PROTEIN COMPOSITION OF NUCLEAR MATRIX PREPARATIONS FROM HeLa CELLS: AN IMMUNOCHEMICAL APPROACH

RON VERHEIJEN1*, HELMA KUIJPERS1, PETER VOOIJS1, WALTHER VAN VENROOIJ2 AND FRANS RAMAEKERS1

1Department of Pathology, Radboud Hospital, and 2Department of Biochemistry, University of Nijmegen, Geert Grooteplein Zuid 24, 6525 GA Nijmegen, The Netherlands

SUMMARY

Procedures for the isolation of HeLa S3 nuclear matrices were re-examined with special emphasis on the use of various nucleases and detergents as well as on the ionic strength of the final salt extraction.

The protein composition of the resulting nuclear matrix preparations was analysed by one- and two-dimensional gel electrophoresis and found to be extremely reproducible. By means of co-electrophoresis several typical cytoskeletal proteins (actin, vimentin and cytokeratins) and heterogeneous nuclear RNA (hnRNA)-associated core proteins (hnRNP) were shown to be present in such nuclear matrix preparations. The nature of some other protein components was elucidated using two-dimensional immunoblotting and immunofluorescence. For this purpose mouse monoclonal antibodies to cytoskeletal components (vimentin, cytokeratins), small nuclear RNP (70x10^6 M_r protein of U1-RNP), hnRNP (C1/C2) and the pore-complex lamina (lamins A, B and C) were used next to human autoimmune sera obtained from patients with connective tissue diseases and directed against the residual nucleoli and the internal fibrillar mass. These antibodies enabled us to identify a number of proteins present specifically in the nuclear matrix and to show that part of the cytoskeletal proteins are still present in the isolated structures.

INTRODUCTION

When isolated nuclei are depleted of their membranes, soluble molecules and chromatin by means of subsequent treatments with detergents, nucleases and high-salt solutions, a structural framework, mostly referred to as the nuclear matrix, remains (reviewed by Agutter & Richardson, 1980; Kaufmann & Shaper, 1984).

The isolated nuclear matrix consists of three morphologically distinguishable structural elements: (1) a peripheral layer, which represents the remainder of the nuclear envelope and contains pore-complexes in association with a lamina; (2) residual nucleoli; and (3) internal fibrillar structures.

The peripheral pore-complex lamina has been isolated separately and its polypeptide composition has been determined (Franke, Scheer, Krohne & Jarasch, 1981). In higher eukaryotes three distinct polypeptides, lamins A, B and C (60 to

*Author for correspondence.

Key words: nuclear matrix, cytoskeleton, hnRNP, monoclonal antibodies, autoantibodies.
can be discerned. Studies dealing with the chromatin-depleted nucleolar residue indicate that it contains a unique subset of proteins within nuclear matrix preparations (Peters & Comings, 1980; Comings & Peters, 1981; Krohne et al. 1982).

Little is known about the structural polypeptides forming the intranuclear fibrillar network, but the few studies that have been done indicate that it is very complex (Peters & Comings, 1980; Capco, Wan & Penman, 1982; Fischer, Berrios & Blobel, 1982). Experimental evidence showing which polypeptides form the structural backbone is lacking, although some reports have shown that actin, apparently in a non-filamentous form, is the main component of the nuclear matrix (Clark & Rosenbaum, 1979; Krohne & Franke, 1980; Capco et al. 1982; Nakayasu & Ueda, 1983; Staufenbiel & Deppert, 1984).

There is good evidence that this internal matrix represents a structure that also exists in the intact cell (van Eekelen & van Venrooij, 1981; Kaufmann, Coffey & Shaper, 1981; Brasch, 1982; Capco et al. 1982; van Eekelen et al. 1982; Fischer et al. 1982). The internal matrix structure of HeLa cells, for example, seems not to depend on RNA or DNA for its structural integrity. Even so, disulphide bridge formation is not likely to be responsible for its formation (van Eekelen et al. 1982). Furthermore, specific proteins can be found in the isolated matrix (Fey, Wan & Penman, 1984).

Studies in which the three-dimensional structural organization of isolated matrices was viewed using electron microscopy on whole mount preparations instead of thin sections corroborate the hypothesis of the existence of an internal nuclear protein structure. These studies also showed a distinct interaction between the cytoskeleton and the nuclear matrix (Capco et al. 1982; Capco, Krochmalnic & Penman, 1984; Fey et al. 1984).

