Isolation and Characterization of Bovine Lymphocyte Nuclear Matrix

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ABSTRACT. A nuclear matrix was obtained from isolated nuclei of bovine lymphocyte by digestion with DNase I and subsequent extractions with 0.4 M and 2.0 M NaCl. 91.1 % of the nuclear protein, 99.5 % of the DNA and 83.1 % of the RNA were removed from isolated nuclei by these treatments. with the following nuclease digestion, almost all residual DNA and RNA was removed leaving the nuclear protein matrix.

Ultrastructural analysis of the nuclear matrix revealed a spherical structure containing a peripheral lamina, an internal fibrogranular network and residual nucleoli.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the nuclear matrix demonstrated three major polypeptides of 68,000, 53,000 and 43,000 dalton as well as more than 50 minor polypeptides of various molecular weight classes. The 68,000 dalton polypeptide corresponds to lamin B, one of three predominant polypeptide components of rat liver nuclear matrix. However, polypeptides corresponding to lamin A and C were not detectable in the bovine lymphocyte nuclear matrix.

Berezney and Coffey (4, 6) were the first to propose that a proteinaceous network, termed the nuclear matrix, serves as the skeletal foundation which determines the overall organization of rat liver nuclei. Since then, some proteinaceous structures, resembling a nuclear matrix, have been prepared from HeLa S₃ cell (15), *Tetrahymena* (30), Friend erythroleukemia cell (19), *Physarum polycephalum* (25), 3T6 fibroblast (9) and others. These proteinaceous structures are very similar to each other, composed of three components: peripheral lamina, internal fibrogranular structure and residual nucleoli. The nuclear matrix appears to be a ubiquitous structure that is present in almost all eukaryotic cells. Much evidence is accumulating to suggest that the nuclear matrix not only may function as the skeleton of the nucleus but also may be associated with many dynamic aspects of nuclear function: nuclear swelling (30, 31), DNA replication (5, 7, 13, 16, 27), RNA transcription (1, 11, 23), hormone binding (3), T-antigen binding (9), processing and transport of RNA (22, 29), *etc.*

To know the real function of the nuclear matrix, it is important to examine the matrix in cells from a variety of organisms and tissues. In this paper, we first report the isolation method and characterization of the bovine lymphocyte nuclear matrix.

Abbreviations: SDS, sodium dodecyl sulfate, PMSF, phenyl methyl sulfonyl fluoride; DEP, diethyl pyrocarbonate; GTM buffer, 50 mM Tris-HCl (pH 7.5) containing 25 % (v/v) glycerol and 2 mM MgCl₂.

MATERIALS AND METHODS

Lymphocyte nuclei. Lymphocytes used in these experiments were isolated from bovine submaxillary lymph nodes. Routinely 3–6 lymph nodes from adult cattle were dissected from surrounding tissue and finely minced with a cutting knife. The minced tissue was suspended in STM buffer (50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and 5 mM MgCl_2) and gently stirred. Then, connective tissue and other debris were removed by filtration through four layers of gauze. The lymphocyte suspension thus obtained was centrifuged at $100\times g$ for 10 min. The resulting cell pellet was washed once in the buffer. The washed lymphocytes (50 mg DNA) were suspended in 25 ml of STM buffer, containing 1% Triton X-100, 1 mM PMSF and 0.1% DEP. The cells were disrupted by passing the suspension through a syringe needle (0.65×30 mm) four times. Then the homogenate was centrifuged at $200\times g$ for 5 min, and the supernate (Triton X-100 supernate) was removed. The resulting nuclear pellet was resuspended in the STM buffer, containing 1% Triton X-100, 1 mM PMSF and 0.1% DEP, and subjected to cell homogenization in the same manner for two more times and finally centrifuged at $200\times g$ for 5 min. The nuclei obtained were essentially free of cytoplasmic contamination as judged by electron microscopy.

Nuclear matrix. The procedure for the isolation of bovine lymphocyte nuclear matrix is presented in detail in the RESULTS section. All mediums for isolation contained 1 mM PMSF and 0.1% (v/v) DEP to inhibit some protease and RNase activity, respectively. The preparation steps were conducted at 0–4°C. Rat liver nuclear matrix prepared as previously (20) was kindly provided by Dr. H. Ueyama, of our laboratory.

