BIOPHYSICS AND BIOCHEMISTRY

Lamin B Content in Chromatin Fractions after Purification of Nuclear Matrix from Cells of Different Types

M. A. Lapshina, I. I. Parkhomenko, R. I. Papina, and A. A. Terentiev

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 146, No. 11, pp. 515-519, November, 2008
Original article submitted November 19, 2007

We analyzed the content of lamin B, one of the main proteins of the nuclear membrane, in different chromatin fractions obtained during purification of the nuclear matrix from different cell types. Depending on cell type and nuclear matrix preparation technique, lamin B was found in different not associated with matrix chromatin compartments. This effect was observed after chromatin extraction with ammonium chloride after nucleolysis and after chromatin extraction with sodium chloride before nuclease treatment. These findings suggest that the structure of the nuclear matrix is destabilized in certain extraction procedures and that studies of subcompartmentalization of nuclear macromolecules require additional control of nuclear matrix integrity.

Key Words: *lamin B; soluble chromatin; nuclear matrix; purification*

In the context of complicated genome organization and great number of structural and regulatory factors functioning in cell nucleus, subcompartmentalization, *i.e.* correct spatial organization, is a biological property of nuclear macromolecules as important as their activity. Nuclear matrix (NM) is now considered to be the main component of the cell nucleus, which can provide spatial coordination of intracellular molecules and biochemical processes. It is known that apart from structural functions, *e.g.* formation of chromatin loop domains, NM is involved in transcription, replication, reparation of DNA and transport of macromolecules [6,10,12].

Despite large body of evidence on NM structure and functions, there are serious problems related to methods of NM preparation [3]. Several methods of NM preparations were proposed [5,8,11]. These methods in different variations are widely applied in the studies of NM structure and functions as well as for investigation of subcompartmentalization of nuclear proteins. The main approach for NM preparation is extraction of chromatin not associated with the matrix with high ionic strength solutions in combination with hydrolysis of nuclear DNA (or DNA and RNA). This chromatin extraction stage faces serious criticism, because high salt concentrations can impair intermolecular interactions and introduce artifacts in protein-nucleic composition as well as in the structure of NM [4]. In light of this, the maintenance of NM integrity during its preparation is an important problem.

The aim of this study was to investigate the resistance of NM to different conditions of extraction of non-matrix-associated chromatin. Since NM

Laboratory of Molecular Biology, Institute of Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka, Russia. ***Address for correspondence***: lapshina@icp.ac.ru. M. A. Lapshina

0007-4888/08/1465-0580

properties depend on protein composition that notably varies in different cell types, we conducted a comparative analysis of NM integrity in artificially transformed human and animal cells and in human tumor cells. The integrity of NM was evaluated by the content of lamin B, a major NM protein, in the nuclear fractions.

MATERIALS AND METHODS

Experiments were carried out on HeLa (human cervical adenocarcinoma), MCF-7 (human breast adenocarcinoma), HEK293 (Ad5-transformed human embryonic kidney epithelial cells) and COS-7 cells (SV40-transformed African green monkey kidney fibroblast-like cells). The cells were cultured in DMEM medium containing 10% newborn calf serum (PAA Laboratories) at 5% CO_2.

Cell nuclei were purified as described elsewhere [7]. The cells were lysed in 0.25 STC buffer (0.25 M sucrose, 50 mM tris-HCl pH 9.0, 5 mM $CaCl_2$, 20 mM NH_4Cl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol) containing 0.2% Triton X-100. The nuclei were purified by double centrifugation of the suspension in 0.25 STC through a layer of 1 STC buffer (identical to 0.25 STC buffer, but containing 1 M sucrose). Final washing was performed in 0.25 STC buffer.

NM preparation *in vitro* by the DN method (DNase — NaCl) was conducted as described elsewhere [11] with modifications. Purified nuclei were treated with DNase I (Serva) in concentration 250 U/mg chromatin (chromatin concentration in the suspension was 6 mg/ml) at 4°C for 30 min and the nuclease fraction (N) was collected. Additional chromatin fractions were obtained by sequential extraction of the nuclei with TM buffer containing 10 mM tris-HCl (pH 7.5), 0.2 mM $MgCl_2$, 2 mM PMSF, and 1 mM dithiothreitol (TM fraction), TM buffer containing 2 M NaCl (high-salt fraction, HS). Then NM was washed with TM buffer (washout fraction, W). After repeated washout with TM, the final sediment, NM, was obtained.