Additional indications of the existence of an intranuclear structural framework can be deduced from studies of its functional aspects in, for example, DNA replication (McCready et al. 1980; Pardoll, Vogelstein & Coffey, 1980; Vogelstein, Pardoll & Coffey, 1980; Berezney & Coffey, 1985), hormone binding (Barrack & Coffey, 1980), association with viral tumour antigens (Staufenbiel & Deppert, 1983), and processing and transport of RNA (Herman, Weymouth & Penman, 1978; Miller, Huang & Pogo, 1978; Maundrell, Maxwell, Puvion & Scherrer, 1981; Jackson, Caton, McCready & Cook, 1982; Mariman et al. 1982a; Mariman, Hagebols & van Venrooij, 1982b; Ben-Ze'ev & Aloni, 1983; Mariman, van Beek-Reinders & van Venrooij, 1983).

The primary transcript of DNA in eukaryotic cells is heterogeneous nuclear RNA (hnRNA). This hnRNA is present in the cell nucleus as fibrillar ribonucleoprotein (RNP) particles and granules (Holoubek, 1984; Wilk et al. 1985). The major protein components of hnRNP complexes are the core proteins of about 30 to 41×10^3 M_r. The nomenclature proposed for these proteins by Beyer, Christensen, Walker & LeStourgeon (1977) has recently been extended by Wilk et al. (1985) as a result of their two-dimensional gel analyses of the core proteins from isolated 35–40 S hnRNP complexes.
In our approach to identify the nature of the protein components that participate in the nuclease- and high-salt-resistant nuclear structure we have examined the effects of several methods of preparation on the polypeptide pattern of this matrix, including different types of nuclease treatments and high-salt extractions. After the establishment of such a routine procedure, and verification of its reproducibility, several of the polypeptides present in such preparations were identified using immunochemical techniques.

In this paper we use the terminology of Kaufmann & Shaper (1984) and define the nuclear matrix as the detergent-, nuclease-, and salt-resistant entity composed of components of the nucleolus, a non-histone intranuclear meshwork, and a peripheral layer composed of the lamina with its pore-complexes.

**Materials and Methods**

**Cell culture and labelling**

Tissue culture media and calf sera were purchased from Flow Laboratories Ltd, Irvine, Scotland. HeLa S3 cells (human cervix carcinoma) were grown in suspension at 37°C at densities ranging from $0.5 \times 10^6$ to $10^6$ cells ml$^{-1}$ on Suspension Minimal Essential Medium supplemented with $10\%$ newborn calf serum and $1.5 \text{ g l}^{-1}$ lactalbumin hydrolysate (van Eekelen et al. 1982). Cellular protein was labelled by incubating the cells for 16 h with $5-10 \mu\text{Ci ml}^{-1} [35\text{S}]$methionine (Amersham U.K. ± 1000 Ci mmol$^{-1}$) at densities of $10^6$ to $2 \times 10^6$ cells ml$^{-1}$. For the first 2–3 h the cells were incubated in tissue culture medium that contained only labelled methionine, then $0.1 \text{ vol.}$ of complete medium was added.

**Cell fractionation and purification of nuclear matrices**

All chemicals were of analytical grade. Buffers were boiled in the presence of $0.02\%$ diethylpyrocarbonate and then autoclaved. Cell fractionations were carried out in the presence of $0.5 \text{ mM}$ phenylmethylsulphonyl chloride (PMSC) and $5 \text{ mM- } N$-ethylmaleimide (MalNEt) to reduce proteolytic degradation and disulphide bridge formation, respectively. These agents were added from freshly prepared stocks. Ribonuclease A (RNase A) (Sigma Chemical Co., München) was pre-incubated for 15 min at 100°C to reduce possible protease activity. Centrifugation steps were carried out at 5 min at 800 $\text{g}$ and 2°C.

