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Research Article

Separation of nuclear protein complexes by blue native polyacrylamide gel electrophoresis

The nucleus is a highly structured organelle with distinct compartmentalization of specific functions. To understand the functions of these nuclear compartments, detailed description of protein complexes which form these structures is of crucial importance. We explored therefore the potential of blue native PAGE (BN-PAGE) method for the separation of nuclear protein complexes. We focused on (i) solubility and stability of nuclear complexes under conditions prerequisite for the separation by BN-PAGE, (ii) improved separation of native nuclear protein complexes using 2-D colorless native/blue native PAGE (CN-/BN-PAGE), and (iii) mass spectrometric analysis of protein complexes which were isolated directly from native 1-D or from 2-D CN/BN-PAGE gels. The suitability of BN-PAGE for nuclear proteomic research is demonstrated by the successful separation of polymerase I and polymerase II complexes, and by mass spectrometric determination of U1 small nuclear ribonucleoprotein particle composition. Moreover, practical advice for sample preparation is provided.

Keywords: Actin / Blue native polyacrylamide gel electrophoresis / Nuclear protein complexes / RNA polymerase / Small nuclear ribonucleoproteins

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1 Introduction

The nucleus of eukaryotic cells is functionally and structurally compartmentalized; however, the function of various compartments is still not completely revealed. To uncover this relationship, a great need exists for tools enabling the separation and the analysis of specific nuclear components under physiological (native) conditions. As the tendency of many nuclear proteins toward nonspecific aggregation under nonphysiological conditions (for a review, see *e.g.*, [1]) might lead to misleading results, the conditions of such isolation procedure should be adjusted as close as possible to the physiological state [2].

The blue native PAGE (BN-PAGE; [3]) has a potential to separate protein complexes in the native state (up to the size of 1.5 MDa). The method was primarily designed for

separation of highly hydrophobic mitochondrial membrane protein complexes [3]. Later, it was exploited for the separation of soluble protein complexes of mitochondrial matrix [4], plasma membrane protein complexes [5–7], or whole cell lysates [8]. Moreover, an adaptation of the technique to agarose gel matrices with a higher separation limit was utilized for separation of very large protein complexes such as pyruvate dehydrogenase complex [4] or virus particles [9]. The procedure has several advantages that make the BN-PAGE suitable also for separation of nuclear protein complexes. The most important is the neutral pH ensuring physiological stability of protein complexes during separation. CBB G-250 used as a “charge shift” compound also limits self-aggregation of proteins during the sample preparation and electrophoresis [3]. In addition, the composition of separated complexes can be determined directly by MS. Importantly, components of separated complexes identified by MS can be further detected by specific antibodies essentially by the same technique combined with denaturing SDS electrophoresis in the second dimension (as described in [3]).

The progress of MS techniques in protein identification has enabled a direct analysis of large protein complexes or highly complex samples such as cell organelles (reviewed in [10] and [11]). A well-designed subfractiona-

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Abbreviations: 6-AcA, 6-aminocaproic acid; **BN-PAGE**, blue native-PAGE; **CN-PAGE**, colorless native PAGE; **NDH II**, nuclear DNA helicase II; **Pol I**, DNA-dependent RNA polymerase I; **Pol II**, DNA-dependent RNA polymerase II; **snRNP**, small nuclear ribonucleoprotein; **UBF**, upstream-binding factor

tion technique coupled with MS identification of proteins can serve, therefore, as a powerful tool for analysis of nuclear compartments. This was reported for, *e.g.*, nucleolus [12, 13] or spliceosome [14].

In this study, we explored the potential of BN-PAGE for separation of protein complexes extracted from purified nuclei. We analyzed the conditions for sample preparation with respect to the solubility and the stability of nuclear protein complexes. We found that efficient separation of nuclear protein complexes (*e.g.*, DNA-dependent RNA polymerase I (Pol I) and DNA-dependent RNA polymerase II (Pol II), upstream-binding factor (UBF), or nuclear DNA helicase II (NDH II)) by BN-PAGE can be achieved. Using the analysis of U1 small nuclear ribonucleoprotein (snRNP) particle, we demonstrated that the BN-PAGE technique can be coupled with MS determination of the protein complex composition. To obtain complexes separated strictly from each other, we also employed 2-D separation of nuclear protein complexes by combination of colorless native PAGE (CN-PAGE) with BN-PAGE [15] that is particularly useful in situations of imperfect 1-D separation of nuclear lysates from certain tissues. In conclusion, we found the BN-PAGE method to be a very effective tool for studying protein–protein interaction in the nucleus.

2 Materials and methods

2.1 Chemicals

Acrylamide, Bis, CBB G-250 (Serva Blue), sucrose, leupeptin, and pepstatin were from Serva (Heidelberg, Germany). 6-Aminocaproic acid (6-AcA) was purchased from Fluka (Buchs, Switzerland). Bistris, Tricine, and all other chemicals, unless stated otherwise, were obtained from Sigma (St. Louis, MO, USA).

