

Preparation of Nuclear Matrices from Cultured Cells: Subfractionation of Nuclei In Situ

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ABSTRACT Analyses of the different structural systems of the nucleus and the proteins associated with them pose many problems. Because these systems are largely overlapping, in situ localization studies that preserve the in vivo location of proteins and cellular structures often are not satisfactory. In contrast, biochemical cell fractionation may provide artifactual results due to cross-contamination of extracts and structures. To overcome these problems, we have developed a method that combines biochemical cell fractionation and in situ localization and leads to the preparation of a residual cellular skeleton (nuclear matrix and cytoskeletal elements) from cultured cells. This method's main feature is that cell fractionation is performed in situ. Therefore, structures not solubilized in a particular extraction step remain attached to the substrate and retain their morphology. Before and after each extraction step they can be analyzed for the presence and location of the protein under study by using immunological or cytochemical techniques. Thereby the in vivo origin of a protein solubilized in a particular extraction step is determined. The solubilized protein then may be further characterized biochemically. In addition, to allow analyses of proteins associated with the residual cellular skeleton, we have developed conditions for its solubilization that do not interfere with enzymatic and immunological studies.

Eucaryotic nuclei contain non-chromatin structural systems that can be prepared from isolated nuclei by extraction of the DNA, RNA, and most of the proteins (1–21). The proteinaceous residual structures obtained are insoluble in buffers containing nondenaturing detergents and of both high and low ionic strength. These structures are composed of a peripheral lamina with associated residual pore complexes (pore complex lamina) (1, 22), and, depending on the method of isolation, they may also contain residual nucleoli and additional intranuclear material (23–27). Together, these three substructures form the nuclear matrix (4, 5; for review, see Berezney [28]). It has been postulated that the nuclear matrix, aside from being of structural importance (for references, see above), is involved in many biological functions, such as DNA replication (29–38), RNA synthesis, and RNA processing (39–45). A regulatory role for the nuclear matrix is suggested by its association with hormone receptors (3, 46) and viral tumor antigens (31, 47–49), which act as pleiotropic regulator molecules (50). In addition, virus maturation seems to occur at this structure (11, 31, 51, 52). However, one has to be aware that many of the biological functions ascribed to

the nuclear matrix (for reviews, see Berezney [28] and Hancock [53]) may involve additional nuclear constituents (e.g., the chromatin). Consequently, functional studies can not be restricted to this structure alone; a role for other nuclear components has to be considered, too.

A promising approach to the analysis of functions of nuclear structures is to study the proteins that either take part in or interfere with nuclear processes. Knowledge of the exact subnuclear location of such a protein gives hints as to the function of the structure with which it is associated. However, the subnuclear location of a protein is difficult to determine in unfractionated cells, in that different nuclear constituents are located closely together and obscure one another. Therefore, cells and nuclei need to be fractionated. For nuclear fractionation and for the preparation of nuclear matrices, cells grown in tissues have been used widely, because they can be obtained easily in large amounts (for examples, see references 1, 3–6, 8, 9, 12, 13, 16, 18, 22, 25, 26). In addition, nuclear matrices isolated from cells of solid tissues, such as liver are virtually free of contamination with cytoplasmic intermediate filaments (see references above and our earlier report [54]).

However, functional analyses are greatly facilitated if the cells can be manipulated readily before fractionation by, e.g., radioactive labeling, drug treatment, synchronization, or viral infection. For this purpose, cultured cells offer considerable advantages over cells grown in tissues. Yet we recently found that nuclei and nuclear matrices isolated from cultured cells contain cytoplasmic intermediate filaments as their major proteins (54, 55); this would complicate studies using nuclear matrices from cultured cells. The elaborate filament systems of the cells (Fig. 1a) collapse onto isolated nuclei (55) and during further extraction form an aggregate with the nuclear matrix (Fig. 1b), as is shown here for vimentin. A discrimination between cytoplasmic filaments and the nuclear matrix and the proteins tightly associated with these structures then no longer is possible. The origin of proteins in the nuclear matrix fraction, therefore, cannot be determined with certainty. Problems of cross-contamination, however, are inherent in all cellular fractions. For example, the detergent extraction employed to lyse cells solubilizes cytoplasmic, nucleoplasmic, and membraneous material together. Similarly, other biochemical extracts contain various cellular constituents.

In general, biochemical cell fractionation procedures hardly yield homogeneous biological structures, because the molecules are not extracted by biological criteria but according to their solubility properties. Consequently, the *in vivo* location of a protein cannot be defined with certainty by analyzing the

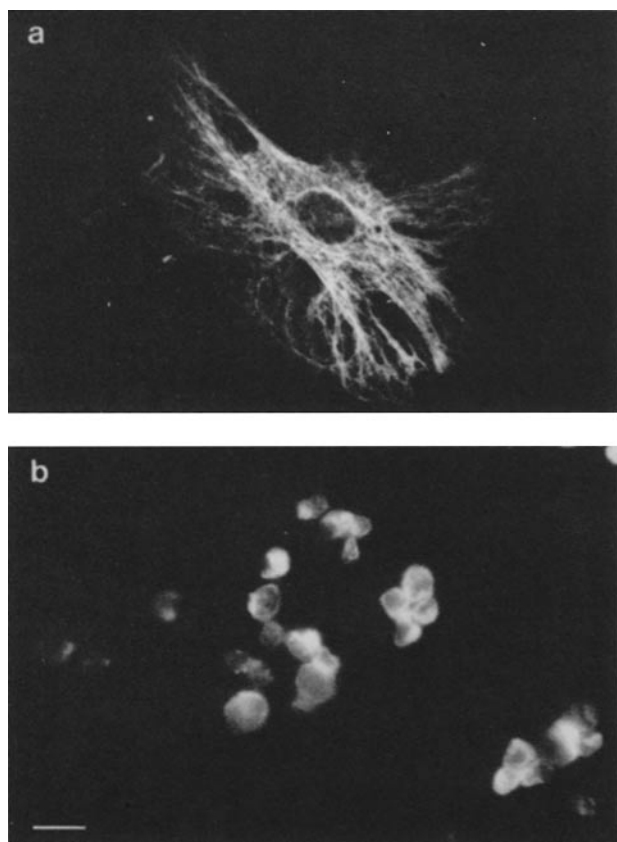


FIGURE 1 Vimentin filaments in cultured cells and nuclear matrix fractions. Vimentin is visualized by immunofluorescence using affinity purified guinea pig vimentin antibodies (55). (a) Unfractionated mouse fibroblasts (3T3) cultured on a coverslip and fixed. (b) "Nuclear matrices" isolated in suspension from mouse fibroblasts (3T3) as described (54), settled onto a coverslip, and fixed. Bar, 20 μ m. \times 350.