The procedure that we have established for the isolation of nuclear matrices, carried out at 0–4°C, is as follows: cells were harvested on frozen NKM buffer ($130 \text{ mM-NaCl, 5 mM-KCl, 1.5 mM-MgCl}_2$), pelleted by centrifugation, washed twice with isotonic NKM solution and pelleted again. Each of the following steps in the procedure was preceded by washing the pellet twice with reticulocyte suspension buffer (RSB) ($10 \text{ mM-NaCl, 10 mM-Tris-acetate, pH 7.4, 1.5 mM-MgCl}_2$). Subsequently, the cell pellet was suspended in hypertonic buffer (RSB with $0.3 \text{ M-sucrose}$) and after addition of $0.05 \text{ vol.}$ of $10 \%$ Triton X-100 in RSB the suspension ($4 \times 10^7 \text{ cells ml}^{-1}$) was gently swirled in ice for about 1 min and centrifuged to sediment the cytoskeletons. After washing these were resuspended in RSB ($4 \times 10^7 \text{ cells ml}^{-1}$) and, after addition of $0.1 \text{ vol.}$ of a freshly prepared solution of $5\%$ sodium deoxycholate (DOC)/$10\%$ Tween-40 in RSB, homogenized by 10 strokes of a motor-driven Teflon pestle in a Potter tissue homogenizer (Kontes Co., Vineland, N.J.). The nuclei were pelleted, washed and resuspended in HRSB ($110 \text{ mM-NaCl, 10 mM-Tris-acetate, pH 7.4, 1.5 mM-MgCl}_2$) at a density of $1 \times 10^8$ nuclei ml$^{-1}$ and incubated with $800 \mu\text{g ml}^{-1}$ deoxyribonuclease I (DNase I) (Sigma) and $25 \mu\text{g ml}^{-1}$ RNase A (Sigma) for $15 \text{ min}$ at 20°C. During this digestion step MalNEt was omitted, but immediately after the incubation it was added again to a final concentration of $5 \text{ mM}$.

The DNA-depleted nuclei were spun down, washed and gently resuspended in $0.4\text{ M-(NH}_4)_2\text{SO}_4$, $50 \text{ mM-Tris-acetate, pH 7.4, 1.5 mM-MgCl}_2$. The matrices were pelleted, washed and resuspended in RSB.
Gel electrophoresis

Samples were prepared for gel electrophoresis as described by van Eekelen & van Venrooij (1981). After pelleting, the nuclear matrices were immediately dissolved in sodium dodecyl sulphate (SDS) sample buffer. SDS/polyacrylamide gel electrophoresis (SDS/PAGE) was performed using the Laemmli (1970) buffer system.

For two-dimensional gel electrophoresis under non-equilibrium isoelectric focusing conditions in the first dimension, the procedure of O’Farrell was used (O’Farrell, Goodman & O’Farrell, 1977), and electrophoresis was performed for 1800 Vh. For the second dimension 10% SDS/polyacrylamide gels were used.

For the identification of non-muscle actin and vimentin on two-dimensional gels a cytoskeletal preparation from bovine lens cortical fibres was comigrated with [35S]methionine-labelled HeLa proteins. For the localization of cytokeratin spots (cytokeratins 7, 8, 18 and 19, according to the nomenclature of Moll et al. 1982) a cytoskeletal preparation of the human bladder carcinoma cell line T24 was used.

The hnRNA-associated core proteins were identified by comigration with unlabelled core proteins of 35–40 S hnRNP complexes (Wilk et al. 1985).

Blotting and detection of proteins

Transfer of proteins from 10% polyacrylamide gels onto nitrocellulose sheets was performed as described by Habets et al. (1983). After transfer the blots were dried and stored at room temperature. Detection of the antigens on the blots was essentially performed as described (Habets et al. 1985). For detection of labelled proteins on the gels the procedure of Bonner & Laskey (1974) was used.

Immunofluorescence microscopy

HeLa cell nuclear matrix preparations were immunolabelled in suspension essentially as follows: about 5×10⁶ matrices were centrifuged (5 min, 800 g, 4°C), the pellet washed twice with 200 μl phosphate-buffered saline (PBS) containing 5% foetal calf serum (FCS) and pelleted again. The matrices were resuspended in 50 μl of the primary antibodies in the appropriate dilutions and incubated for 45 min at 4°C, with occasional stirring. Table 1 summarizes the antibody preparations used for immunoblotting and immunofluorescence studies. Subsequently the