Preparation of an RNase-depleted DNase. Commercial pancreatic DNase (DN-CL, purchased from Sigma) was further purified to remove a contaminating RNase. This was carried out by affinity chromatography on Agarose-UMP (obtained from Miles laboratories) according to the procedure of Brison and Chambon (8). The purified preparation of DNase was essentially "RNase-free".

Electron microscopy. Samples were prepared for electron microscopy by fixation in 3.2% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.2, 5 mM MgCl_2) at 4°C overnight. They were then centrifuged at $400\times g$ for 5 min and the pellets were post-fixed with 1% osmium tetroxide (0.1 M sodium cacodylate buffer, pH 7.2, 5 mM MgCl_2) at 0°C for 2 h. The specimens were dehydrated with ethanol and embedded in an Epon 812 mixture. Thin sections were double-stained with uranyl acetate and lead citrate solutions. Electron micrographs were made with a Hitachi H-600 electron microscope at an original magnification ranging from $\times 5,000$ to $\times 20,000$.

SDS-Polyacrylamide gel electrophoresis. All electrophoretic analyses were performed in 1 mm thick slab gels according to the procedure of O'Farrell (26). The gels used were composed of a 4.5% acrylamide stacking gel overlaying a 13.5% acrylamide separation gel. The acrylamide/bisacrylamide ratio was maintained at 1 : 0.027 in both the stacking and separation gels. Lymphocyte and lymphocyte nuclei were solubilized (about 4 mg protein/ml) in lysis buffer (0.1 M Tris-HCl buffer, pH 7.5, containing 2% SDS and 4 M urea) then centrifuged at 60,000 rpm for 16 h to precipitate the bulk of the DNA. The other samples were solubilized in the lysis buffer without centrifugation. They were boiled at 100°C for 3 min with β -mercaptoethanol (10%) and bromophenol blue (0.01%). Following electrophoresis, the slab gels were stained with coomassie blue R-250 and destained in 10% (v/v) acetic acid, containing 20% (v/v) methanol.

Determination of DNA, RNA and Protein. DNA, RNA and protein were determined by the method of Burton (10) with calf thymus DNA as standard, by orcinol reaction (28) with yeast RNA as the standard and by the method of Lowry *et al.* (21) with bovine serum

albumin as the standard, respectively. However, the DNA content of the lymphocytes was analyzed fluorometrically (18) in order to rapidly prepare the nuclear matrix.

RESULTS

Isolation of bovine lymphocyte nuclear matrix and nuclear protein matrix. Berezney and Coffey (4) first described the isolation of nuclear matrix from rat liver. The bulk of nuclear DNA was removed during low magnesium treatment in that case. However, bovine lymphocyte nuclei were burst away by treatment in low magnesium buffer. Thus, we employed a DNase treatment in the presence of 2 mM MgCl₂ in the first step. The latter method always resulted in the successful preparation of lymphocyte nuclear matrix as follows: Lymphocyte nuclei (50 mg DNA) were suspended in 20 ml of GTM buffer (50 mM Tris-HCl buffer, pH 7.5, containing 25% (v/v) glycerol and 2 mM MgCl₂). The suspension was incubated at 25°C for 30 min with 1 mg of purified DNase I. Then the suspension was centrifuged at 400 × g for 30 min and the supernate (Ds) was removed. The white pellet (DNase treated nuclei) was suspended in 8 ml of GTM buffer by Pasteur pipette. To this suspension, 32 ml of GTM buffer containing 0.5 M NaCl was slowly added, with gentle shaking, to a final concentration of 0.4 M. After incubation on ice for 10 min, the suspension was centrifuged at 600 × g for 90 min. The supernate (Hs 1) was carefully removed and the resulting translucent pellet (low salt nuclear matrix) was mildly suspended in 8 ml of GTM buffer containing 2 M NaCl. It was incubated on ice for 10 min and centrifuged at 600 × g for 20 min. The supernate (Hs 2) was removed and the resulting pellet (nuclear matrix) was suspended in 2.5 ml of GTM buffer. The nuclear matrix was stored at -20°C for a few days without structural alteration.