extracts alone. A complementary means is necessary to determine the location and possible place of function of a protein. This led us to develop an *in situ* cell fractionation procedure which allows the comparison of cells and structures before and after each extraction step. Then a correlation can be made between a protein in a particular extract and its association with a certain subcellular structure which reflects its *in vivo* location. This is especially important for proteins found in several subcellular locations as is the case with many nonstructural and regulatory proteins, e.g., viral tumor antigens or oncogene products (31, 49, 56). Until recently, to solubilize the proteins associated with the nuclear matrix, strong denaturing detergents such as sodium dodecyl sulfate have been used that impede further biochemical analyses. Our procedure allows solubilization of nuclear matrix proteins by the use of the zwitterionic detergent Empigen BB under conditions that are relatively mild in that they retain enzyme activities (57) and permit immunological analyses (49, 58).

MATERIALS AND METHODS

Cell Culture and Radioactive Labeling: Cell lines of the following origin were used: HeLa/human cervix carcinoma; 3T3/mouse Balb/c fibroblasts; TC7/African green monkey kidney. They were grown on petri culture dishes in Dulbecco's modified Eagle's medium, Boehringer, Mannheim, Federal Republic of Germany; No. 210048). For phase-contrast and immunofluorescence analyses, glass coverslips (ϕ 12 mm), which had been washed with ethanol and sterilized, were included in the culture. For electron microscopic analyses, cells were grown on polyester foils in test chambers (Bachofer, Reutlingen, Federal Republic of Germany; No. TCSC-1).

For radioactive labeling of the DNA, RNA, and phospholipids, 300 μ Ci of [3 H]thymidine (20 Ci/mmol), [3 H]uridine (27 Ci/mmol), or methyl- [3 H]choline chloride (60 Ci/mmol) was added to 5 ml of the culture medium (Dulbecco's). Proteins were labeled with 50 μ Ci 14 C-protein hydrolysate (56 mCi/mg atom) in 5 ml of culture medium (Dulbecco's) containing only 20% of the amino acids. Isotopes were obtained from Amersham-Buchler (Braunschweig, Federal Republic of Germany). Confluent and subconfluent cell monolayers on plates (ϕ 5 cm) were labeled for 4 or 16 h. No significant differences in the results were observed.

Cell Fractionation: Cells on plates either grown to confluency or subconfluent monolayers were washed three times with Kern-matrix buffer (KM buffer): 10 mM *N*-morpholinoethanesulfonic acid, pH 6.2; 10 mM NaCl; 1.5 mM MgCl₂; 10% glycerol; 30 μ g aprotinin (200 kIU; Trasylol, Bayer, Leverkusen, Federal Republic of Germany). For the first extraction step, KM buffer containing 1% nonidet P40 (NP40), 1 mM ethyleneglycol-*bis*-(β -Aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), and 5 mM dithiothreitol (DTT) was used. 2 ml was added per plate (ϕ 9 cm), incubated for 3 min on ice, and removed. Then another 4 ml was added and incubated on ice for 27 min. Immediately after each incubation, phenylmethylsulfonyl fluoride (1 mM) was added and the extracts were frozen. They were combined later to give the first extract. This was necessary to prevent degradation by lysosomal enzymes. To all other extracts phenylmethylsulfonyl fluoride was added; the extracts were then frozen immediately. After the first extraction, structures on plates were washed three times with KM buffer and incubated for 15 min at 37°C with 50 μ g/ml of deoxyribonuclease I (DNase I) (Sigma Chemical Co., München, Federal Republic of Germany, No. D-5010) in KM buffer (2 ml/plate; ϕ 9 cm). After removal of this extract, KM buffer containing 2 M NaCl, 1 mM EGTA, and 5 mM DTT was added and incubation was for 30 min in the cold. Then structures were washed three times with KM buffer and incubated for 30 min at 37°C with 50 μ g/ml each of DNase I and ribonuclease A (RNase A) (Sigma, No. R-5500) in KM buffer (3 ml/plate; ϕ 9 cm). The structures prepared were washed three times with KM buffer. In some preparations, DTT and EGTA were omitted and the buffers were saturated with disulfiram (Sigma Chemical Co., No. T-1135) instead.

Microscopy: For microscopic analyses, all structures were washed three times with phosphate-buffered saline (PBS) (140 mM NaCl; 3 mM KCl; 8 mM Na₂HPO₄; 1.5 mM KH₂PO₄; pH 7.4). The phase-contrast image was viewed

¹ *Abbreviations used in this paper:* DNase, deoxyribonuclease; DTT, dithiothreitol; KM buffer, Kern-matrix buffer; NP40, Nonidet P40; PBS, phosphate-buffered saline; RNase, ribonuclease; TK buffer, Tris/KCl buffer.

without fixation in a Zeiss photomicroscope III. For immunofluorescence analyses, structures were either fixed in methanol/acetone or kept unfixed. The immunofluorescence procedure and the antibodies have already been described (54, 55). Staining of the DNA was performed using a saturated solution of the intercalating dye 4,6-diamidino-2-phenylindol (Serva, Heidelberg, Federal Republic of Germany) in PBS or PBS containing 20% dimethylsulfoxide. The stain ethidiumbromide (Serva) was used at a concentration of 20 $\mu\text{g}/\text{ml}$ or 200 $\mu\text{g}/\text{ml}$ in PBS. For electron microscopic analyses, structures attached to polyester foils were fixed in the test chamber for 2 h at room temperature with 3.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, VT; No. 20100) in PBS. They were washed with PBS, postfixed, and stained with 2% OsO_4 in PBS for 1 h at room temperature. After washing they were dehydrated through a series of ethanol steps, stained with uranyl acetate (saturated solution in ethanol) for 30 min at room temperature and embedded in Epon. After removal from the test chambers, the polyester foils were stripped off the Epon blocks, leaving the structures intact. Thin sections (600–800Å) were cut, stained with lead citrate (1 mg/ml H_2O) for 1 min, and viewed in a Philips EM 301 electron microscope.