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Reference</th>
<th>Code in Fig. 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGE53/CK18-2</td>
<td>M Cytokeratin 18</td>
<td>Ramaekers et al. (1983b)</td>
<td>18</td>
</tr>
<tr>
<td>pKer</td>
<td>R Human callus keratins</td>
<td>Ramaekers et al. (1983a)</td>
<td></td>
</tr>
<tr>
<td>RV201</td>
<td>M Bovine lens vimentin</td>
<td>Unpublished</td>
<td>V,V*,V**</td>
</tr>
<tr>
<td>pVim</td>
<td>R Bovine lens vimentin</td>
<td>Ramaekers et al. (1983a)</td>
<td></td>
</tr>
<tr>
<td>4IC14</td>
<td>M Lamin A,B,C</td>
<td>Burke et al. (1983)</td>
<td>Lα,Lε</td>
</tr>
<tr>
<td>LN43</td>
<td>M Lamin B</td>
<td>Unpublished</td>
<td>Lb</td>
</tr>
<tr>
<td>T100</td>
<td>H Nucleolar proteins</td>
<td>Unpublished</td>
<td>Nα,Nε</td>
</tr>
<tr>
<td>J26</td>
<td>H Nucleolar proteins</td>
<td>Unpublished</td>
<td>Nα,Nε</td>
</tr>
<tr>
<td>T5</td>
<td>H Nucleolar proteins</td>
<td>Unpublished</td>
<td>Nδ</td>
</tr>
<tr>
<td>Z3</td>
<td>H 86×10³ Mₑ</td>
<td>van Venrooij et al. (1985)</td>
<td>Iα</td>
</tr>
<tr>
<td>L15</td>
<td>H 56, 70 (×10³ Mₑ)</td>
<td>Unpublished</td>
<td>Iα,Iε</td>
</tr>
<tr>
<td>4F4</td>
<td>M C₁/C₂ core proteins</td>
<td>Choi &amp; Dreyfuss (1984)</td>
<td>Iδ</td>
</tr>
<tr>
<td>2/73</td>
<td>M 70×10³ Mₑ/U1-RNP</td>
<td>Billings et al. (1982)</td>
<td>Iδ</td>
</tr>
</tbody>
</table>

M, mouse monoclonal antibody; R, polyclonal rabbit antiserum; H, human autoimmune serum; L, lamin; N, nucleolar proteins; I, proteins localized in the internal fibrillar structure.
Immunochemical characterization of nuclear matrix proteins

Fig. 1. Estimation of the percentages of high-salt extracted [35S]methionine-labelled proteins from HeLa nuclei as a function of ionic strength of the extraction buffer. The values given are the average of four experiments showing deviations of ± 2%. The ionic strength $I$ of a solution is given by the relation $I = 1/2 \sum C_i Z_i^2$, where $C_i$ is the concentration of an ion of type $i$ and $Z_i$ the number of charges carried by this ion.

matrices were pelleted, washed twice with 200 μl PBS/5% FCS and incubated for 45 min at room temperature in 50 μl of the appropriate fluorescein isothiocyanate (FITC)-conjugated second antibodies (Nordic, Tilburg, The Netherlands). These included: FITC-conjugated rabbit anti-mouse immunoglobulin G (IgG) (heavy and light chains) for detection of the monoclonal primary antibodies, FITC-conjugated goat-anti-rabbit IgG (heavy and light chains) for the detection of the polyclonal rabbit primary antibodies and FITC-conjugated goat-anti-human Ig (heavy and light chains) for the human autoimmune antibodies.

After this incubation the matrices were washed as described above and suspended in 50 μl PBS/glycerol (1:1, v/v). The fluorescent matrix samples were diluted 1:25 in PBS containing 10% normal goat serum and spun down onto coverslips using a Cytospin centrifuge. The samples were dried overnight at room temperature, mounted in Gelvatol (Monsanto, St Louis, Missouri, U.S.A.) containing 100 mg ml⁻¹ 1,4-diazobicyclo-[2,2,2]-octane (DABCO; Janssen Pharmaceutica, Beerse, Belgium) and viewed with a Leitz Dialux 20 EB microscope equipped with epifluorescent illumination using appropriate filters for fluorescein fluorescence. Pictures were taken on Tri-X film (Kodak) with an automatic camera using an ASA setting of 400.

RESULTS

Effects of high-salt extraction

In studying the effects of salt solutions on the protein patterns of the nuclear matrices, DNA-depleted nuclei were extracted with salt solutions of various ionic strengths in the presence of 50 mM-Tris-acetate (pH 7.4) and 1.5 mM-MgCl₂ (Fig. 1). An increase in ionic strength leads to an increase of the amount of extractable protein, with the maximal amount of extractable proteins being about 55% in the case of (NH₄)₂SO₄ and about 60% when NaCl is used. The results of extraction with KCl were similar to those of NaCl (data not shown).
The various high-salt extracts were analysed on one-dimensional SDS/polyacrylamide gels (Fig. 2) showing that although some variation in the relative amounts of extracted proteins can occur, there is no striking qualitative difference between the patterns of extracted proteins or between the protein patterns of the remaining nuclear matrices. Only minor differences were observed between the protein patterns of the matrix obtained after treatment with (NH₄)₂SO₄ or NaCl. In particular, somewhat more of a protein that is probably identical to actin could be extracted using (NH₄)₂SO₄ (arrows in Fig. 2).

