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Identification of nuclear envelope proteins and glycoproteins which co-isolate with the nuclear protein matrix

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Nuclear envelopes and nuclear matrices were isolated from rat liver nuclei. Although differences in polypeptide composition of the structures are evident on SDS gel electrophoresis, they have an almost identical distribution of concanavalin A-binding glycoproteins. These matrix-associated concanavalin A-binding glycoproteins derive entirely from the nuclear envelope and are recovered almost quantitatively in the matrix. They constitute easily identifiable markers for nuclear envelope association with matrix or other nuclear subfractions. Surface labelling of nuclei with ^{125}I using solid-phase lactoperoxidase further confirmed that a large number of envelope-associated nuclear surface proteins co-isolate with the matrix. Protein kinase activity, as well as endogenous substrates for the kinase(s) are shown to be the same in both envelopes and matrix. Envelope-derived proteins and glycoproteins may comprise a substantial proportion of total matrix protein.

Introduction

In recent years a number of functions previously thought to be associated with the nuclear envelope have been ascribed to the nuclear matrix [1,2]. Although electron microscopy reveals a residual nuclear envelope and nuclear pores at the periphery of the matrix [3] and two-dimensional gel electrophoresis has shown a number of proteins common to both structures [4], there are few reports in the literature concerning the extent to which nuclear envelope proteins are associated with the nuclear matrix. The three lamins: A, B and C are the only well described proteins which co-isolate with both the envelope and matrix [5] All three proteins are associated with the inner nuclear membrane and do not extend into the nucleus [6].

Most matrix isolation techniques involve a step designed to solubilize the nuclear envelope while leaving the internal matrix intact. This is usually accomplished by using a nonionic detergent to solubilize the two nuclear membranes, leaving the pore complexes and lamina associated with the matrix [7]. A number of authors have, however, questioned the effectiveness of nonionic detergents in the removal of nuclear envelope proteins [8–10]. They find that while these detergents extract over 95% of lipid, only 20% of envelope protein is removed. Kaufmann and Shaper [11] have recently devised a method which appears to effectively separate the envelope from the internal matrix structure. This, however was achieved at the expense of the integrity of the matrix. The

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internal nuclear matrix was selectively solubilized using 20 mM dithiothreitol in 1 M NaCl The nuclear membrane-lamina complex remained insoluble and was enriched in the three lamins. The solubilized material comprised up to 30% of total nuclear protein, formed a complex pattern on two-dimensional gel electrophoresis and did not include the lamins. One of the major proteins identified appears to be a 38 kDa nucleolar phosphoprotein [12]. The authors note, however, that a number of the solubilized polypeptides may well derive from the envelope and that their topographical distribution requires further clarification

Although very detailed studies of nuclear subfractions by two dimensional gel electrophoresis have been performed [4], the characterization of the polypeptides does not go beyond their identification as proteins; no specific structural or functional features are identified. These gels also do not always easily reveal the extent of cross contamination of one nuclear subfraction by another. Although many polypeptides are present in a number of nuclear subfractions, the subfraction from which they originate can often not be identified. Such information is essential for the more detailed characterization of functional domains in the nucleus. In this study we have attempted to identify bona fide envelope and envelope-associated proteins, other than those belonging to the lamina, and to establish to what extent they coisolate with the matrix We show that the nuclear matrix isolated by standard techniques contains a large number of nuclear envelope proteins and glycoproteins. These proteins can make up a substantial proportion of total matrix protein.

Materials and Methods

Isolation of nuclei, nuclear envelopes and nuclear protein matrices

Nuclei were isolated essentially according to the method of Blobel and Potter [13] as previously described [14], except that the final wash with Triton X-100 was omitted.

Nuclear envelopes were prepared using heparin (sodium salt, Sigma) according to the method of Bornens [15]. Material banding at approximately 37% sucrose ($d = 1.18–1.20$) was diluted 5-fold with 2 mM sodium phosphate (pH 7.8) and pelleted by centrifuging at $50000 \times g$ for 30 min. This was taken as pure envelope

Nuclear protein matrix was isolated according to the method of Berezney and Coffey [3]. All manipulations were at +4°C. The matrix was stored in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5)/200 μM $MgCl_2$ (TM buffer) in liquid nitrogen until used

Adenylate cyclase assay

Plasma membranes were prepared by the method of Aronson and Touster [16] and the post-mitochondrial supernatant (PMS) by centrifuging the liver homogenate at $120000 \times g$ for 2 h.