For preparation of the nuclear protein matrix, the pellet of nuclear matrix obtained was suspended in 8 ml of GTM buffer without the addition of DEP, and centrifuged at 600 × g for 20 min. This washing was repeated once more to remove DEP which inhibits the RNase activity. The pellet was suspended in 8 ml of GTM buffer, then 0.5 mg of purified DNase, 0.5 mg of RNase A and PMSF (at a final concentration of 1 mM) were added to the suspension. After incubation at 25°C for 20 min, the suspension was centrifuged at 600 × g for 20 min. The final pellet (nuclear protein matrix) was carefully suspended in 2.5 ml of GTM buffer. A flow diagram for isolation of the nuclear protein matrix is shown in Fig. 1.

During the subsequent extraction procedure, 95–99% of the total nuclear DNA

Fig. 1. Scheme for the subfraction of isolated lymphocyte nuclei yielding a nuclear matrix and a nuclear protein matrix. For detail, see RESULTS.

TABLE 1. STEPS IN THE ISOLATION OF NUCLEAR MATRIX

Step	Consecutive extractions	Resultant nuclear sphere	Total nuclear material extracted		
			Protein (%)	DNA (%)	RNA (%)
0.	Isolation of nuclei	Isolated nuclei	0	0	0
1.	DNase treatment	DNase treated nuclei	10.1 $\pm$ 0.9	18.5 $\pm$ 0.4	33.4 $\pm$ 3.8
2.	0.4 M NaCl treatment	Low salt nuclear matrix	77.7 $\pm$ 5.7	95.3 $\pm$ 0.2	70.7 $\pm$ 2.8
3.	2 M NaCl treatment	Nuclear matrix	91.1 $\pm$ 0.1	99.5 $\pm$ 0.1	83.1 $\pm$ 1.8
4.	Nucleases treatment	Nuclear protein matrix	92.0 $\pm$ 1.4	>99.9	98.7 $\pm$ 0.3

Each value is the mean $\pm$ standard deviation of three determinations.

was removed (Table I). The residual DNA, however, was tightly bound to the nuclear matrix, which is resistant to the exhaustive DNase digestion, as shown in Fig. 2. The residual DNA was removed by the following DNase-RNase treatment, while a small amount of RNA remained attached to the nuclear protein matrix. The procedure for isolation of the matrix removed most of the histones, leaving about 10% of the total nuclear proteins. The recovery of proteins from nuclei during consecutive extractions is summarized in Table I, and the polypeptide composition of each subfraction will be described later.

Phase-contrast microscopy and electron microscopy studies. Figure 3 shows the lymphocyte and nuclear spheres observed with a phase-contrast microscope at each

Fig. 2. The remains of DNA in the low salt nuclear matrix and nuclear matrix after digestion with DNase I. Lymphocyte nuclei (5.6 mg DNA) were suspended in 2 ml of GTM buffer, containing 1 mM PMSF and 0.1% DEP and incubated at 25°C for 30 min with an indicated amount of purified DNase I. The low salt nuclear matrix and the nuclear matrix were prepared as described in RESULTS. Remaining DNA in the low salt nuclear matrix (—○—) and the nuclear matrix (—●—) were determined by the method of Burton (10). The values are represented as a percentage of the total nuclear DNA.

step of the above extraction procedure. The round shape was maintained throughout the steps. The presence of glycerol in the extraction medium was essential for holding the structures. Without glycerol, considerable nuclear spheres turned to fibrous fragments because of destabilization. As shown in Fig. 3, the mean diameter $\pm$ standard deviation of lymphocyte, isolated nuclei, DNase treated nuclei, low salt nuclear matrix and nuclear matrix were 2.94 ± 0.40 , 2.53 ± 0.30 , 1.73 ± 0.29 , 1.45 ± 0.27 and $1.62 \pm 0.32 \mu\text{m}$, respectively. The shape of the nuclear protein matrix was not mentioned in Fig. 3, since it showed too faint an image with phase-contrast microscopy. But the diameter of the nuclear protein matrix was roughly similar to that of the nuclear matrix. Significant decrease in the diameter of nuclei was observed by DNase treatment. It is surprising that DNase treated nuclei held their round shape in spite of considerable shrinking (about 30% of diameter and 70% of volume) due to digestion.