Histones were almost completely removed from DNA-depleted nuclei by ionic strengths of 0·9 and higher. From these data we have chosen a salt concentration in our extraction procedure of 0·4 M·(NH₄)₂SO₄.

Effects of nucleases

The effects of different nucleases were studied by incubating isolated nuclei either with DNase I (RNase-free) (Fig. 3A), DNase I/RNase A (Fig. 3B) or with micrococcal nuclease/RNase A mixtures (Fig. 3C). No striking differences between the protein patterns of the three types of nuclear matrix preparations could be found, except that RNase treatment seemed to reduce the amount of hnRNA-associated proteins in the nuclear matrix (compare Fig. 3A with B and C) and micrococcal nuclease treatment seemed to remove more of some unidentified polypeptides, as indicated by the open arrowheads in Fig. 3B.

Two-dimensional gel electrophoretic analysis of nuclear matrix proteins

The routine procedure that we use for the isolation of nuclear matrices on the basis of the foregoing experiments does not differ essentially from methods described earlier by van Eekelen et al. (1982) and Fey et al. (1984). In the procedure of van Eekelen et al. the Triton X-100 step was omitted while the digested nucleic acids and their associated proteins were extracted in two steps. Fey et al. (1984) used 0·25 M·(NH₄)₂SO₄ in the high-salt extraction instead of the 0·4 M·(NH₄)₂SO₄ as used by us.

Fig. 4 shows the one-dimensional SDS/polyacrylamide gel patterns and Fig. 5 the two-dimensional patterns of the different fractions obtained after the subsequent extraction steps. One of the major protein components that occurs only in the nuclear matrix fraction consists of a number of protein spots in the 65–72×10³ Mr region, with isoelectric points ranging between 8 and 8·5. This group of apparently closely related polypeptides is shown in detail in Fig. 6. The identity of this cluster, which consists of about 15–17 polypeptide spots, is unknown. Fig. 7 shows the electronmicroscopic appearance of a nuclear matrix isolated by the routine procedure.

Cytoskeletal proteins

Comparing all the protein patterns shown in Fig. 5, it is obvious that a 43×10³ Mr polypeptide, comigrating in both dimensions with actin from bovine lens (indicated as a) occurs in all fractions in relatively high amounts. Polypeptide spots comigrating
Immunochemical characterization of nuclear matrix proteins

With the intermediate filament proteins vimentin (indicated by v in Fig. 5) and the four HeLa cytokeratins (i.e. nos 7, 8, 18 and 19, indicated as such in the gels) were observed in all fractions except in the soluble fraction and in the DNase/RNase incubation supernatant. In the DNase/RNase extract, however, a small amount of a polypeptide comigrating with cyto keratin 8 can be detected. The polypeptide migrating in the vicinity of vimentin (asterix in Fig. 5A) may represent either a tubulin subunit or a vimentin breakdown product, which has a molecular weight slightly lower than vimentin and behaves in a slightly more acidic way on NEpHGE gels.

hnRNP

hnRNA-associated proteins (hnRNP) can be subdivided into three classes. The main components are represented by the A1, A2, B1a, B1b, B2, C1, C2, C3 and C3x polypeptides (Wilk et al. 1985), which are indicated as such in Fig. 5c,D,E.

As a result of DNase/RNase treatment, proteins A1, A2, B1a and B1b are partly released. After the subsequent high-salt treatment part of C1, B2 and probably B1c are released next to a remainder of A1, A2, B1a and B1b. By using comigration we were unable to indicate the positions of proteins C2, C3 and C3x in these fractions. However, as will be shown below, using immunoblotting we could show their presence in the nuclear matrix fraction.

Identification of nuclear matrix proteins by the immunoblotting method

Two-dimensional gels of [35S]methionine-labelled nuclear matrix preparations were blotted onto nitrocellulose sheets and used for the immunochemical detection and characterization of several nuclear proteins. All prominent protein spots, present on the autoradiographs of the gels, also occurred on the autoradiographs of such protein blots, indicating an optimal transfer of polypeptides in all regions of molecular weight and isoelectric point (not shown). Using several monoclonal antibodies as well as human autoantibodies directed to specific components of the cytoskeleton and the nucleus (see Table 1), on these [35S]methionine-labelled protein blots we were able to identify a number of proteins present specifically in the nuclear matrix (Fig. 8).

Monoclonal antibodies to the intermediate filament proteins vimentin and cytokeratin 18 clearly recognized these proteins in the blots (Fig. 8A). The immunofluorescence studies with these monoclonal antibodies and the rabbit antisera directed against keratin and vimentin stain a fibrillar network apparently surrounding the nuclear matrix (Fig. 8a,c).