Adenylate cyclase was assayed for essentially according to the method of Drummond and Severson [17]. Nuclei or membrane preparations were incubated for 45 min at 37°C with 0.75 mM [8-^{3}H]-ATP (Amersham, 20 Ci/mmol) in 50 mM Tris-HCl (pH 7.5) containing 25 mM KCl, 1.5 mM $MgCl_2$, 20 mM KF, 10 mM theophylline, 25 mM GTP, 2 mM creatine phosphate, 12.5 μg/ml creatine kinase, 37 5 μg/ml bovine serum albumin and 20 μl post-mitochondrial supernatant. The incubation was terminated by the addition of 10% trichloroacetic acid containing 2mM ATP, ADP, AMP, cAMP and adenosine. The supernatant was extracted three times with water-saturated diethyl ether and cAMP determined after fractionation on a 1×30 cm acriflavin-Sephadex column as described by Egly and Porath [18].

Radioiodination

A modification of the method of Richardson and Maddy [10] was followed. Nuclei were suspended in 250 mM sucrose in 50 mM Tris-HCl (pH 7.5)/25 mM KCl/1.5 mM $MgCl_2$ (TKM buffer) (pH 7 5) and KI added to a final concentration of 20 μM. To this was added 30 μl of 1 mM H_2O_2 and 10 μl of Na ^{125}I (10 mCi/ml). To initiate the reaction, 50 μl of Sepharose-bound lactoperoxidase was added The mixture was incubated with gentle shaking for 2 h at +4°C or 15 min at room temperature and the reaction terminated by addition of 100 μl of 2 mM sodium metabisulphite in 250 mM sucrose, 10 mM Tris-HCl (pH 7.2). Sepharose was allowed to settle under gravity and the supernatant carefully re-

moved. Unreacted ^{125}I was removed by pelleting the nuclei or nuclear envelopes and removing the supernatant. The pellet was washed until the supernatant contained 0.5% of radioactivity present in the pellet. Concanavalin A was purified [19] and raidoiodinated to a specific activity of 100 μCi/μg.

Incubations with [^{32}P]ATP

Samples (200 μg protein) were suspended in a buffer containing 10 mM $MgCl_2$, 3.6 mM dithiothreitol, 100 μM phenylmethylsulphonyl fluoride (PMSF), 70 mM Tris-HCl (pH 8.0). 2 μCi of [γ^{32}P]ATP was added to a final concentration of 10 μM and samples were incubated at 25°C for 10 min. Sodium metabisulphite was then added to 100 mM and samples pelleted, washed, counted and solubilized in sample application buffer for two-dimensional gel electrophoresis.

Gel electrophoresis

One-dimensional 10% SDS slab gels were run according to Laemmli [20]. Two dimensional gel electrophoresis was performed according to Peters and Comings [4]. Gels were stained for protein with Coomassie brilliant blue. Protein was transferred from slab gels to nitrocellulose sheets by electroblotting according to Towbin et al. [21] Blots were blocked with 10% bovine serum albumin and incubated with ^{125}I-labelled concanavalin A (5 μCi/μg), with or without 0.25 M α-D-methyl mannoside, dried and autoradiographed.

Analytical procedures

Protein was determined according to Lowry et al. [22] and DNA according to Burton [23]. Phospholipid was determined as phosphate according to Chen et al. [24]. Carbohydrate was determined using anthrone.

Results

Nuclear envelopes purified from heparinized nuclei banded at their characteristic density of 1.18–1.20 [14,25] on centrifugation in a 25–50% sucrose gradient. The yield (with respect to protein) of envelopes and matrix from nuclei was 3.5% and 4.8%, respectively. The envelope comprised 62.5% protein, 33.3% phospholipid and less than 1% DNA while the matrix comprised 94.5% protein and less than 1% DNA and phospholipid. The carbohydrate content of the envelope and matrix was 3.2% and 5%, respectively. These results are similar to those obtained by others [3,26,27].