Fig. 3. Phase-contrast microscopy of lymphocytes (A), isolated nuclei (B), DNase treated nuclei (C), low salt nuclear matrix (D) and nuclear matrix (E). One or two drops of these samples were suspended in 1 ml of STM buffer immediately after preparation. Bar = $10 \mu\text{m}$. The mean diameters $\pm$ standard deviation (from 100 spheres) of these fractions: lymphocyte, isolated nuclei, DNase treated nuclei, low salt nuclear matrix and nuclear matrix, are 2.94 ± 0.40 , 2.53 ± 0.30 , 1.73 ± 0.29 , 1.45 ± 0.27 and $1.62 \pm 0.32 \mu\text{m}$, respectively.

Fig. 4. Electron microscopy of lymphocyte (A), isolated nuclei (B), DNase treated nuclei (C), low salt nuclear matrix (D), nuclear matrix (E) and nuclear protein matrix (F). All samples were fixed and stained as described in MATERIALS AND METHODS. Bar = 2 μ m.

Ultrastructural morphologies of lymphocyte and nuclear spheres were shown in Fig. 4. Electron micrographs of these fractions also revealed the maintenance of round nuclear shapes. The attachment of granular particles to the surface of spheres is shown in DNase treated nuclei (C). These particles may be material released by DNase digestion. The surface lamina, internal fibrogranular structure and residual nucleoli are clearly shown in the nuclear matrix (E) and in the low salt nuclear matrix (D). However, densely stained nucleoli are hardly shown in the nuclear protein matrix (F).

The nuclear pore complex was easily shown in electron micrographs of untreated (B) and DNase treated nuclei (C). However, it was rather difficult to distinguish the pore complex from surface structures in the electron micrographs of the low salt nuclear matrix (D), the nuclear matrix (E) and the nuclear protein matrix (F). Internal fibrogranular structures are recognizable first in the low salt nuclear matrix (D). The quantity of the fibrogranules decrease as the extraction steps proceed. This may be related to a removal of nucleic acids from low salt nuclear matrix by salt washing and by nucleases digestion.

SDS-polyacrylamide gel electrophoresis of matrix proteins. SDS-polyacrylamide gel electrophoresis of nuclear matrix and other subfractions are compared in Fig. 5. Low salt (0.4 M NaCl) extraction caused complete loss of H 1 histone with only a partial loss of histone cores from DNase digested nuclei. Almost complete removal

Fig. 5. SDS-polyacrylamide gel electrophoresis of the nuclear matrix polypeptides. Each sample was treated before electrophoresis as described in the text. A. 1 and 9; molecular weight markers, 2; lymphocyte, 3; Triton X-100 supernate, 4; isolated nuclei, 5; Ds, 6; Hs 1, 7; low salt nuclear matrix, 8; nuclear matrix. Histone bands are indicated by asterisks. B. 1 and 4; molecular weight markers, 2; bovine lymphocyte nuclear matrix, 3; rat liver nuclear matrix. Rat liver nuclear lamins are indicated by arrows (lamin A, 70,000, lamin B, 68,000, lamin C, 65,000). The molecular weight markers used were phosphorylase-a (94,000), bovine serum albumin (67,000), Ig G heavy chain (50,000), ovalbumin (45,000), chymotrypsinogen (25,000), trypsin inhibitor (21,000), myoglobin (17,000) and cytochrome *c* (12,000).

of these cores was obtained by subsequent high salt (2.0 M NaCl) treatment, leaving matrix proteins. Low salt nuclear matrix (lane 7) and nuclear matrix (lane 8) are characterized by three major polypeptide bands with a molecular weight of 68,000, 53,000 and 43,000 dalton. The 68,000 polypeptide had the same mobility with lamin B from rat liver nuclei on SDS-polyacrylamide gel electrophoresis as shown in Fig. 5B. The results suggest that the 68,000 polypeptide is related to lamins which are the major components of nuclear matrix from several sources (6, 9, 14). One of the prominent proteins in the nuclear matrix (43,000 dalton) is also clearly observed in lymphocytes (lane 2), Triton X-100 supernatant (lane 3), isolated nuclei (lane 4) and Ds (lane 5). This abundant protein may be actin, which is a major protein component of lymphocyte. The electrophoretical pattern of the nuclear protein matrix is not shown in Fig. 5, but it was extremely similar to that of the nuclear matrix (lane 8).