Solubilization of Nuclear Matrices: Residual cellular skeletons were prepared in situ as described above. For solubilization a plate (ϕ 9 cm) was incubated for 60 min at 0°C with 5 ml of TK buffer (50 mM Tris, pH 9.0; 25 mM KCl; 10% glycerol; 30 μg aprotinin) containing 5 mM DTT, 1 mM EGTA, and 0.5, 1, 2, or 3% Empigen BB (Albright and Wilson, Frankfurt, Federal Republic of Germany). From these extracts proteins could be immunoprecipitated directly. However, to ensure quantitative immunoprecipitation they were diluted to 0.5% Empigen BB with TK buffer containing 1% NP40. Immunoprecipitation and SDS PAGE were as described (55).

RESULTS

Strategy of the In Situ Fractionation

Our aim was to avoid an alteration in the morphology of unextracted cellular constituents during fractionation, especially a collapsing of intermediate and other filament systems onto nuclear structures. This can be achieved best by leaving the structures attached to the substratum. In situ cytoskeletal preparations previously have been obtained this way using more or less isotonic buffers of about neutral pH containing nonionic detergents (59–61). But if these structures are incubated for longer periods of time or extracted further using the relatively severe conditions of the nuclear matrix preparation, they usually detach from the substratum. This can be avoided if the first extraction is performed at a lower pH and at low ionic strength (KM buffer). Then during the following extractions, structures will not detach from the substratum even if rounded cells (e.g., virus-infected, transformed, drug-treated, or mitotic cells) are used. Fractionation at a relatively low pH also ensures that DNA-binding proteins are not solubilized in the first extraction. Except for these restrictions in the first step, considerable variations in the fractionation procedure are possible. In our fractionation scheme, a DNase I digestion (50 $\mu\text{g}/\text{ml}$) is performed as the second step to avoid disruption of nuclear structures by the unfolding chromatin. This otherwise may occur when subsequently histones are released from the DNA with high salt (2 M NaCl). In addition, this digestion helps to keep the structures attached to the substratum during histone extraction, although it is not absolutely required for this purpose. The extraction conditions here are kept virtually identical to the conditions described for nuclear matrix preparations in suspension (54), but because centrifugation is not necessary, the in situ fractionation is much faster and gives a higher yield and a better preservation of structures. A detailed description of the method is given in Materials and Methods.

Morphological Changes during Course of Fractionation

Our fractionation scheme consists of four consecutive ex-

traction steps: (a) nonionic detergent/low salt; (b) DNase I/low salt; (c) high salt; and (d) DNase I/RNase A/low salt. Phase-contrast micrographs of unfixed human HeLa cells and of structures derived from them after each step are shown in Fig. 2, a, c, e, g, and i. For electron microscopic analysis of cells and structures, fixation, embedding, and sectioning have been performed in situ as described in Materials and Methods. This ensures that an optimally preserved monolayer is investigated (Fig. 2, b, d, f, h, and j). In addition, serial sections are possible starting from a defined side of the structures (the side of attachment to the substratum).

The main extraction of cytoplasmic constituents occurs during the first fractionation step, leaving behind a cytoskeletal framework (61) with associated polysomes (62). In addition, extracted nuclear structures lacking membranes and some internal material (nucleoplasm) remain attached to the substratum (Fig. 2, c and d). After DNase I digestion the nucleoli and the cytoskeleton are largely unchanged (Fig. 2, e and f). In phase contrast the intranuclear material is still visible but appears somewhat more granular than before the digestion (Fig. 2, e). Surprisingly, however, when analyzed in the electron microscope, most of the intranuclear material no longer is visible; only some fibrillogranular structures are seen (Fig. 2f). Except for the appearance of the nucleoli, the nuclear structure already is similar to the nuclear matrix (Fig. 2j). This is in obvious contradiction not only to the phase-contrast images of the same structures but also to biochemical analyses that show that virtually no protein is extracted during this digestion step (see below and Discussion). The subsequent high salt extraction solubilizes part of the cytoskeleton with the remaining polysomes (Fig. 2, g and h). The nucleoli expand significantly as is already seen with high-magnification phase-contrast optics. Although no additional changes are visible in the electron microscope (Fig. 2h), a considerable decrease in intranuclear material is apparent when the structures are analyzed by phase-contrast microscopy (Fig. 2g). The final DNase I/RNase A extraction only changes the structure of the nucleolar spheres giving rise to small and compact residual nucleoli. The preparation yields structures that represent an extended, remnant skeleton of the cell designated here as residual cellular skeleton (Fig. 2, i and j). It is composed of some cytoplasmic filaments (residual cytoskeleton) that surround the extracted nuclear skeleton (nuclear matrix). The nuclear matrix is bounded by a residual nuclear lamina. Under the conditions of preparation, i.e., in the presence of DTT/EGTA, the interior appears to be almost empty with the exception of occasionally visible residual nucleoli. A similar morphology was obtained by Kaufmann et al. (26) after preparation of nuclear matrices from rat liver in the presence of reducing agents. However, after using oxidizing conditions (e.g., disulfiram) during extraction, a complex system of granules and fibers was observed inside the nuclear matrix (26). Structures equivalent in morphology were also prepared with our method under these conditions, (data not shown). Fig. 2, i and j, demonstrates that single unbroken structures are obtained, in that centrifugation and other mechanical forces are avoided. No significant shrinking or collapsing of nuclear or cytoplasmic residual structures is observed; in particular, nuclear morphology and size are well preserved. The expanded state of the structures ensures an optimal extraction.