In vitro isolation of NM using DA method (DNase — ammonium sulfate) was conducted as described previously [5] with modifications. Hydrolysis of nuclear DNA was performed as described above, then 1 M $(NH_4)_2SO_4$ was added to the suspension dropwise to a final concentration of 0.25 M, the suspension was incubated at room temperature for 5 min and the supernatant (nuclease and high-salt fraction, NHS) was separated. The sediment was washed with TM buffer (fraction W) and after repeated washing with TM buffer, final sediment, NM, was obtained.

In vitro NM preparation by ND method (NaCl — DNase) was performed as described earlier [8] with modifications. Purified nuclei were resuspended in TM buffer containing 1.5 M NaCl (chromatin concentration in the suspension was 6 mg/ml), incubated for 5 min at 4°C, and the supernatant was separated (HS fraction). Then chromatin was extracted with TM buffer (TM fraction), the sediment was resuspended, and DNA hydrolysis was carried out as described above (fraction N). NM was washed with TM buffer (fraction W) and after repeated washout with TM buffer, the sediment (NM) was obtained.

Electrophoresis of nuclear fraction proteins was conducted in 7% or 10% denaturating PAAG with consequent silver staining.

Immunoblotting was conducted after electrophoresis of the prepared nuclear fractions in 10% denaturating PAAG using goat polyclonal antibodies to lamin B and HRP-conjugated rabbit antibodies to goat IgG (Santa Cruz Biotechnology).

NM was prepared *in situ* as follows. The cells were cultured on coverslips treated with 0.01% polylysine solution (Sigma). Specimens were sequentially incubated in extraction buffers on ice with slow mixing. Cell lysis, purification of cell nuclei, and preparation of NM samples by DN, DA and ND methods were conducted *in situ* as described above. DNA hydrolysis was carried out with 250 U/ml DNase I.

Immunofluorescent staining of specimens was conducted after fixation with 2% paraformaldehyde and treatment with 0.5% Triton X-100. Specimens were preincubated in BB buffer (5% newborn calf serum in sodium phosphate buffer, pH 7.4), incubated with antibodies to lamin B (as described above), washed with BB buffer, incubated with Texas Red-conjugated mouse antibodies to goat IgG (Santa Cruz Biotechnology), and washed with BB buffer and then with phosphate buffer.

RESULTS

Electrophoresis of proteins from different NM samples collected from HeLa cells *in vitro* (Fig. 1, *a,b*) showed that irrespective of the preparation method some proteins were present in NM: this is most distinctly notable for polypeptides with molecular weights of 40-43, 67-70 and 200 kDa. At the same time, different NM samples had different protein composition. In particular, the sample prepared by ND technique was characterized by high content of low-molecular-weight proteins (12-16 kDa) and proteins with molecular weights of 30-35 and 170 kDa. Thus, the polypeptide profiles of NM from the

same source prepared by different techniques were quantitatively and qualitatively different. The differences in polypeptide composition of NM are related to differences in the composition of soluble chromatin fractions.

NM samples obtained by different purification techniques from HeLa cells *in situ* had similar spatial structure [1,5,8,11]. We carried out *in situ* purification of NM from HEK293 cells, were not previously used in these studies. For the analysis of the structure of *in situ* prepared NM, HEK293 cells cultured on coverslips were sequentially washed in all solutions used for isolation of nuclei and NM *in vitro* and then immunofluorescent staining of the prepared specimens with antibodies to lamin B was carried out. Lamin B was chosen as the main subject of the study because this protein is expressed in all tissues and cell types, whereas lamin A/C expression is tissue-specific [2], which can complicate comparative analysis of chromatin fractions obtained from different cell types.

During purification of cell nuclei and subsequent *in situ* preparation of NM, nuclear morphology did not virtually change until DNA hydrolysis: lamin B was located in cell nucleus, typical staining was seen along nucleus periphery (Fig. 2, *a-c*). After high-salt extraction with 2 M NaCl or 0.25 M $(NH_4)_2SO_4$ (DN and DA methods, respectively) the nuclei became smaller, but the NM structure was preserved. Nuclear localization of lamin B was still clearly seen. Peripheral localization of lamin B in these samples was less pronounced, which can be attributed to the loss of nucleus volume and compaction of the nuclear membrane. This leads to intensification of fluorescence in internal zones of the nuclei. At the same time, peripheral localization of lamin B was still observed in some regions of the nuclear membrane (Fig. 2, *d ,e*). If the high-salt extraction was carried out before DNA hydrolysis (ND method), the preparations contained no typically stained cell nuclei, but only amorphously stained regions probably corresponding to fragments of nuclear membrane (Fig. 2, *f*).