The monoclonal antibody 41CC4 to rat liver lamins A, B and C (Burke, Tooze & Warren, 1983) detected, on two-dimensional blots, only lamins A and C of HeLa cells (Fig. 8D). For the detection of lamin B we used the monoclonal antibody LN43 (Fig. 8E). Immunofluorescence studies with these two lamin antibodies revealed a diffuse staining of the whole nuclear matrix structure with a higher fluorescence intensity at the matrix periphery (Fig. 8f).
The core proteins C₁ and C₂ of the 40S hnRNP particle (according to the nomenclature proposed by Beyer et al. 1977) were detected by monoclonal antibody 4F4 (Choi & Dreyfuss, 1984). From Fig. 8G it can be seen that both proteins are present over a relatively broad range of isoelectric points in our blots, probably due to nucleic acid remainders still in tight interaction with these hnRNP core polypeptides. It is likely that the C₁ ($39 \times 10^3 M_r$) and C₂ ($41 \times 10^3 M_r$) correspond to the C₃ and C₃X core proteins, respectively, described by Wilk et al. (1985). In the fluorescence pictures it can be seen that this antibody stains the nuclear matrix rather diffusely except for the nucleoli (Fig. 8H).

The monoclonal antibody 2-73 directed against the $70 \times 10^3 M_r$ protein of U1–RNP (Billings, Allen, Jensen & Hoch, 1982) reacts with three discrete protein spots (two major spots and one weaker spot) in the two-dimensional immunoblots. The three polypeptides migrate together within a small range of molecular weights and pH values (Fig. 8I). These proteins have been described to have a very slow rate of $[^{35}S]$methionine incorporation and can be detected only in Coomassie-Blue-stained gels (Billings & Hoch, 1984). Immunofluorescence shows a dot-like distribution of the $70 \times 10^3 M_r$ antigens in the nuclear matrix (Fig. 8J).

In addition to the mouse monoclonal antibodies, human autoimmune sera from patients suffering from connective tissue diseases were used for immunoblotting and immunofluorescence studies. The carefully selected sera were either directed against the internal fibrillar mass of the nuclear matrix (Fig. 8L) or against the residual nucleoli (Fig. 8N) and showed, on one-dimensional Western blots, a specific reaction with only one or two nuclear proteins.

The human autoimmune serum Z3, which was shown in one-dimensional blots to react with an antigen with an apparent molecular weight of $86 \times 10^3$ that has been suggested to occur specifically in the nuclear matrix (van Venrooij et al. 1985), reacts on a two-dimensional blot with an antigen having an isoelectric point of about 8.4 (Fig. 8K). Immunofluorescence studies with this serum on isolated nuclear matrix preparations revealed a diffuse staining reaction, with exclusion of the nucleolar remainder (Fig. 8L).

In the immunoblots the three nucleolar antibodies T5, J26 and T100 each reacted with different proteins, apparently specific for nucleoli (see, e.g., the reaction of T5 in Fig. 8M,N).

Fig. 9 summarizes all our immunoblotting data. The schematic drawing of the autoradiograph shows the typical nuclear matrix protein pattern and the identification of some components by immunoblotting. The indications used for the different protein spots correspond to the code used in Table 1 and permits a direct correlation between antiserum and proteins recognized on the immunoblots.

Fig. 2. Analysis of $[^{35}S]$methionine-labelled proteins in the high-salt extracts of HeLa nuclei and the corresponding remaining nuclear matrices. The proteins were extracted with: A, $(NH_4)_2SO_4$; or B, NaCl and analysed on a 13% SDS/polyacrylamide gel. Lanes 1 to 5 show the proteins extracted with salt solutions with ionic strengths of 0.3, 0.6, 0.9, 1.2 and 1.8, respectively. Lanes 1* to 5* show the corresponding proteins in the remaining structures after these salt treatments. The arrow indicates the position of actin.
Fig. 3

hnRNP proteins

Mr x $10^{-3}$
Immunochemical characterization of nuclear matrix proteins

Fig. 4. One-dimensional gel electrophoretic analysis of protein fractions of the extraction steps obtained during the isolation of nuclear matrices. The gel is a 13% SDS/polyacrylamide gel. Lanes A, Triton-soluble fraction of HeLa cells; B, DOC/Tween-soluble fraction; C, proteins released after DNase I/RNase A incubation; D, high-salt extractable fraction after nuclease treatment; and E, nuclear matrix preparation; M, molecular weight markers. [\(^{14}\text{C}\)]-methylated marker proteins (Amersham) used \(10^{-3}M\) were: lysozyme (14-3), carbonic anhydrase (30), ovalbumin (46), bovine serum albumin (69) and phosphorylase b (93).