Immunofluorescence analyses of the structures confirm that all the different systems of the residual cytoskeleton still

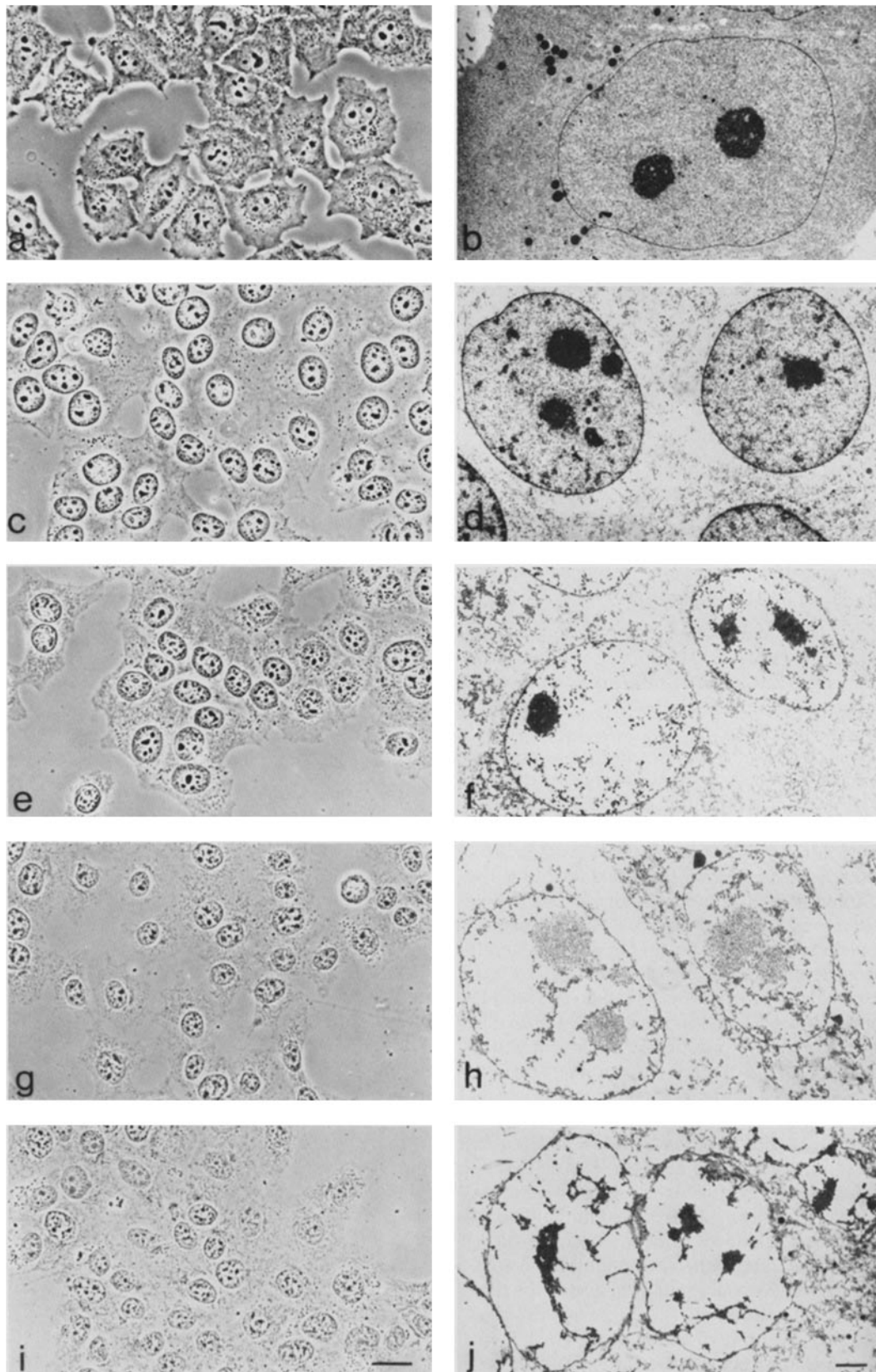


FIGURE 2 Morphology of cells and structures obtained during fractionation. HeLa cells grown on coverslips (for phase-contrast microscopy) or on plastic foils (for electron microscopy) were fractionated and processed for microscopic analyses as described in Materials and Methods. Micrographs of the structures obtained after each extraction step are shown. (a and b) Cells. (c and d) NP40-extracted structures. (e and f) DNase I-digested structures. (g and h) High salt-extracted structures. (i and j) DNase I/RNase A-digested structures (residual cellular skeletons). (a, c, e, g, and i) Phase-contrast images: bar, 20 μm ; $\times 325$. (b, d, f, h, and j) Electron microscopic images: bar, 2 μm ; $\times 2,500$.