2.2 Antibodies

Antiactin “pan-reactive” mAb recognizing the C-terminal actin fragment was obtained from Dr. J. L. Lessard (Division of Developmental Biology, Cincinnati, USA), and mAb against the C-terminal domain of the largest subunit of Pol II was a gift from Dr. M. Vigneron (Institute of Genetics and Molecular and Cell Biology, Illkirch, France). Rabbit polyclonal antibody against Pol I and antibody against UBF were obtained from Dr. I. Grummt (Division of Molecular Biology of the Cell II, Heidelberg, Germany). Rabbit polyclonal antibody against nuclear DNA helicase II (NDH II) was a gift from Dr. S. Zhang (Institute of Molecular Biotechnology, Jena, Germany; [16]). ANA human reference serum 5 recognizing two proteins of

snRNP core particle was obtained from Department of Health and Human Services, Centers for Disease Control (Atlanta, USA; [17]).

2.3 Isolation of HeLa cell nuclei

Suspension culture of HeLa cells was grown to a density of 5×10^5 cells/mL in minimum essential medium for suspension culture (S-MEM, Prague) supplemented with 5% v/v heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY, USA) at 37°C in humidified atmosphere containing 5% CO₂. Cells were collected by centrifugation at $400 \times g$ for 5 min at 4°C, washed three times in ice-cold PBS, resuspended in 20 volumes of lysis buffer (100 mM sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1.5 mM MgCl₂, 0.4% NP 40), and incubated for 5 min on ice. Crude nuclei were centrifugated at $1000 \times g$ for 10 min at 4°C. The nuclear pellet was washed once in 10 mL of Buffer 1 (100 mM sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1.5 mM MgCl₂) and then in Buffer 2 (210 mM sucrose, 45 mM Bistris, pH 7.0). Protease inhibitors (1 mM Pefabloc SC, Roche Diagnostics GmbH, Mannheim, Germany; 1 µg/mL leupeptin; 1 µg/mL pepstatin) were added to all buffer solutions before use. Nuclei were pelleted by centrifugation at $400 \times g$ for 5 min at 4°C and used immediately for BN-PAGE.

2.4 Isolation of rat liver nuclei

Nuclei were isolated from rat liver according to the Kihlmark and Hallberg method [18]. Briefly, liver (10 g) from 1-month-old Wistar rats was cut into small pieces (1 mm³) and homogenized in two volumes of sucrose buffer (0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 50 mM triethanolamine-HCl, pH 7.5) by 15 strokes of a tight-fitting Potter–Elvehjem homogenizer. Nuclei were further purified by centrifugation in SW28 rotor (Beckman) at $124\,000 \times g$ for 1 h at 4°C through a sucrose cushion (2.3 M sucrose, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 50 mM triethanolamine-HCl, pH 7.5). Nuclear pellet was then resuspended in one volume of 50 mM Bistris, pH 7.0, 20% glycerol, and kept frozen at –70°C until use. Protease inhibitors were included in all solutions as described above.

2.5 Preparation of nuclear extracts for BN-PAGE

Nuclei isolated from HeLa cells or from rat liver tissue were dissolved in native sample buffer (50 mM Bistris, pH 7.0 supplemented with protease inhibitors). One volume of native sample buffer with different concentration of 6-AcA (0.075–2 M) was then added to an aliquot corresponding to 4×10^6 HeLa cells or 500 mg rat liver tissue,

and the samples were incubated on ice for 20 min. The nuclear extracts were then clarified by centrifugation at $17\,100\times g$ for 30 min, and protein concentration was assessed by Bradford protein assay (BioRad, Hercules, CA, USA). CBB G-250 dissolved in 500 mM 6-AcA was added to nuclear extracts to final concentration of 0.5%. The samples were incubated for 5 min on ice and then loaded onto the gel.

2.6 BN-PAGE

BN-PAGE was performed essentially as described in [3] with the following modifications. Electrophoresis was run on 4–16% polyacrylamide gradient gel ($105\times 140\times 1$ mm; containing 125 mM 6-AcA, 50 mM Bistris, pH 7.0) at 50 V for the first 30 min at 4°C, and then at a constant current of 5 mA until the end of electrophoresis. When the tracking dye reached the second third of the gel height, the electrophoresis was interrupted and the blue cathode buffer (50 mM Tricine, 15 mM Bistris, pH 7.0, 0.02% CBB G-250) was replaced by colorless cathode buffer (50 mM Tricine, 15 mM Bistris, pH 7.0). Electrophoresis was stopped when the tracking line of CBB G-250 dye had left the edge of the gel.

2.7 Preparation of nuclear extracts for 2-D CN-/BN-PAGE

Nuclei isolated from rat liver tissue were dissolved in one volume of native sample buffer. One volume of 3 M 6-AcA was then added (final concentration was 1.5 M 6-AcA). Extracts were further processed as described above with one exception: CBB G-250 dye was substituted with 0.001% Ponceau S.

2.8 2-D CN-/BN-PAGE

2-D CN-/BN-PAGE was performed as described [15]. Briefly, protein samples corresponding to 500 mg rat liver were separated on 4–16% gradient gel (1 M 6-AcA, 50 mM Bistris, pH 7.0) in the first dimension (CN-PAGE, colorless cathode buffer) until Ponceau S left the gel. The gel was then cut into individual strips, which were placed onto 4% acrylamide glycine stacking gel (100 mM glycine, 20 mM Bistris, pH 8.1) of the 2-D gel (4–16% native gradient gel, 1 M 6-AcA, 50 mM Bistris, pH 7.0). The second dimension (BN-PAGE) was started in cathode glycine buffer (100 mM glycine, 20 mM Bistris, pH 8.1, 0.002% CBB G-250), which was replaced after 60 min of electrophoresis with blue cathode buffer containing 0.002% CBB G-250. The setting of both electrophoretic runs was the same as described for 1-D BN-PAGE.