One-dimensional gel electrophoresis of envelopes and matrix is shown in Fig. 1a. As found by other authors [2,28,29] lamins A, B and C with molecular weights of 72, 68 and 66 kDa, respectively, are major components of the matrix. The lamins are present but less prominent in the envelope. This is a characteristic of envelopes obtained from heparinized nuclei as a substantial proportion of the lamins are solubilized during heparinization [30]. Envelopes prepared using heparin are thus enriched in membrane components rather than in those deriving from the lamina such as the lamins.

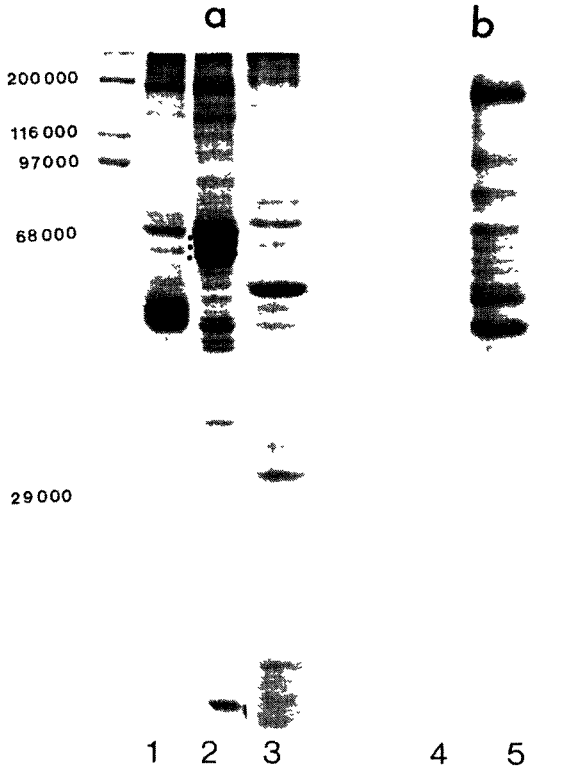

Fig 1 One-dimensional SDS gel electrophoresis of nuclear envelope (1,5), nuclear protein matrix (2) and plasma membrane (3,4) (a) Stained with Coomassie brilliant blue, (b) Blotted onto nitrocellulose and stained with ^{125}I-labelled concanavalin A Dots indicate the lamins. The left hand lane contains molecular weight markers

To establish that our preparations were free of plasma membranes which are a rich source of glycoproteins, we assayed for the presence of adenylate cyclase, an established plasma membrane marker enzyme [31]. No adenylate cyclase could be detected either in nuclei or nuclear membrane fractions (Table I). Furthermore blots of envelope and plasma membrane probed for carbohydrate with ^{125}I-labelled concanavalin A (Fig. 1b) revealed a completely different qualitative and quantitative distribution of concanavalin A-binding polypeptides in the two fractions confirming the lack of contamination of nuclear envelopes by plasma membrane polypeptides.

In order to assess the extent of association of nuclear surface proteins with the nuclear matrix, the method of Richardson and Maddy [10] was chosen whereby selective labelling of surface proteins in intact nuclei could be achieved. When nuclei were labelled with ^{125}I using lactoperoxidase immobilized on Sepharose 4B beads an average of 2% of input radioactivity was incorporated into nuclear protein and of this, 75% was associated with isolated nucelar envelopes (Table II). Envelope lipids accounted for less than 3% of radioactivity, whereas histones, as indicators of the exposure of intranuclear proteins, bound only 5% of label (Table II). When nuclei were sonicated prior to labelling, only 13% of label was recovered in the envelope. These results confirm the specificity of the labelling method for the nuclear surface.