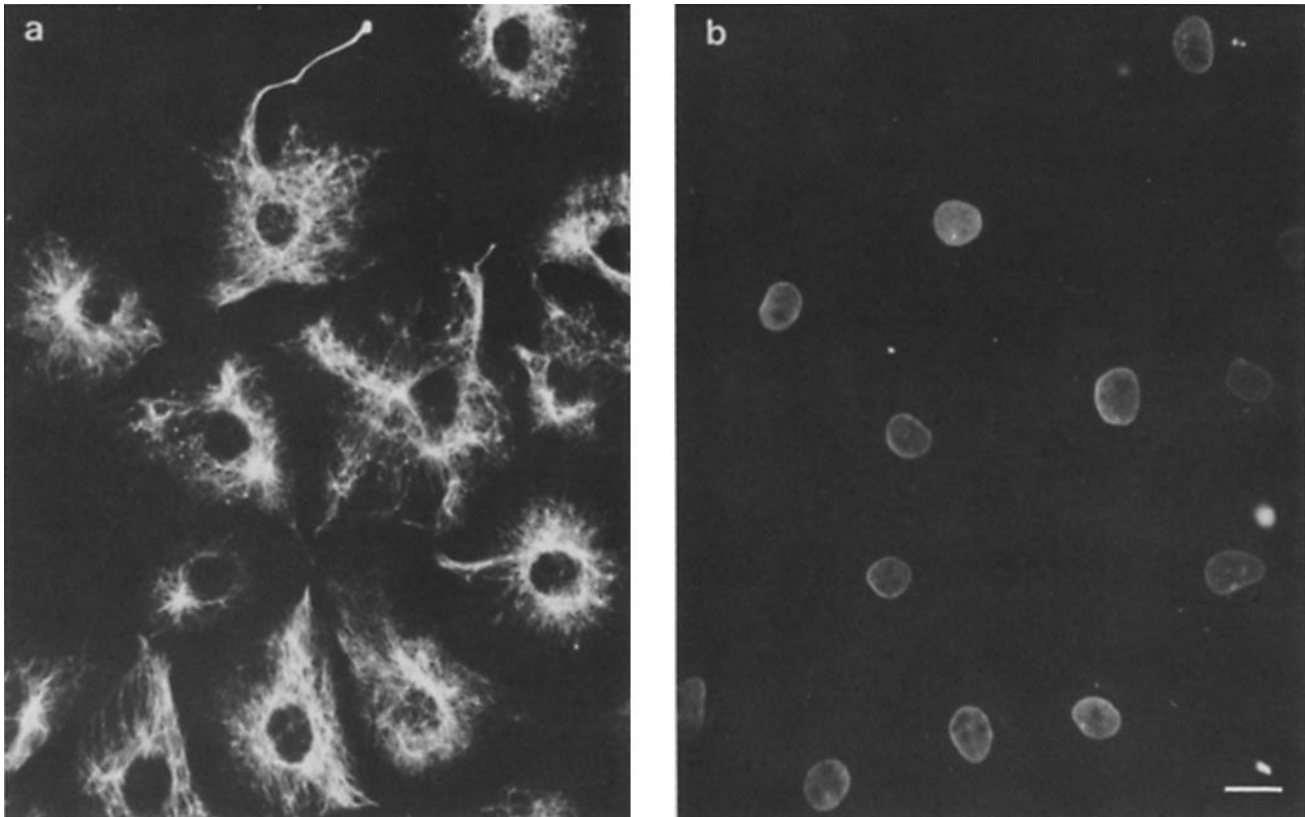


FIGURE 3 Display of vimentin filaments and nuclear lamina in residual cellular skeletons. Residual cellular skeletons were prepared from monkey kidney TC7 cells grown on coverslips as described in Materials and Methods. The unfixed structures were stained for double immunofluorescence microscopy using affinity purified vimentin (a) and lamin B (b) antibodies (for antibodies see references 54, 55). The same structures are shown in a and b. Bar, 20 μm . $\times 400$.

display the same morphology as in unfractionated cells. The intermediate filaments are stained as brightly as in whole cells. Some stress fibers are left, whereas microtubules cannot be detected using immunofluorescence. Fig. 3 shows a double immunofluorescence labeling of the residual cellular skeleton prepared in situ from monkey kidney TC7 cells. As an example, the extended vimentin filaments are stained (Fig. 3 a) and the peripheral part of the nuclear matrix, the residual lamina, is visualized using lamin antibodies (Fig. 3 b). The staining of these structures is indistinguishable from that of unfractionated cells. Therefore, a clear differentiation between the residual cytoskeleton and the nuclear matrix is already possible at the level of the light microscope. With these well-spread structures, nonspecific fluorescence is low. In addition, it usually can be distinguished from a specific reaction, because different locations within the structure are still discernible (compare Fig. 1 b with Fig. 3). Similar immunofluorescence analyses are possible with all structures obtained during the different steps of the fractionation procedure. It should be noted that fixation and permeabilization of these structures are not necessary.

Extraction of Lipids, DNA, RNA, and Protein during Fractionation

To follow the extraction of biological macromolecules during fractionation, HeLa cells were labeled with [^3H]thymidine, [^3H]uridine, methyl- ^3H]choline chloride, and a ^{14}C -protein hydrolysate, respectively. They were fractionated in situ and the percentages of radioactivity in the different extracts and

the residual cellular skeleton were determined (Table I). No significant differences were obtained when cells were labeled for 4 or 16 h. The cellular phospholipids are extracted quantitatively in the first fractionation step. Over 99% of the DNA is solubilized mainly by the action of DNase I and by the subsequent high salt treatment. Only a very small amount of DNA remains associated with the nuclear matrix. Extraction of the cellular RNA occurs during all steps of fractionation. The largest amount, however, is released during high salt treatment. About 10% of RNA is still associated tightly with the nuclear matrix after DNase I/high salt treatment. The majority of this RNA then is removed by the subsequent RNase A digestion. Only a small fraction remains associated with the nuclear matrix. Protein is extracted mainly during the NP40/low salt extraction step and by high salt treatment. The first DNase I digestion solubilizes very little protein. As has been reported (54), almost no protein is removed by the DNase I/RNase A digestion. The residual cellular skeleton makes up $\sim 10\%$ of the cellular protein. This value is higher than reported for rat liver nuclear matrix fractions (5). The difference obviously is caused by the associated residual cytoskeleton that is missing in the liver nuclear matrix fraction (54). We have previously estimated that the intermediate filament polypeptides make up $\sim 20\text{--}25\%$ of the protein in this fraction, whereas the major nuclear matrix polypeptides, the lamins, account for only 10–15% (reference 54 and Fig. 5, g and h).