2.9 2-D BN/SDS-PAGE

The first dimension (BN-PAGE) was performed as described above. The gel was then cut into individual strips and incubated in denaturing buffer (1% SDS, 1% β -mercaptoethanol) for 30 min. Eight percent SDS acrylamide gel and 4% stacking gel was poured under the gel strip. The second separation (SDS-PAGE) was done at 80 V for 45 min and then at 20 mA/gel. Proteins were transferred onto an NC membrane and detected by specific antibodies as described above.

2.10 Protein visualization and immunoblotting

Separated proteins were visualized by CBB R-250 staining, or transferred from the gel onto a PVDF membrane using semidry blotting. If necessary, the excess of CBB G-250 dye on the PVDF membrane was removed by washing in 40% methanol/10% acetic acid five times for 5 min with 0.1% SDS in the last incubation. After blocking, the membrane was incubated with primary antibodies (90 min, room temperature) followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (goat antirabbit, goat antimouse, BioRad; goat antihuman, Sigma). Proteins were detected by ECL (Pierce Biotechnology, Rockford, IL, USA). M_r of protein complexes separated by BN-PAGE was estimated according to high-molecular-weight standards (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.11 In-gel digestion and MS

CBB R-250-stained protein bands were cut from the gel, destained, and digested directly in the gel by trypsin as described previously [19]. More complicated peptide mixtures arising from the digestion of protein complexes (1-D BN-PAGE or 2-D CN-/BN-PAGE) were analyzed by HPLC-MS/MS on an IT (LCQ DECA, ThermoFinnigan, CA). The analyses and data processing were done according to [20].

3 Results and discussion

3.1 Solubility of nuclear protein complexes in blue native sample buffer

In the original protocol of Schagger and von Jagow [3], 6-AcA was selected as an appropriate protein solubilization agent preventing protein aggregation during electrophoresis, since no migration of this compound occurred in the electrical field. Mitochondrial membrane protein complexes, for which the method was designed, are well

solubilized in the presence of a detergent and 750 mM 6-AcA without the effect on their integrity [3]. However, we detected differences in the solubility and stability of several nuclear complexes in 750 mM 6-AcA (not shown). It was not unexpected since differences in solubility of individual nuclear proteins are well known; e.g., proteins of the nuclear matrix are insoluble even in 2 M NaCl [21]. Furthermore, we found that the total protein amount released from nuclei into the supernatant of native sample buffer increased in parallel with the increasing concentration of 6-AcA. On the other hand, we have not observed any effect of detergents such as 1% Triton X-100, 1% NP 40 or 1% lauryl maltoside on solubility or stability of nuclear protein complexes analyzed. Therefore, the detergents were omitted from the following experiments.

We investigated the extractability of specific nuclear proteins from rat liver purified nuclei in a relation to increasing concentration of 6-AcA in native sample buffer (Fig. 1). Note that actin appeared in supernatant only at higher concentration of 6-AcA (750 mM), while Pol I or Pol II trace amounts are seen already on the blot even if no 6-AcA in the sample buffer was used. However, all three proteins showed salt-dependent solubility and all were nearly completely solubilized at the highest (2 M) 6-AcA concentration. On the contrary, nuclear proteins prepared from HeLa cells by a different isolation method (see Section 2) were approximately 50% soluble (lamin A/C, actin, and snRNP proteins) or 20% soluble (Pol II and NDH II) at 500 mM 6-AcA. This ratio changed only slightly with increasing concentration of 6-AcA up to 2 M, as shown for snRNP proteins (Fig. 1D). The only exception was Pol I whose solubility significantly increased up to 2 M 6-AcA.

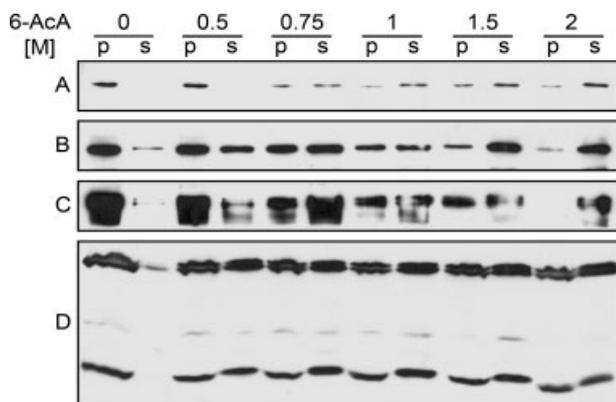


Figure 1. Solubility of nuclear proteins in native sample buffer. After solubilization in varying concentrations of 6-AcA (0–2 M), nuclear lysates were subjected to SDS-PAGE, blotted, and immunodetected with specific antibodies against actin (A), Pol I (B), and Pol II (C) of rat liver nuclei and against snRNP proteins extracted from HeLa cell nuclei (D) to assess the distribution of proteins in the insoluble (p) and soluble (s) fractions.