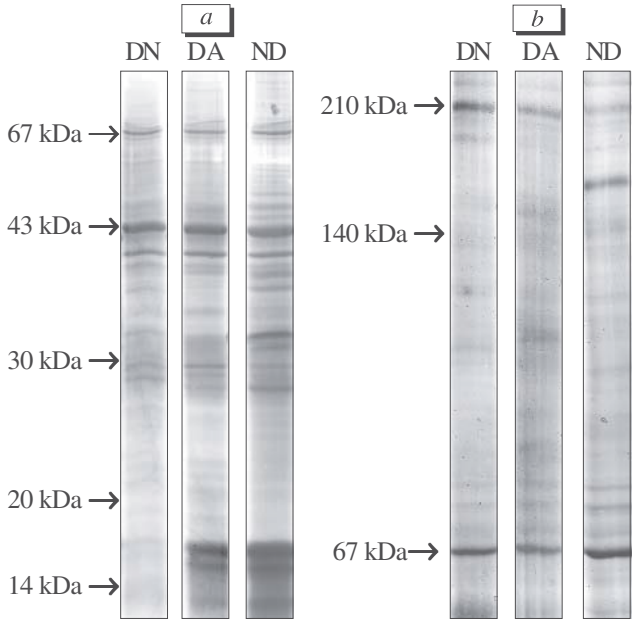

Fig. 1. Polypeptide composition of NM samples prepared using different techniques. Proteins from NM samples purified from HeLa cells using different techniques *in vitro* were separated in 10% (*a*) and 7% (*b*) PAAG; 4 μg of each sample was used for silver staining.

Thus, *in situ* preparation of NM can be accompanied by its partial degradation and appearance of its structural components in soluble chromatin fractions. For evaluation of the integrity of skeletal structures of the cell nucleus, we studied the content of lamin B in all nuclear fractions obtained during NM purification. Lamin B was detected in purified cell nuclei and in NM samples prepared with any of the applied methods (Fig. 3, *a-h*), which agrees with the data on its localization [13]. However, analysis of lamin B content in soluble chromatin fractions showed that the integrity of NM structure depended on the purification method. Lamin B was virtually absent in soluble chromatin fraction (Fig. 3, *a, d, g*) after preparation of NM from all cell types by DN method, while at different stages of purification of NM from HEK293 cells and MCF-7 by DA and ND methods, an appresiable portion of nuclear lamin B pool appeared in soluble not-matrix-associated chromatin fractions (Fig. 3, *b, c, e, f*).

After extraction of chromatin with NaCl without preliminary DNA hydrolysis (ND method) transition of lamin B into soluble fractions (Fig. 3, *c, f*) was probably determined by strong DNA-protein interactions. The appearance of an appreciable part of lamin B in not-matrix-associated chromatin fractions after high-salt extraction without preliminary DNA hydrolysis was associated with partial degradation of the nuclear membrane. At the same time, the high-salt fraction prepared without DNA hydrolysis is characterized by high viscosity, which hinders sedimentation of insoluble chromatin during centrifugation. For reducing the viscosity of high-salt fraction and promoting sedimentation of insoluble chromatin, chromatin concentration in this fraction was decreased, DNA was mechanically broken, and the rate of centrifugation of this fraction was increased. In all cases, lamin B was found in the high-salt fraction. The only condition for efficient separation of non-matrix-associated chromatin from NM was destruction of strong bonds between chromosome DNA and NM. Even short-