An important characteristic of a cell fractionation procedure is that during a particular extraction step distinct classes of molecules are quantitatively removed. Additional material

TABLE I
Extraction of Phospholipids, DNA, RNA, and Protein during the Different Fractionation Steps

	NP40/low ionic strength	DNase I/low ionic strength	High ionic strength % of total radioactivity	DNase I/RNase A low ionic strength	Nuclear matrix/residual cytoskeleton
Phospholipids	>99 (ND)	<0.1 (—)	<0.1 (ND)	<0.1 (ND)	<0.1 (—)
DNA	2.2 (2.1;0.1;<0.1)	54.4 (—)	43.2 (38.5;4.3;0.4)	0.2 (0.16;<0.1;<0.1)	<0.1 (—)
RNA	25.8 (23.7;1.8;0.3)	9.8 (—)	55.7 (48.7;5.9;1.1)	8.4 (7.5;0.8;0.1)	0.3 (—)
Protein	56.6 (50.4;4.9;1.3)	3.8 (—)	29.2 (25.4;3.2;0.6)	0.6 (0.4;0.1;0.1)	9.8 (—)

Phospholipids, DNA, RNA, and proteins of HeLa cells were labeled for 16 h as described in the text. Cells were fractionated and the percentage of radioactivity in each fraction was determined. The upper values give the percentage of material extracted in each step (sum of values in parentheses). The lower values in parentheses give the percentage of material solubilized by each of the repeated extractions. ND: not determined.

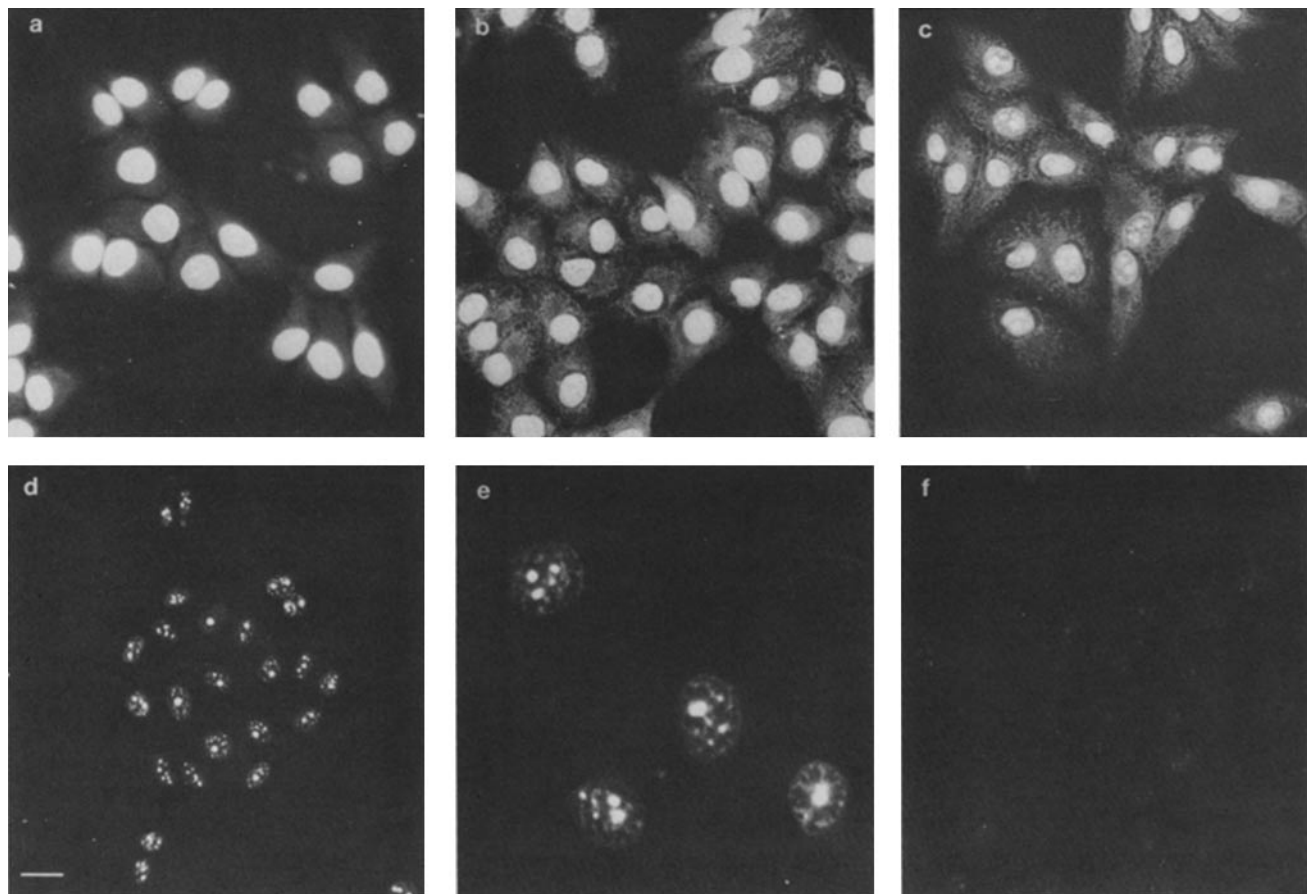


FIGURE 4 Visualization of nucleic acids in cells and structures obtained during fractionation. HeLa cells were grown on coverslips, fractionated, fixed, and stained with ethidium bromide as described in Materials and Methods. (a) Cells. (b) NP40-extracted structures. (c) DNase I-digested structures. (d and e) High salt-extracted structures. (f) DNase I/RNase A-extracted structures (residual cellular skeletons). (a-d and f) Bar, 20 μ m; \times 350. (e) Bar, 8 μ m; \times 750.

should be extracted only when different extraction conditions are employed. Therefore, we have repeated the detergent, high salt, and DNase I/RNase A extraction steps three times and determined the percentage of radioactivity in each extract. Table I shows that only trace amounts of the different biological macromolecules are released in the reextraction steps. We, therefore, conclude that the extractions are exhaustive

and that distinct classes of molecules are solubilized in each step.

The extraction of nucleic acids was also followed morphologically after the staining of cells and structures with ethidium bromide which reacts with both DNA and RNA (Fig. 4). In the cytoplasm cells are stained brightly (Fig. 4a), while this staining is slightly weaker after detergent extraction (Fig. 4b).