These data indicate that the solubility of individual nuclear proteins in the native sample buffer depends on 6-AcA concentration and on the method of sample preparation or on the type of tissue. Taken together, the conditions for sample preparation should be tested for each specific nuclear protein individually respective to the tissue and the isolation method used.

3.2 Stability of nuclear protein complexes in blue native sample buffer

Although our data indicate that the solubility of particular protein depends on the concentration of 6-AcA in the native sample buffer and also on the cellular origin, the procedure described above obviously cannot demonstrate the stability of a complex consisting of examined proteins. We investigated, therefore, the stability of selected multi-protein complexes in extracts, which were prepared under conditions described above and then separated by BN-PAGE. The Western blot of the gel probed by antibodies against actins, Pol I, Pol II, UBF, snRNPs, and NDH II (Fig. 2) showed that except labile actin complexes, the complexes of other tested proteins were fairly stable. The specificity of antibody labeling on native gel blots was confirmed by 2-D BN/SDS-PAGE, where the coordinates of detected protein on 2-D gel maps corresponded well to the migration distance of denatured subunit (y axis) and to the migration distance of the intact complex separated in the first dimension (x axis). Note that even in 1.6 M 6-AcA, there was no detectable breakdown of Pol I 540 kDa and Pol II 640 kDa complexes, as individually migrating a large subunit of Pol I (M_r 116 kDa, see Fig. 2A) or Pol II (M_r 230 kDa, see Fig. 2C) were not detected.

Pol I was resolved as a major single band complex migrating at $M_r \sim 540$ kDa. A certain portion of the Pol I-positive material was also retained at the top of the gel, which might indicate the presence of other high-molecular-weight Pol I complexes, or formation of high-molecular-weight aggregates caused by the high ionic strength of 6-AcA. The abundance of the Pol I 540 kDa signal increased in parallel with the concentration of 6-AcA (Fig. 2A). The molecular mass of the the Pol I complex assessed by BN-PAGE corresponds well with previously reported results, in which the apparent molecular mass of the Pol I complex has been estimated to be 550 kDa by sedimentation in a sucrose gradient [22]. In addition, the size exclusion (gel filtration) chromatography revealed active Pol I fractions at 500 kDa [23] or in the range of 500–900 kDa [22]. Moreover, a subpopulation of Pol I (10%) was eluted as a large well-defined complex with $M_r > 2000$ kDa [22, 23]. Likewise, UBF component of Pol I transcription initiation complex [24] migrates in the native gel with the same mobility as Pol I (see Fig. 2B). Our data

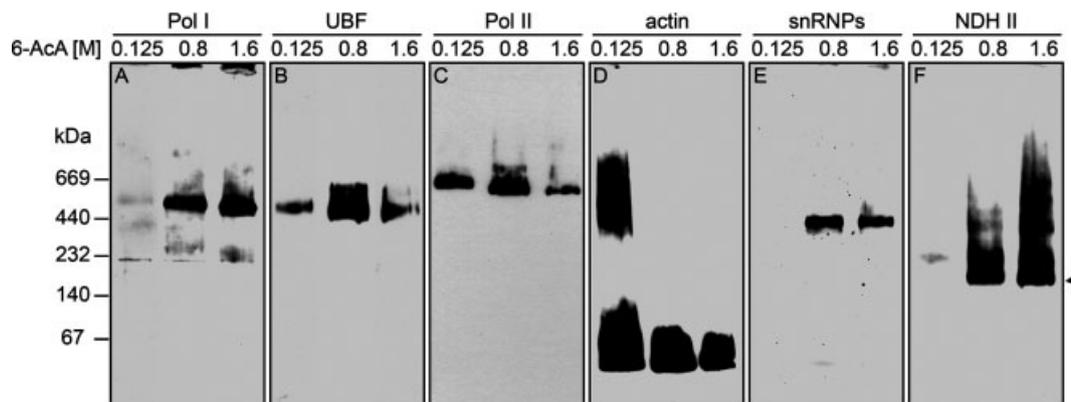


Figure 2. Solubility and stability of nuclear protein complexes separated under native conditions. Extracts of nuclei isolated from 4×10^6 HeLa cells were loaded *per* each lane of the native gel (corresponding to the total protein amount of 30 μ g in 125 mM 6-AcA, 70 μ g in 800 mM 6-AcA, and 90 μ g in 1.6 M 6-AcA) and subjected to BN-PAGE followed by immunoblotting with antibodies against Pol I (A), UBF (B), Pol II (C), actin (D), snRNP proteins (E), and NDH II (F). The monomer of NDH II is marked by arrowhead.

indicate that the signal of Pol I at 540 kDa really stems from the intact Pol I protein complex, even though UBF was previously found to copurify with the gel chromatographic the appearing at >2000 kDa and not with the low-molecular-weight fraction [22]. This can be explained by differences in the solubilization protocol, since the high salt extraction (420 mM NaCl) of nuclei was used for preparation of nuclear extracts for size exclusion chromatography [22]. Our data indicate that UBF might be present also in the low-molecular weight Pol I complex, if extracted from cells under milder conditions. In support of this, a high concentration of 6-AcA (1.6 M) in native sample buffer leads to a significant decrease in UBF presence in the 540 kDa complex (Fig. 2B).