Fig. 3. Two-dimensional gel analysis of nuclear matrix preparations treated with different nucleases. The following treatments were tested:

A. Digestion with DNase I only, under RNase-free conditions. For these experiments RNase-free DNase I was used (DPFF quality; Worthington Biochemical Corp.). The nuclei \((1\times10^8/\text{ml})\) were incubated for 15 min at 20°C in HRSB containing 800 \(\mu\text{g}\) ml\(^{-1}\) DNase I and 0.5 mM-PMSC.

B. Digestion with DNase I/RNase A. The nuclei \((1\times10^8/\text{ml})\) were incubated for 15 min at 20°C in HRSB containing 800 \(\mu\text{g}\) ml\(^{-1}\) DNase I (Sigma), 25 \(\mu\text{g}\) ml\(^{-1}\) RNase A (Sigma) and 0.5 mM-PMSC.

C. Digestion with micrococcal nuclease/RNase A. The nuclei \((1\times10^8/\text{ml})\) were incubated for 15 min at 10°C in RSB containing 200 U ml\(^{-1}\) micrococcal nuclease (P-L Biochemicals, Inc., Milwaukee, Wis.), 25 \(\mu\text{g}\) ml\(^{-1}\) RNase A (Sigma), 0.5 mM-PMSC and 1 mM-Ca\(^{2+}\). v, vimentin; a, actin; 7, 8 and 18, the different HeLa cytokeratin subunits.
DISCUSSION

In this study we have tried to identify proteins of the detergent-, nuclease-, and salt-resistant fraction of HeLa cells, usually referred to as the nuclear matrix (reviewed by Agutter & Richardson, 1980; Kaufmann & Shaper, 1984). The method that we now use routinely for the preparation of nuclear matrices has been developed from studies in which several conditions of nuclease and high-salt treatments were tested. The final procedure, however, is comparable to those described earlier by van Eckelen et al. (1982) and Fey et al. (1984).

Identification of nuclear matrix proteins in this study has been achieved mainly by a combination of two-dimensional gel electrophoresis and immunoblotting studies using mouse monoclonal antibodies and human autoimmune sera directed against nuclear and cytoskeletal components (Table 1). In this way several proteins present in nuclear matrix preparations could be identified.

Over the past years actin has been identified in many studies as a major protein in isolated nuclear fractions, but in most cases the possibility that actin was present as a cytoplasmic contamination could not be excluded (Comings & Harris, 1976; LeStourgeon, 1978). Yet, it has been demonstrated that manually isolated and cleaned nuclei of amphibian oocytes contain large amounts of actin (Clark & Rosenbaum, 1979; Krohne & Franke, 1980) and, recently, Scheer, Hinsen, Franke & Jockusch (1984) have shown that nuclear actin of amphibian oocytes might be involved in the transcription of lampbrush chromosomes.

Fig. 5. Two-dimensional gel electrophoretic analysis of fractions A to E from Fig. 4. A, Triton-soluble fraction of HeLa cells; B, DOC/Tween-soluble fraction; C, proteins released by DNase I/RNase A treatment; D, high-salt extractable fraction after nuclease treatment; and E, nuclear matrix preparation. A1, A2, B1a, B1b, B1c, and C1 indicate the hnRNA-associated core proteins according to the nomenclature of Wilk et al. (1985).
In nuclear matrix preparations from HeLa cells we find a major component of $43 \times 10^3 M_r$, which comigrates with bovine actin on two-dimensional gels. Our preliminary results with rhodamine-conjugated phalloidin applied to nuclear matrix preparations indicate that filamentous actin may be present in these preparations. However, the exact localization of the staining reaction cannot be determined on the basis of these light-microscopic studies.