It is more structured due to the extraction of RNA not bound to the cytoskeletal framework (62). During DNase I digestion, the cytoplasmic fluorescence does not change (Fig. 4*c*), but it is completely absent after high salt treatment (Fig. 4*d*) suggesting that a large part of cytoplasmic RNA is extracted in this step. The specificity of the cytoplasmic staining for RNA is demonstrated by the fact that it is eliminated by RNase A treatment (data not shown). The nucleus of unfractionated cells is stained strongly with the nucleoli being most prominent (Fig. 4*a*). Detergent extraction results in a slightly weaker staining (Fig. 4*b*), probably reflecting the extraction of some nuclear RNA. After DNase I digestion, the morphology and the intensity of the nuclear fluorescence have changed drastically (Fig. 4*c*). Staining is associated with only the nucleoli, the nuclear lamina, and some material inside the nucleus. A large amount of the intranuclear DNA has been removed. After high salt treatment, the nuclear lamina, the nucleoli, and little internal material are still weakly stained (Fig. 4, *d* and *e*). This fluorescent material corresponds to the intranuclear structures seen in phase-contrast microscopy (Fig. 2*g*). It is removed by RNase A or DNase I/RNase A (Fig. 4*f*) but not by DNase I alone (data not shown) and, therefore, represents RNA. The intranuclear structures, however, remain visible in the phase-contrast microscope. These interpretations were substantiated using the DNA-specific dye 4,6-diamidino-2-phenylindol (data not shown). A bright nuclear staining but only a weak nucleolar fluorescence were visible with cells and detergent-extracted structures. DNase I treatment reduced the fluorescent staining drastically. The same structures were stained as with ethidium bromide. This fluorescence, however, was gone after high salt extraction.

The polypeptide patterns of extracts and structures prepared in situ from HeLa cells are shown in Fig. 5. Major bands of the residual cellular skeleton (Fig. 5*g*) are the lamins A, B,

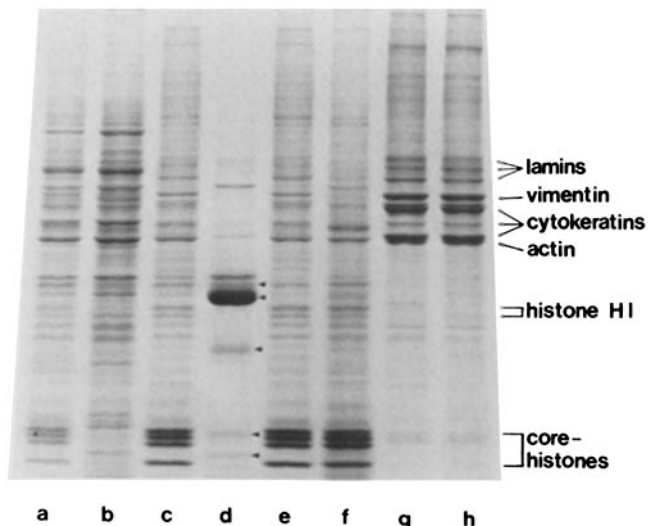


FIGURE 5 SDS polyacrylamide gel analysis of extracts and structures obtained during fractionation. HeLa cells were fractionated in situ (a-g) or in suspension (h) and the polypeptides from extracts and structures were separated on a 8.5–15% hyperbolic gradient gel. Sample order is the following: (a) Homogenate. (b) NP40 extract. (c) NP40-extracted structures. (d) DNase I extract; polypeptides labeled with arrowheads belong to the DNase I preparation added. (e) DNase I-digested structures (f) High salt extract. (g) Residual cellular skeleton prepared in situ. (h) Residual cellular skeleton prepared in suspension as described (54).

and C (72, 68, and 62 kilodaltons [kD]) and the intermediate filament polypeptides (vimentin, 57 kD; cytokeratin I, 52 kD; cytokeratin II, 44 kD) (54). These bands are visible already in the nuclear (Fig. 5*c*) and the DNase I-treated nuclear fraction (Fig. 5*e*). The lower 42-kD polypeptide present in all fractions represents actin. No significant differences in the corresponding polypeptide patterns of a fractionation performed in suspension (54) can be seen; in particular, the residual cellular skeletons prepared in situ are virtually identical to those prepared in suspension (cf. Fig. 5, *g* and *h*). Histones are extracted almost completely and exclusively by the high salt treatment (Fig. 5, *f* and *g*). This is somewhat astonishing, in that a large part of DNA is already removed by DNase I digestion (Table I). We, therefore, have tested how much DNA can be extracted from nuclei with DNase I and whether histones are removed at some stage. Repeating the DNase I digestion six times resulted in removal of >90% of the DNA. Yet, at that point still no histones were extracted (data not shown). DNase I treatment, however, specifically releases some polypeptides of unknown identity which are not present in the DNase I preparation added (Fig. 5*d*).

Solubilization of Nuclear Matrices

The nuclear matrix resists extraction by high and low salt concentrations and by nonionic detergents and can be solubilized only after strong denaturation using the detergent SDS (5) or high concentrations of urea (63). When testing the solubility of the nuclear matrix under various conditions, we found the zwitterionic detergent Empigen BB to be most effective. Optimal conditions for solubilization require sufficient reduction during nuclear matrix preparation. A relatively high pH, as well as omission of divalent cations during solubilization with Empigen BB are advantageous (TK buffer). Under these conditions, Empigen BB concentrations >1% extensively solubilize the residual cellular skeleton by the following criteria: (a) Solubilized proteins remain in solution after centrifugation at 100,000 *g*. (b) The polypeptide patterns of the residual cellular skeleton and of the Empigen BB extracts are indistinguishable (data not shown). (c) Structures are no longer visible in phase-contrast and in electron microscopy (even after staining with phosphotungstic acid or tannic acid) (data not shown). Because enzyme activities are well retained (57), Empigen BB does not seem to denature proteins during solubilization. Furthermore, proteins can be immunoprecipitated readily from Empigen BB extracts (49, 58). We, therefore, conclude that Empigen BB extracts of the residual cellular skeleton largely contain the nuclear matrix components solubilized in a state that allows biochemical and immunological analyses.