Pol II-positive material migrated at M_r of about 640 kDa, which is close to the previous estimation of Pol II complex M_r as 500–600 kDa [25]. The intensity of Pol II signal increased in parallel with increasing concentration of 6-AcA up to 800 mM and then decreased (Fig. 2C). A slightly increased mobility of Pol II (600 kDa) in 800 mM 6-AcA sample compared to 125 mM 6-AcA sample probably reflects partial dissociation of the Pol II complex.

Actin complexes migrated as a broader band spanning the M_r region of 400–750 kDa of the gel when a low concentration of 6-AcA was used (Fig. 2D). Apparently, this indicates the participation of actin in several protein complexes of similar molecular weight (see below) and their lower stability. This is further supported by the complete dissociation of actin from these complexes when 6-AcA was higher than 200 mM. The similar migration of actin with Pol I and Pol II complexes indicates that actin might be present in both these complexes. This is in

agreement with previous observations that actin is required for DNA transcription performed by both polymerase I and II complexes [26, 27].

Anti-snRNP antibody labeled one distinct complex with M_r 450 kDa (Fig. 2E). The composition of the snRNP complex was further determined by MS (see below).

Another protein of interest, nuclear DNA helicase II, was a part of three complexes when separated by BN-PAGE (M_r 220, 380, and 550 kDa, see Fig. 2F). Moreover, at concentrations of 6-AcA exceeding 200 mM, 142 kDa monomers of NDH II were also present in the gel. However, unlike actin, apparent breakdown of high-molecular-weight NDH II complexes was not observed with increasing salt concentration. This indicates that under physiological conditions, NDH II might associate with protein complexes larger than the pore limit of 4% polyacrylamide gel. A higher concentration of 6-AcA in the sample might lead to a partial disruption of complexes, which could then enter the gel. Although several interacting partners of NDH II have been already described [28], the nature of the NDH II protein complexes has not been revealed up to date.

Altogether, our data show that both protein solubility and stability are dependent on the concentration of 6-AcA used in the native sample buffer. A similar effect of 6-AcA on stability of mitochondrial membrane pyruvate dehydrogenase complex was described by Holt *et al.* [4, 29]. As already mentioned, the concentration of 6-AcA should be therefore adjusted for each protein complex of interest individually in order to find the optimal equilibrium between its solubility, stability, and nonspecific aggregation.

3.3 Mass spectrometric determination of nuclear protein complexes after 1-D BN-PAGE

The direct MS identification of protein complex composition separated in native state by nondenaturing LC followed by native electrophoresis was applied previously for heme-binding protein complexes [30]. Here we used a similar approach to identify the composition of the whole nuclear protein complex isolated directly from the 1-D native gel after separation by BN-PAGE (*i.e.*, without preceding separation of individual protein complex subunits by denaturing electrophoresis in the second dimension before MS).

The band marked by an arrow (M_r 450 kDa, Fig. 3A) was excised after a tryptic digestion analyzed by HPLC-MS/MS (see Section 2). Six snRNP core proteins (Sm proteins SmD1, SmD2, SmD3, SmE, SmG, and SmB/B') that are constant parts of almost all snRNP particles [31], and two proteins specific for U1 snRNP particle (U1-A and U1-70 K) were identified as components of the 450 kDa band (Table 1A). U1 snRNP is an evolutionary highly conserved nuclear ribonucleoprotein complex, which assembles with other snRNP particles on the pre-mRNA in order to form a spliceosome [31, 32]. Intriguingly, except SmF and U1-C subunits, most of the U1 snRNP particle subunits were present, indicating that the

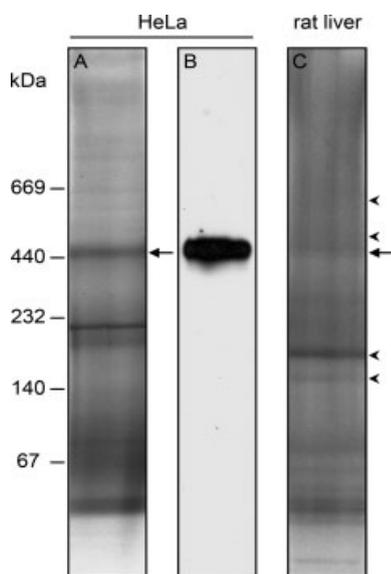


Figure 3. Nuclear protein complexes separated by 1-D BN-PAGE. (A) BN-PAGE gel of HeLa nuclear extract was stained by CBB R-250. Band at 450 kDa (arrow) was excised and analyzed by MS (see Table 1). (B) Immunoblot of the gel prepared as in (A) and immunodetected with anti-snRNP antibody. (C) Gel slices (1 mm, arrowheads) and CBB R-250-stained band at 450 kDa (arrow) of BN-PAGE gel (rat liver) were excised and the proteins were identified by MS (see Table 1).

450 kDa complex represents predominantly the U1 snRNP particle. Additionally, the 450 kDa band identified by MS as a snRNP particle corresponds well to the 450 kDa complex specifically labeled by anti-snRNP antibody on the immunoblot of the native gel (Fig. 3B). In sum, both findings confirm the identity of the protein complex as snRNP particle. To further verify these results and to exclude the possibility of an aberrant mobility of dissociated snRNP monomer proteins in the native, 2-D BN/SDS-PAGE followed by an immunoblot probed with an antibody specific for snRNP proteins was performed (Fig. 4). This antibody [17] detected two spots corresponding to M_r of approximately 28 and 13 kDa, both migrating at the 450 kDa position of the 1-D native gel. These data demonstrate that the coupling of BN-PAGE and MS analysis enables a direct identification of protein complex composition.