When matrix was isolated from ^{125}I-labelled nuclei, 42% of label was recovered (Table II) indicating extensive association of nuclear surface proteins with the matrix. When envelopes isolated from ^{124}I-labelled nuclei were extracted with 2 M NaCl and 1% Triton X-100 as for the preparation of nuclear matrix, only one third of radioactivity was removed (Table II). These results show that a large proportion of nuclear surface proteins resist high salt and detergent extraction and persist in the matrix-like material obtained from the envelope

As surface iodination of nuclei is likely to also label the nuclear pore complexes which are well described components of the matrix, the results in Table II may represent pore complex labelling rather than that of nuclear membrane protein. We therefore probed the matrix for glycoproteins which have been shown to be components of the nuclear membranes [26] rather than the pore complex or the lamina [32].

The envelope has three characteristic strongly concanavalin A-binding glycoproteins, one at 190 kDa and a doublet between 50 and 54 kDa (Fig. 1b, Fig. 2). About eight other characteristic concanavalin A-binding bands occur between 70 and 150 kDa. The nuclear matrix showed a distribu-

TABLE I

ADENYLATE ACTIVITY IN NUCLEI OR NUCLEAR MEMBRANE FRACTIONS

The quantities of the various fractions are expressed as μg protein

Fraction		Adenylate cyclase activity (pmol cAMP/min)
Plasma membrane	(50 μg)	3 14
Nuclei	(900 μg)	< 0 1
Nuclear envelope	(50 μg)	< 0 1
Nuclear envelope	(300 μg)	< 0 1

TABLE II

DISTRIBUTION OF PROTEIN BOUND RADIOACTIVITY IN NUCLEAR ENVELOPE AND NUCLEAR MATRIX FROM ^{125}I-LABELLED NUCLEI

	Bound radioactivity	
	dpm ($\times 10^{-5}$)	%
Nuclei	12 [a]	100
Nuclear envelope	9	75
Nuclear envelope (subjected to steps 1, 2 and 3 of matrix preparation purification)	6 1	51
Nuclear matrix	5	42
Lipid	0.34	3
Histones	0 50	4
Nuclear envelope (isolated from sonicated nuclei)	1 6	13
Histones (isolated from sonicated nuclei)	2 2	18

[a] Input radioactivity was 1 10^8 dpm which resulted in the incorporation of 1 2·10^6 dpm into nuclei These nuclei were used as starting material for the production of matrix and envelope Where sonicated nuclei were iodinated an aliquot of 1 2 10^6 dpm was used to isolate either envelope or histones The results above represent the average of three determinations

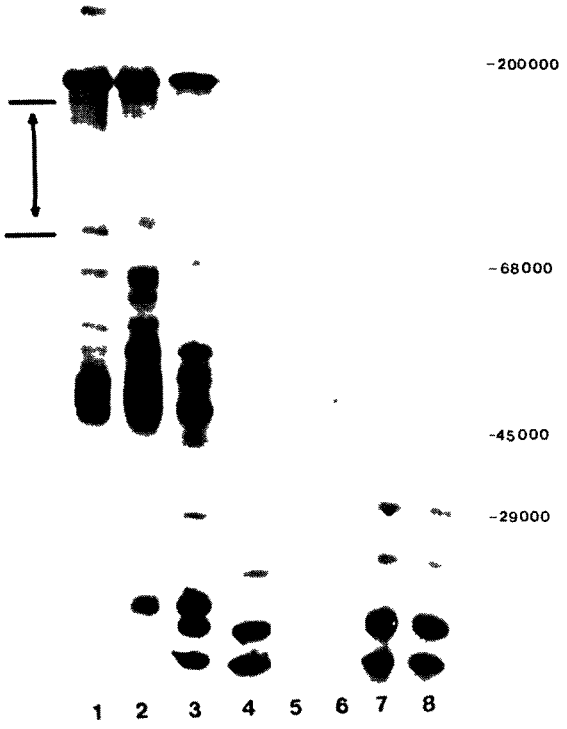

Fig 2 Detection of concanavalin A-binding glycoproteins Samples (100 μg protein) were subjected to one-dimensional SDS electrophoresis, transferred to nitrocellulose, incubated with ^{125}I labelled concanavalin A, and autoradiographed (Further details in Materials and Methods) 1–4 Staining with ^{125}I-labelled concanavalin A in the absence of α-D-methyl mannoside 5–8 Staining with ^{125}I-labelled concanavalin A in the presence of 0 25 M α-D-methyl mannoside 1,5 Nuclear matrix, 2,6 nuclear envelope, 3,7 nuclei; 4,8 heparin supernatant after removal of nuclear envelopes