DISCUSSION

Knowing the exact intracellular location of a protein is often a prerequisite for understanding its function in the cell. In principle, two different approaches to the determination of the location of a protein are employed: (a) in situ studies using immunological or cytochemical methods or (b) biochemical cell fractionation. Both approaches have inherent advantages and drawbacks that limit their use. Although under appropriate fixation conditions, the in vivo location of a protein is largely preserved during in situ studies, a clear assignment of an association with a cellular structure often is not possible, because overlapping or closely aligned structures

are not resolved. In addition, quantitation and further biochemical characterization of the protein under study is not possible. On the other hand, the separation of cellular structures sought during biochemical cell fractionation often creates artifacts due to cross-contamination of the extracts and structures thus prepared. These problems are especially intriguing in analyzing the different structural systems of the nucleus, because they are largely overlapping and, therefore, *in situ* localization studies are not satisfactory. In addition, these systems cannot be easily prepared by biochemical cell fractionation, because, for example, cytoplasmic intermediate filament systems collapse onto nuclei and co-purify with nuclear matrices (54, 55), whereas nuclear membranes and nucleoplasmic constituents are extracted together with the cytoplasm during cell lysis. We have therefore tried to combine the advantages of both approaches by developing a method for the *in situ* preparation of nuclear matrices; in our method structures not solubilized in a particular extraction step remain attached to the substrate. The localization of unsolubilized cellular components during sequential extractions is not altered as is shown here for the residual cytoskeleton and for the nuclear lamina. This method, therefore, offers the advantage that expanded structures preserved in their morphology can be analyzed before and after each extraction step for the presence and location of the protein under study by using immunological and cytochemical methods. This *in situ* analysis of biochemically fractionated cells thereby allows a determination of the *in vivo* origin of a protein solubilized at any step of the preparation.

Cytoskeletal preparations preserving the *in vivo* morphology of cytoskeletal systems have been described previously (59–61). However, upon further subfractionation these structures detach from the substrate. This is especially true for the rather severe conditions employed during nuclear matrix preparation. Attachment of structures under these conditions is largely dependent on the first extraction step. If this step is performed at low ionic strength and at relatively low pH, attachment is stabilized for some reason(s) unknown to us. Considerable variations can then be introduced in subsequent preparation steps without affecting the attachment of the structures.

Preparation of well preserved nuclear matrices requires an enzymatic digestion of nuclear DNA before the extraction of chromatin proteins, probably to prevent nuclear structures from breaking during unfolding of the DNA. By using the *in situ* preparation we have analyzed further the DNase I digestion step. By repeating DNase I treatments >90% of the DNA can be solubilized. Together with DNA only some distinct polypeptides are removed selectively. However, no histones are extracted. This demonstrates that under our conditions the majority of the chromatin proteins still remains associated with the extracted nuclei even after solubilization of almost all the DNA. It suggests that chromatin proteins, besides binding to DNA, are able to interact with one another and/or with (an) other structural system(s) of the nucleus.

Analysis of the structures obtained during *in situ* preparation by electron microscopy shows the major decrease in electron density in the nucleus already after DNase I digestion (Fig. 2*f*). With the exception of nucleoli little additional intranuclear material is visible and these structures already are quite similar to the nuclear matrices obtained in the final preparation step. This is in apparent contradiction to the corresponding phase-contrast images that show no decrease

in optical density and to the finding that almost no protein is extracted during the DNase I digestion. Inasmuch as both biochemical and phase-contrast analyses of all fractionation steps correlate well, it appears that after DNase I digestion the intranuclear material is not seen in the electron microscope for some technical reason. It might be that this material is no longer stainable or that it is obscured by the embedding plastic as has been suggested for the internal nuclear matrix (24). This, however, appears unlikely here, in that almost no protein has been extracted as compared with the structures before the DNase I digestion that are stained heavily. In addition, DNase I-digested nuclei can be stained when prepared using oxidizing conditions (see below). We, therefore, assume that the intranuclear material has been lost after fixation during processing of the structures for electron microscopy. Then, after removal of the DNA the remaining intranuclear structures must be rather labile under our preparation conditions that include DTT and EGTA. However, omission of chelators and the use of oxidizing reagents (e.g. disulfiram) leads to DNase I-treated structures containing considerable internal material (data not shown). It seems reasonable that such conditions stabilize the intranuclear structures, whereas reducing conditions and chelators have the opposite effect (for discussion see references 15 and 26). By analogy to the DNase I-digested nuclei, a similar effect may also occur with isolated nuclear matrices. Mild oxidizing reagents such as disulfiram might be necessary to stabilize the intranuclear structures during sample preparation for the electron microscope. This could explain part of the differences observed with the intranuclear material, when nuclear matrices prepared under different conditions are analyzed. In addition, the similarity of the polypeptide patterns of nuclear matrices prepared with DTT/EGTA or disulfiram (data not shown) suggests that disulfiram does not simply act by unspecifically cross-linking proteins.

In this context we emphasize that the fractionation scheme described cannot be regarded as a standard procedure but has to be adopted for each specific problem. The nuclear matrices prepared here constitute a minimal residual structure due to the presence of reducing agents and chelators. It seems reasonable that proteins weakly associated with the nuclear matrix are solubilized during extraction under these conditions and, therefore, are assigned to other nuclear structures. The conditions employed here, however, are necessary to allow the solubilization of the residual cellular skeleton by the use of Empigen BB at a relatively high pH. Inclusion of chelators and reducing agents in only the final step is not sufficient; instead they have to be added from the beginning on to yield a quantitative solubilization. The conditions used for the solubilization of the residual cellular skeleton allow biochemical and immunological analyses of the extracted proteins. This is especially important in view of the fact that many regulatory proteins such as viral tumor antigens (31, 47–49), hormone receptors (3, 46), or heat shock proteins (64), seem to perform their functions in association with the nuclear matrix.

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