3.4 MS determination of nuclear protein complexes separated by 2-D CN-/BN-PAGE

Although MS analysis of nuclear protein complexes isolated from HeLa cells separated by BN-PAGE is feasible as described above, we encountered several problems using similar analysis for nuclear protein complexes isolated from rat liver tissue. When subjecting the 450 kDa band from the rat liver nuclear extract separated by 1-D BN-PAGE (see Fig. 3C, arrow) to MS, prevalently histones and hnRNP proteins were identified (see Table 1B). Histones were also found in other areas of the gel (see Fig. 3C, arrowheads) forming rather high background, which completely disabled MS analysis of otherwise well-separated protein complexes (as verified by immunoblot, data not shown). The artificial migration of lysine- and arginine-rich histone proteins could be explained by aggregation and disaggregation of these basic proteins in the presence of CBB G-250 during electrophoretic run [33]. Another possible reason for nonuniform mobility of some proteins can be weak stability of protein complexes during the electrophoretic separation. Both mechanisms can cause final location of proteins or protein complexes in the areas of the 1-D native gel, which do not correspond to their correct molecular weight. The problem of non-specific background can be overcome by 1-D separation (in the absence of CBB G-250) followed by a separation in perpendicular direction. Theoretically, the “well-resolved” protein complexes are expected to reside on a diagonal line in the resulting 2-D gel, whereas the incorrectly resolved proteins/protein complexes will migrate – in the ideal case – under the diagonal to the position reflecting their correct molecular weight. The presented silver-stained gel of rat liver nuclear extract separated by 2-D CN/BN-PAGE (Fig. 5A) shows a typical result of described separation. As anticipated, the well-resolved protein

Table 1. MS analysis of U1 snRNP complexes separated by 1-D BN-PAGE or 2-D CN/BN-PAGE

Sample	Identified protein	Peptide sequence ^{a)}	NCBI no.	
A	SmE	VMVQPINLIFR	62667426	
	SmG	GNSIIMLEALER	6094212	
	SmD1	NGTQVHGTITGVDVSMNTHLK	51338665	
		NREPVQLETLSIR	51338665	
		EPVQLETLSIR	51338665	
	SmD2	GDSVIVVLR	51338666	
	SmD3	VAQLEQVYIR	51338667	
	SmB/B'	VLGLVLLR	134545	
	U1-A	EVSSATNALR	134092	
		GQAFVIFK	56605690	
	U1-70 K	RQQEVETELK	13633918	
		MWDPHNDPNAQGDAFK	13633918	
		EFEVYGPIK	13633918	
		LGGGLGGTR	13633918	
		DPIPYPPEK	13635663	
		GGGGGQDNGLEGLGNSDR	13635663	
		TVLDPVTGDLSDR	19863446	
	SF3b130			
	B	SmE	VmVQPINLIFR	62667426
		SmD3	FLILPDmLK	51338667
Histone H1.4		KASGPPVSELITK	18959218	
		ASGPPVSELITK	18959218	
		SGVSLAALK	18959218	
		SGVSLAALKK	18959218	
		ALAAGGYDVEK	111782	
Histone H1.b		GVGASGSFR	288640	
Histone H1(0)		VTIAQGGVLPNIQAVLLPK	121986	
Histone H2A.m		HLQLAIR	62663816	
Histone H2A.1		NDEELNKLLGK	122009	
		AGLQFPVGR	62663816	
Histone H2A.Z		GDEELDSLK	55562729	
		ATIAGGGVIPHIK	55562729	
Histone H2B		AmGIMNSFVNDIFER	477676	
		AMGImNSFVNDIFER	477676	
		AmGImNSFVNDIFER	477676	
		KESYSVYVYK	399856	
Histone H3, family 3B		YRPGTVALR	38648851	
		DNIQGITKPAIR	204601	
Histone H4		TVTAmDVVYALKR	204601	
		TVTAMDVVYALK	204601	
		ISGLIYEETR	204601	
		VFLENVIR	204601	
		DAVITYTEHAK	204601	
		IDTIEITDR	6647752	
		GGGGNFGPGPGSNFR	6647752	
		GFAFVTFDDHDTVDK	51980308	
hnRNP A3		VFIGNLNLTLVK	20455507	
		SDVEAIFSK	20455507	
		GFAFVQYVNER	20455507	
hnRNP C1/C2		mIAGQVLDINLAAEPK	20455507	
	mYSPAR	20455507		
hnRNP L	NDQDTWDYTNPNLSGQGDPGSNPDKR	133274		