Protein components, which make up the intermediate filament cytoskeleton in HeLa cells (vimentin and cytokeratins), could also be identified as major spots in nuclear matrix preparations in the two-dimensional gels and by immunoblotting. However, the immunofluorescence patterns indicate that these proteins occur as a network structure around the nucleus, which supports the idea that these intermediate filament proteins are firmly attached to nuclear matrix components as was suggested earlier (Woodcock, 1980; Granger & Lazarides, 1982; Peters, Okada & Comings, 1982; Capco et al. 1984; Fey et al. 1984). They are possibly involved in...
Immunochemical characterization of nuclear matrix proteins

the positioning of the nucleus (Virtanen, Kurkinen & Letho, 1979; Virtanen, Vartio & Letho, 1982). The major protein components occurring exclusively in the nuclear matrix fraction are represented by a cluster of basic polypeptides migrating in the $65-72 \times 10^3 M_r$ region. Also, Kaufmann & Shaper (1984) have found that the intranuclear material of their nuclear matrix preparations of rat liver cells contain a
Fig. 9. Autoradiograph and schematic representation of a two-dimensional separation of nuclear matrix proteins containing the immunoblotting data. Next to the components summarized in Table 1 actin (a) and cytokeratins 7 and 8 are also indicated. Note that the spot indicated as V* is only partly composed of vimentin breakdown product (cf. Fig. 8A).
Immunochemical characterization of nuclear matrix proteins

A series of basic (pI > 8.0) 60–70×10^3 Mr polypeptides, which are not recognized by anti-lamin antisera. It is likely that these proteins correspond to the cluster of polypeptides seen in our two-dimensional gels (Fig. 7). This unidentified set of proteins do not react with any of the antisera used in this study. The typical and extremely reproducible two-dimensional gel pattern of this protein cluster showing about 15–17 discrete spots suggests that some of these proteins are the result of post-translational modifications.

In this study we find that some of the hnRNA-associated core proteins are still found to be present in nuclear matrix preparations (Fig. 3). Dreyfuss, Choi & Adam (1984) showed that the 39×10^3 and 41×10^3 Mr proteins (C proteins) are removed quantitatively from the nuclear matrix at 0.5 M-NaCl after digestion with RNase. Using the same sequence of extraction steps we were, however, unable to remove these C proteins completely from the nuclear matrix.

Small nuclear RNAs (snRNAs) have been described to be integral components of hnRNP particles (Busch, Reddy, Rothblum & Choi, 1982). One of these snRNAs, i.e. U1-RNA, is known to be involved in pre-mRNA splicing (Krämer, Keller, Appel & Lührmann, 1984) and its associated proteins are found in the nuclear matrix fraction. It has recently been suggested that the 70×10^3 Mr snRNP, recognized by the monoclonal antibody 2-73 used in this study, might be involved in binding of U1-RNP to the nuclear matrix, since it is not released by incubation with RNase or DNase of nuclei or nuclear matrices, as are the other U1-RNA-associated proteins (Mariman & van Venrooij, 1985). This could indicate that the 70×10^3 Mr protein interacts directly with components of the nuclear matrix or is an integral part of it. Our immunofluorescence and immunoblotting data support this assumption. The monoclonal anti-70×10^3 Mr serum shows a dot-like distribution of the 70×10^3 Mr antigens in the nuclear matrix. Also, in immunoblots of the matrix preparations we could demonstrate the presence of this 70×10^3 Mr polypeptide, which, however, was not found in the autoradiographs. This can be explained by the finding of Billings & Hoch (1984) that this polypeptide has a very slow rate of incorporation of [35S]methionine, and can be detected only in Coomassie-Blue-stained gels.

In summary, we point out that a combination of two-dimensional gel electrophoretic and immunoblotting techniques with well-defined antisera permits the characterization of nuclear matrix components. These protein constituents of the intranuclear mass may be difficult to study by other techniques because of their highly insoluble character. Future studies using immunoelectron microscopy, in combination with antisera such as described here, may provide valuable information about the localization and interrelationship of nuclear matrix proteins.

This study was supported by the Netherlands Cancer Foundation (Queen Wilhelmina Fund) grant no. NUKC 1984-11. The authors thank Dr G. Warren (Heidelberg) for his gift of the hybrid cell line 41CC4, Dr S. Hoch (La Jolla, Ca) for her gift of the hybrid cell line 2-73, Dr G. Dreyfuss (Evanston) for his gift of the ascites fluid 4F4, Dr K. Schäfer (Bochum) for providing the core proteins of 3S hnRNP complexes, Dr E. B. Lane (London) for the monoclonal antibody LN43 and Dr R. Humbel (Luxembourg) for the human serum T100. Furthermore, we thank...
A. Groeneveld for culturing the cells, Dr P. Sillekens for preparing the sample for electron microscopy, and G. Schaart and A. Huysmans for their excellent help in testing the intermediate filament antisera. Mrs Y. Stammes is thanked for her excellent secretarial help in preparing the manuscript.

REFERENCES


(Received 21 June 1985 – Accepted 4 July 1985)