DISCUSSION

Isolation of nuclear matrix. We have developed a procedure allowing the isolation of a nuclear matrix from a bovine lymphocyte by low and high salt extractions including preincubation with DNase I. In the process of isolation, the nuclear matrix

spheres are significantly decreased in size, while the size of isolated lymphocyte nuclei closely resembles the *in situ* diameters. This shift to smaller sizes occurs after DNase treatment. A similar contraction of the nuclear matrices was also reported in HeLa cells (15) and in rat liver (6). Hodge *et al.* (15) reported that the nuclear matrix had a spherical shape having 70% of the diameter of the original nuclei (HeLa S₃ cell) which is in agreement with our results. Berezney and Coffey also described nuclear matrix shrinking by nuclease digestion (6). Wunderlich and Herlan (30) isolated a nuclear matrix from *Tetrahymena pyriformis* which reversibly contracts at varying concentrations of the bivalent cations, Ca²⁺ and Mg²⁺. Therefore, the possible contractive nature of nuclear matrix may be related to some dynamic transformation of the nuclei.

Electron microscopy reveals that the nuclear matrix obtained from bovine lymphocyte still shows the nuclear sphere shape and is composed of a surrounding nuclear lamina, internal fibrogranular structures and residual nucleoli. These ultrastructures of the nuclear matrix are extremely similar to those of other sources: nuclear matrix from rat liver (6), HeLa S₃ cells (15), Friend erythroleukemia cells (19) and 3T3 fibroblasts (27).

The nuclear matrix obtained was mainly composed of proteins containing small amounts of DNA and considerable amounts of RNA. These results agree with those of nuclear matrices from other sources (2, 6, 25, 30). The significance of these residual nucleic acids is uncertain at present, but there are many reports describing the association of a newly synthesized DNA with nuclear matrices (5, 7, 9, 13, 16, 27). On the other hand, Miller *et al.* found heterogeneous nuclear RNA (23) and small nuclear RNA (24) associated with rat liver nuclear skeleton. Clawson and Smuckler (11) also showed the idea that heterogeneous nuclear RNA may be processed after attachment to the nuclear envelope or nuclear matrix. Studies must be advanced on these associated polynucleotides of the nuclear matrix, so that the nuclear matrix may contribute to many nuclear functions.

Proteins of the nuclear matrix. Three major protein bands (68,000, 53,000 and 43,000) are revealed by SDS-polyacrylamide gel electrophoresis of bovine lymphocyte nuclear matrix (Fig. 5A). As indicated above, the 68,000 protein may correspond to one of the three major protein components of rat liver nuclear matrix (69,000, 66,000 and 62,000) (6) or nuclear pore complex-lamina (70,000, 66,000 and 62,000) (14). Likewise in rat liver nuclear matrix, three prominent protein bands (60,000–75,000) related to lamin, were usually detected on SDS-gel analysis of nuclear matrix from other sources (9, 12, 25, 30). However, recent studies of the nuclear matrix polypeptides from other cells indicate possible differences in molecular weights. For example, Long *et al.* reported that only two prominent polypeptide bands (66,000 and 62,000) were detected in isolated matrix from Friend erythroleukemia cells (19), while Krohne *et al.* reported that only one prominent polypeptide (73,000) was detected in nuclear ghosts from oocytes of *Xenopus laevis* (17). Therefore, it is not strange that only one major polypeptide migrates between 60,000–75,000 dalton on SDS-gel analysis of bovine lymphocyte nuclear matrix. These results, however, may not rule out the possible heterogeneity of 68,000 protein(s) of the nuclear matrix.

There are many non-histone protein bands (more than 50 bands) in addition to lamin found on SDS-gel analysis of lymphocyte nuclear matrix protein. For the present, the nature of these proteins is not made sufficiently clear. However, an abundant protein with a molecular weight of 43,000 dalton may be actin as judged by its molecular weight.

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