Table 1. Continued

Sample	Identified protein	Peptide sequence ^{a)}	NCBI no.
C	SmE	GDNITLLQSVSN	134127
		VMVQPINLIFR	62667426
		VmVQPINLIFR	62667426
	SmG	HVQGILR	6094212
	SmD1	YFILPDSLPLDTLLVDVEPK	51338665
	SmD3	VAQLEQVYIR	51338667
	U1-A	GQAFVIFK	56605690
		HDIAFVEFDNEVQAGAAR	56605690
	U1-C	FYCDYCDTYLTHDSPSVR	6094204
	U1-70 K	VLVDVER	13633918
	Histone H2A.1	AGLQFPVGR	62663816
	Histone H4	VFLENVIR	204601
	hnRNP A2/B1	IDTIEIITDR	6647752
	Hemoglobin beta chain	LLVVYPWTQR	
	122699DNase I	YDIVLIQEVV	6015029
D	SmE	GDNITLLQSVSN	134127
		VMVQPINLIFR	62667426
	SmG	HVQGILR	6094212
	SmD1	YFILPDSLPLDTLLVDVEPK	51338665
	SmD2	GDSVIVVLR	51338666
	SmD3	VAQLEQVYIR	51338667
		FLILPDMLK	51338667
	SmB/B'	VLGLVLLR	134545
	U1-A	GQAFVIFK	56605690
		SMQGFPFYDK	56605690
		EVSSATNALR	134092
	U1-C	FYCDYCDTYLTHDSPSVR	6094204
	U1-70 K	EFEVYGPIK	13633918
		VLVDVER	13633918
		LGGGLGGTR	13633918
		GGGGSGQDNGLEGLGSDGR	13633918
		DPIPYLPPLEK	13635663
	E	SmE	VMVQPINLIFR
VmVQPINLIFR			62667426
SmD3		VAQLEQVYIR	51338667
U1-A		GQAFVIFK	56605690
U1-C		FYCDYCDTYLTHDSPSVR	6094204
U1-70 K		DPIPYLPPLEK	13635663
Histone H2B		LLLPGELAK	477676
Histone H4		VFLENVIR	204601
hnRNP C1/C2		MIAGQVLDINLAAEPK	20455507
F		SmE	VMVQPINLIFR
	Histone H2A.1	AGLQFPVGR	62663816
	Carboxyesterase ES-10	FAPPQPAEPWNFVK	119596
		LDLLGNPK	119596
		ESYPFLPTVIDGVVLPK	119596
	Guanylate kinase	VIDKNSKVK	2506792
	Nucleoporin Nup205	YSFIQALVR	41017500

a) Oxidized methionine is shown in lowercase in the peptide sequence.

Peptide sequences of proteins identified by MS analysis of 450 kDa band from HeLa (A) or rat liver (B) native nuclear extract separated by 1-D BN-PAGE are listed and grouped. The table also shows peptide sequences of proteins identified in spot 1 (C), 2 (D), 3 (E), and 4 (F) of rat liver nuclei separated in the CN-/BN-PAGE gel (spots shown on Fig. 4B).

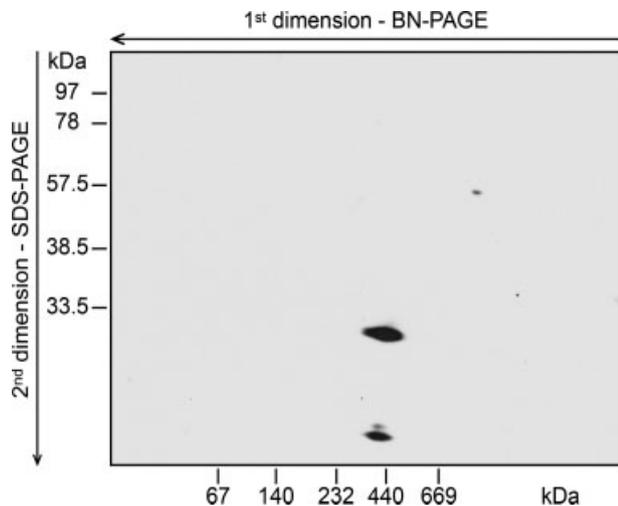


Figure 4. Detection of snRNP proteins in a 2-D BN-/SDS-PAGE gel. Protein complexes extracted by 800 mM 6-AcA from HeLa cell nuclei were separated by 2-D BN-/SDS-PAGE and immunoblotted. Anti-snRNP antibody detected two subunits (M_r around 28 and 13 kDa) of snRNP particle that moved as 450 kDa complex in the first dimension (see Fig. 2).

complexes occur as spots close to the diagonal of the gel, whereas incorrectly separated material creates rather diffuse background under the diagonal.

For MS analysis, CN-/BN-PAGE gels were stained by CBB R-250 and the spots marked by circles in Fig. 5B residing near the diagonal and centered at the mobility area of 450 kDa were selected. The peptides identified by MS analysis are listed in Table 1. Similarly to the results of MS analysis of corresponding material from HeLa cells separated by 1-D BN-PAGE, the peptides found in spots 1–3 (Table 1C–E) were derived from snRNP proteins, namely core Sm proteins and also U1 snRNP specific proteins (U1-A and U1-70 K). This indicates that the U1 snRNP particle migrates under these conditions as several slightly distinct protein complexes of approximate M_r 450 kDa, which is consistent with the M_r of protein complex identified by an antibody against snRNP proteins in 1-D BN-PAGE gel (see Fig. 2E), and with the MS analysis of the 450 kDa band of 1-D BN-PAGE separation of HeLa cell nuclei (see Table 1 for comparison). The MS data were further verified by immunoblotting of the 2-D CN-/BN-PAGE gel with anti-snRNP antibody. The positions of analyzed spots 1–3 correspond exactly to the position of the snRNP antibody signal (Fig. 5C, arrowheads; signal corresponding to spot 3 was visible after prolonged exposure time of ECL, not shown). Spot 4 not so well defined and residing far from diagonal unlike spots 1–3 (Fig. 5B) contained only histone H2A and SmE protein (Table 1F). This indeed supports our

