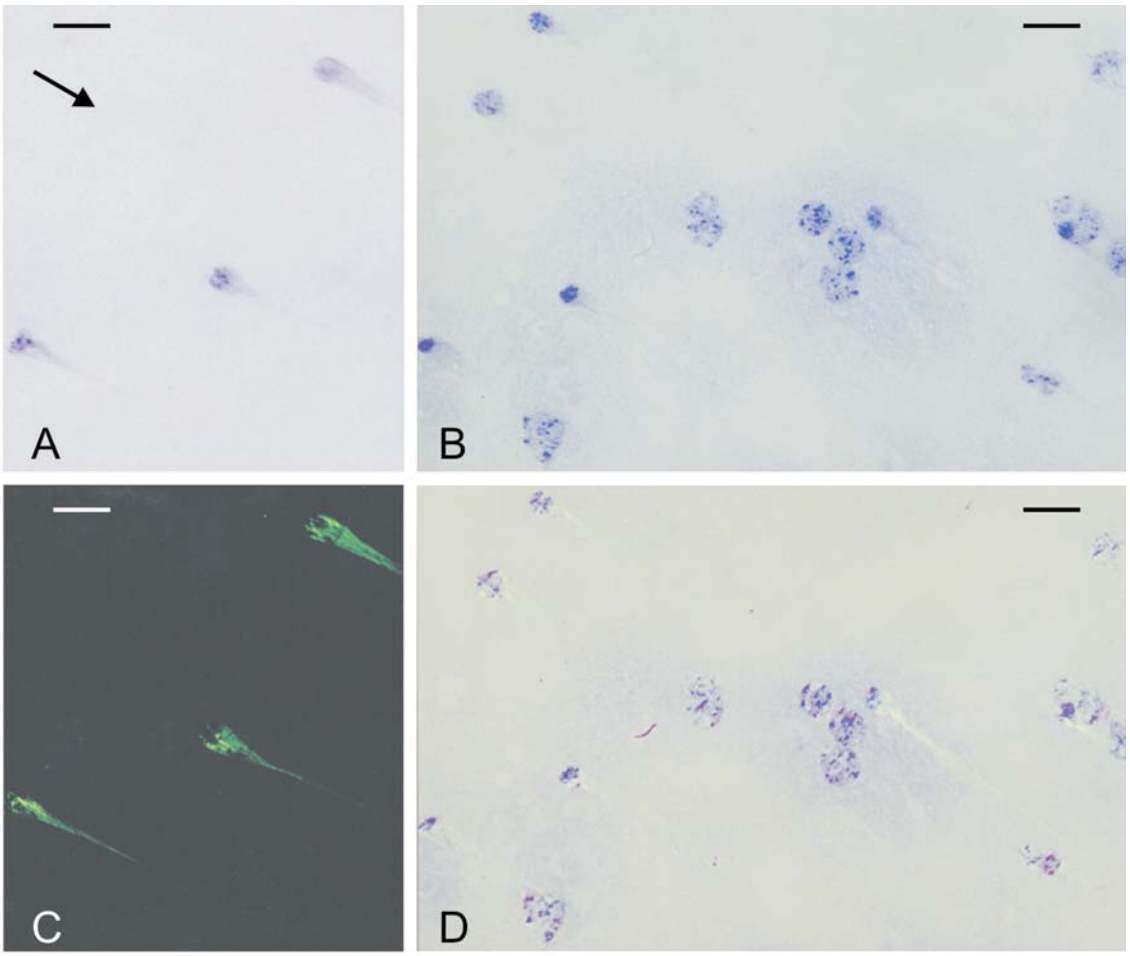

Figure 5

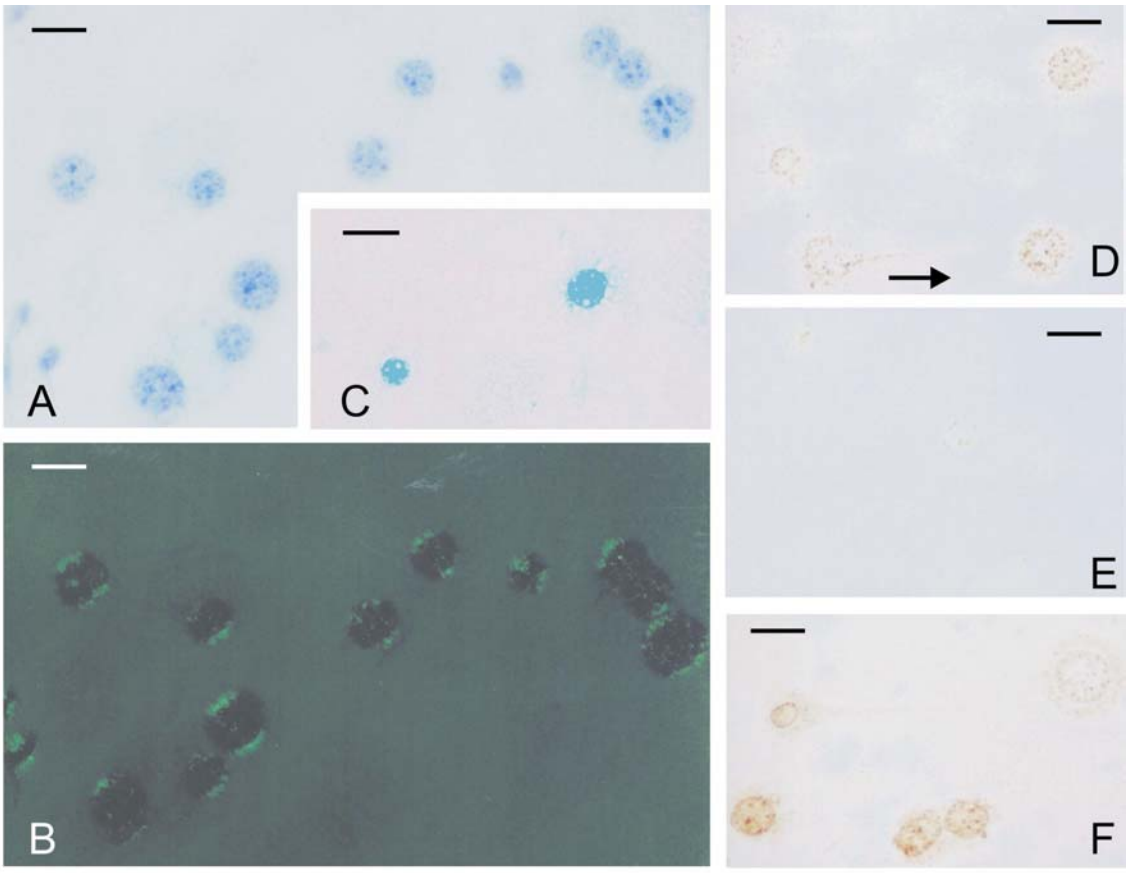

Figure 6

Figure captions

Figure 1. Fixed hepatocyte nuclei from fully nourished (A,B,C,E), starved (D,F) and refed (G) mice. A-D: Toluidine blue staining; E-G: Feulgen reaction. A and B show the same nuclei under non-polarized (A) and polarized (B) light. Bars = 20 μm.

Figure 2. Response to Con-Br in fixed hepatocyte nuclei from fully nourished (A), starved (B) and refed (C,D) mice. Bars = 20 μm.

Figure 3. Unfixed and lysed hepatocyte nuclei from fully nourished mice, positioned horizontally (A,C) and vertically (B,D) and stained with toluidine blue. C and D are polarized light views of A and B, respectively. The arrow indicates the direction of gravity. Bars = 20 μm.

Figure 4. Unfixed and vertically lysed hepatocyte nuclei from fully nourished (D,F) and starved (A,B,C,E,G) mice. A-C: Toluidine blue staining and polarized light views, showing birefringence interference colors (A) and selective absorption of polarized light (B < C). D and E: Feulgen staining; F and G: Response to Con-Br reaction. The arrow indicates the direction of gravity. Bars = 20 μm.

Figure 5. Fixed and vertically lysed hepatocyte nuclei from fully nourished mice. Toluidine blue staining. C and D are polarized light views of A and B, respectively. The arrow indicates the directions of gravity. Bars = 20 μm.