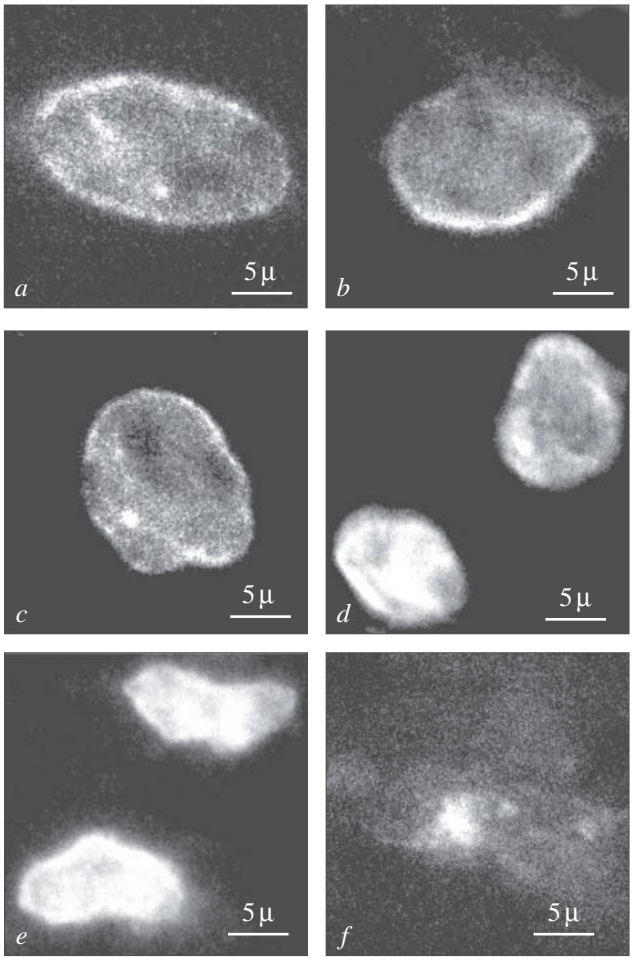

Fig. 2. Immunofluorescent staining of lamin B in cells, nuclei, and NM samples purified using different *in situ* methods from HEK293 cells. *a*) native cell nucleus; *b*) after purification of nuclei; *c*) after DNA hydrolysis; *d-f*) NM after high-salt extraction using DN, DA, ND techniques, respectively.

term incubation of cell nuclei with DNase I before high-salt extraction was enough for retention of lamin B in NM: after preliminary 10-min DNA hydrolysis, lamin B was virtually not detected in the high-salt fraction (Fig. 3, *i*). Thus, the sequence of DNA hydrolysis and high-salt extraction plays the key role in preservation of the structure of prepared NM.

On the other hand, the use of different salts for extraction of non-matrix-associated chromatin provides different degree of structural integrity of NM. We found that non-matrix-associated chromatin obtained after extraction of NM by DN method does not contain lamin B (Fig. 3, *a*, *d*, *g*), whereas after NM purification from HEK293 and MCF-7 cells by DA methods lamin B was detected in the soluble chromatin fraction (Fig. 3, *b*, *e*). Ammonium sulphate used in DA method of NM purification was proposed for high-salt chromatin extraction as an alternative to sodium chloride. Ionic strength of 0.25 M $(NH_4)_2SO_4$ solution is equal to ionic strength of 2 M NaCl solution, which provides a possibility to reduce salt concentration in the extracting buffer and ensure comparable completeness of extraction of non-matrix-associated chromatin [4,5]. However, our findings suggest that ammonium sulphate destabilizes NM.

Extractability of lamin B into soluble chromatin fractions can also depend on the cell type. The studied cell types considerably differ by their physiology and gene expression and protein profiles. These differences manifest in different protein profiles and specific structural features of NM and probably are responsible for different resistance of NM from different cell types to the procedure of fractionation of cell nuclei. Thus, lamin B content in non matrix-associated fractions prepared using