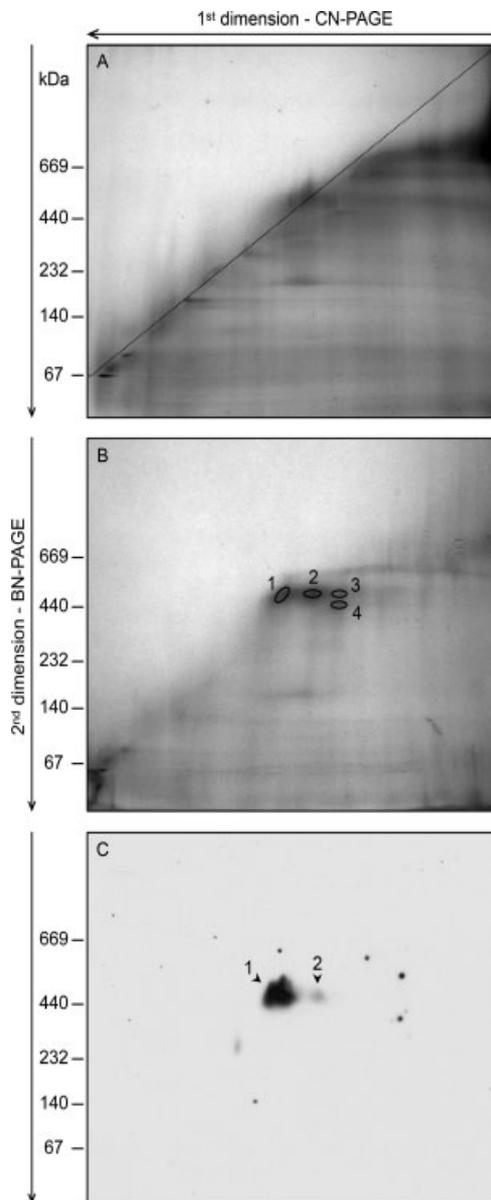


Figure 5. Nuclear protein complexes separated by 2-D CN-/BN-PAGE. (A) The silver-stained gel shows the distribution of proteins and protein complexes extracted from rat liver nuclei and separated by CN-PAGE (horizontal arrow) followed by BN-PAGE in perpendicular direction (vertical arrow). (B) Spots indicated by numbered areas on a CBB R-250-stained gel were excised and subjected to MS analysis (see Table 1). (C) 2-D CN/BN-PAGE gel immunoblotted with anti-snRNP antibody. snRNP-positive spots (marked by numbered arrowheads) in the area centered at 450 kDa correspond to the x-y coordinates of bands excised from the CBB-stained gel (B).

assumption that the area below the diagonal contains proteins possessing either aberrant mobility or breakdown products of less stable complexes.

In summary, we were able to identify complexes consisting of eight to nine out of ten subunits known to assemble the U1 snRNP particle [31] in HeLa or rat liver nuclei by either 1-D BN-PAGE or 2-D CN-/BN-PAGE combined with MS analysis. Recently, the precise stoichiometry of U1 snRNP particle has been determined quantitatively by MS using internal amine-tagged peptide standards [34]. The data revealed that equal amounts of all ten snRNP proteins are present in U1 snRNP particle suggesting a presence of one copy of each snRNP protein in the particle. The theoretical M_r of all subunits in such a complex is approximately 240 kDa. When taking into account the mass contribution of RNA species (165 bp [32]), the electrophoretic migration of the 450 kDa complex is consistent with the assembly model of U1 snRNP particle [32].

Based on the shown data, 2-D CN-/BN-PAGE can be used as an alternative approach to simpler 1-D BN-PAGE when the high background caused by aberrant separation of histones by 1-D BN-PAGE is an issue. Moreover, the separation of certain protein complexes can be further refined using different separation conditions between the first and the second dimension of separation [15].

4 Concluding remarks

Until recently, BN-PAGE has been used with a great benefit in many laboratories most exclusively in the mitochondrial research for its ability to separate native wild and mutant mitochondrial respiratory complexes (see *e.g.*, [35]). Our study shows that in contrast to mitochondrial oxidative phosphorylation complexes, nuclear complexes are much more sensitive to the conditions of nuclear specimen separation. These should be chosen appropriately to reach good protein complex solubility and preserve its stability. This is possible by adjusting the concentration of 6-AcA in the sample and gel buffers.

In conclusion, the potential of the method can be utilized also in the area of nuclear research where the composition of many protein complexes in relation to their function and dynamic changes remains to be identified. For example, Pol I used in this study as one of the model nuclear protein complexes is involved in ribosomal biogenesis by transcription of ribosomal DNA. The biosynthesis of ribosomal components, ribosomal proteins and rRNAs, is tightly regulated. The association of the main transcription activator UBF with the Pol I holoenzyme found in this study opens the possibilities to use BN-PAGE for studies on regulation of rDNA transcription during physiological or pathological conditions such as cellular differentiation or transformation.

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