Figure 6. Fixed and vertically lysed hepatocyte nuclei from starved (A-C, E), fully-nourished (D) and refed (F) mice. A and B: Toluidine blue staining (B is a polarized light view of A); C: Acid fast green staining; D-F: Response to Con-Br reaction. The arrow indicates the direction of gravity. Bars = 20 μm.

Conclusões

- O jejum induz aumento no estado de compactação das regiões mais descondensadas da cromatina de hepatócitos de camundongo, que voltam ao estado de compactação observado nos animais bem alimentados, com a realimentação.
- O jejum induz mudanças estruturais na cromatina que levam a uma drástica diminuição da reatividade dos núcleos de hepatócitos à lectina Con-Br, provavelmente por causa de uma falta de acessibilidade da lectina as glicoproteínas nucleares, com a reorganização dessas proteínas. Essa reatividade também é restaurada com a realimentação.
- A formação de fibras estendidas de cromatina depende do grau de compactação da cromatina, da associação dessa cromatina com os componentes da matriz nuclear, da fixação empregada no material, do tipo de lise empregado e da ação da gravidade. Essas fibras são compostas de DNA e proteínas associadas, inclusive glicoproteínas.
- A alteração da organização nuclear influencia na extensibilidade cromatínica, diminuindo sua freqüência de formação em núcleos de hepatócitos de camundongos submetidos a jejum. O tempo de realimentação empregado não é suficiente para restaurar os valores-controle de freqüência de fibras estendidas de cromatina, mas estes valores claramente se afastam daqueles observados durante a privação de alimento.

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