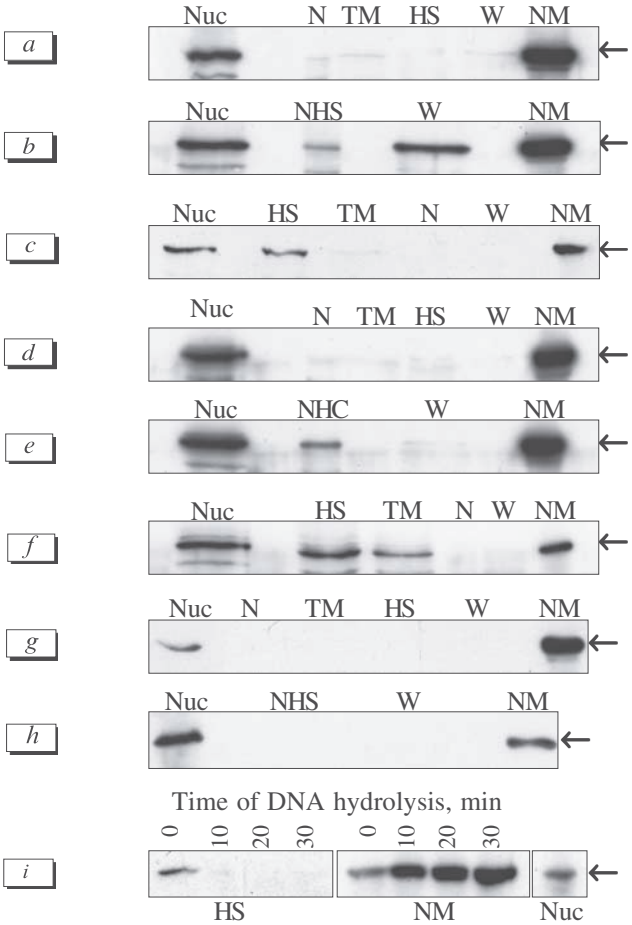

Fig. 3. Content of lamin B in nuclei and nuclear fractions. Nuc: purified cell nuclei. N, TM, HS, NHS, W: soluble chromatin fractions obtained by NM purification using different techniques. Nuclear fractions were prepared from HEK293 cells (*a-c*), MCF-7 (*d-f*) and COS-7 (*g*, *h*) using DN (*a, d, g*), DA (*b*, *e*, *h*) and ND (*c*, *f*) *in vitro*; *i*) content of lamin B in purified cell nuclei (Nuc), high-salt chromatin fraction (HS), and nuclear matrix (NM) obtained from HEK293 cells using DNA hydrolysis of different degree. For immunoblotting, 20 μg protein from each sample was used. Arrows: position of lamin B.

DA (Fig. 3, *b*, *e*) and ND (Fig. 3, *c*, *f*) techniques varied notably between HEK293 and MCF-7 cells nuclei fractionating. Furthermore unlike HEK293 and MCF-7 cells, in COS-7 cells lamin B was not detected in soluble chromatin fractions after NM preparation using DA method (fig. 3, *h*).

Thus, the method of NM preparation considerably affects the structure of the final sample and detection of matrix-associated proteins in soluble chromatin fractions attests to partial destruction of NM during the purification procedure. Our data suggest that chromatin extraction with sodium chloride after DNA hydrolysis preserves NM integrity during its extraction from different cell types. It should be noted that while preparation technique little affected the structure of NM fibrogranular system observed under electron microscope [9], studies of NM endonuclear subcompartmentalization and functioning during coordination of spatial macromolecule distribution require to control the integrity of NM during purification procedure. Different techniques of chromatin fractionation are now used in studies of intranuclear localization of macromolecules, but even the most frequently applied fractionation technique with extraction of non-matrix-associated chromatin with ammonium sulphate can lead to partial destruction of NM. In this context, the studies of intranuclear localization of macromolecules often require the use of various techniques.

REFERENCES

1. Ye. V. Sheval, S. Yu. Kurchashova, E. R. Timirbulatova, *et al*., *Tsytologiya*, No. 47, 77-82 (2005).
2. J. L. Broers, F. C. Ramaekers, G. Bonne, *et al.*, *Physiol. Rev.*, **86**, No. 3, 967-1008 (2006).
3. R. Hancock, *Biol. Cell*., **96**, No. 8, 595-601 (2004).
4. R. Hancock, *Chromosoma*, **109**, No. 4, 219-225 (2000).
5. D. C. He, J. A. Nickerson, S. Penman, *J. Cell Biol*., **110**, No. 3, 569-580 (1990).
6. D. A. Jackson, *Chromosome Res*., **11**, No. 5, 387-401 (2003).
7. M. A. Lapshina, I. I. Parkhomenko, and A. A. Terentiev, *An. N.Y. Acad. Sci.*, **1090**, 177-181 (2006).
8. H. Ma, A. J. Siegel, and R. Berezney, *J. Cell Biol*., **146**, No. 3, 531-541 (1999).
9. M. A. Narang, R. Dumas, L. M. Ayer, and R. A. Gravel, *Hum. Mol. Genet*., **13**, No. 1, 15-23 (2004).
10. J. Nickerson, *J. Cell. Sci*., **114**, Pt. 3, 463-474 (2001).
11. R. Schirmbeck, A. von der Weth, and W. Deppert, *J. Virol*., **67**, No. 2, 894-901 (1993).
12. K. M. Tsutsui, K. Sano, and K. Tsutsui, *Acta. Med. Okayama*., **59**, No. 4, 113-120 (2005).
13. S. Vlcek, T. Dechat, R. Foisner, *Cell. Mol. Life Sci*., **58**, Nos. 12-13, 1758-1765 (2001).