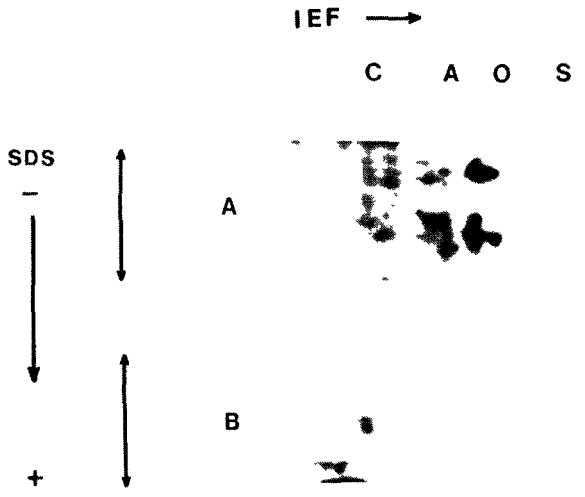

Fig 3 Two-dimensional gel electrophoresis of 400 μg of (A) nuclear envelope and (B) nuclear matrix Gels were blotted, stained with ^{125}I-labelled concanavalin A and autoradiographed Only the sections of the gels corresponding to the molecular weight range demarcated by the arrow in Fig. 2 are shown. Incubation in the presence of α-D-methyl mannoside completely inhibited all binding Standards shown are carbonic anhydrase (C), bovine serum albumin (A), ovalbumin (O) and soybean trypsin inhibitor (S)

tion of concanavalin A-binding components almost identical with that in the envelope (Fig. 2). Although a number of concanavalin A-binding bands migrated in the same region as the lamins, the lamins themselves did not bind concanavalin A as no increased binding in this region was present in the matrix which is enriched in lamins compared to the envelope (Fig. 1). Binding was carbohydrate specific and no binding occurred in the presence of α-D-methyl mannoside (Fig. 2 (5 and 6)). Only a small group of nuclear membrane glycoproteins in the molecular weight range of 75–180 kDa is extracted during matrix isolation (Fig. 3). Nuclei showed an almost identical distribution of concanavalin A-binding components to the envelope and matrix (Fig. 2 (3)) except for the histones (Fig. 2 (7)) which bound concanavalin A nonspecifically as we have previously reported [14]. The supernatant obtained after centrifugation of the nuclear heparin lysate during isolation of envelopes contained virtually no concanavalin A-binding glycoprotein and was enriched in histones (Fig. 2 (4 and 8)), confirming the envelope localization of mannose-rich nuclear glycoproteins.

To quantify nuclear concanavalin A-binding

TABLE III

BINDING OF ^{125}I-LABELED CONCANAVALIN A TO NUCLEI, ENVELOPE AND MATRIX

100 μg of nuclear envelope and nuclear matrix were used per incubation in the case of the nuclei, an aliquot yielding 100 μg nuclear envelope protein was used Values are means ± S E of four determinations

Preparation	Concanavalin A bound (dpm)
Nuclear envelope	5470 ± 150
Nuclear matrix	5020 ± 170
Nuclei	4810 ± 90

sites present in the envelope and matrix, nuclei, envelopes and matrices were incubated with an excess of ^{125}I-labelled concanavalin A and specific binding determined. Results presented in Table III indicate an almost identical number of concanavalin A binding sites in envelope and matrix. These results confirm that not only are the bulk of nuclear concanavalin A-binding glycoproteins recovered in the envelope but over 90% of these survive extraction procedures used to isolate the matrix.

As protein kinase activity towards a number of endogenous substrates has been reported in isolated nuclear envelope [33,34], we investigated whether the kinase(s) and its substrates were retained in the matrix. Envelope and matrix preparations were incubated with [γ-^{32}P]ATP, subjected to two-dimensional gel electrophoresis and autoradiographed. Results presented in Fig. 4 and Table IV indicate that not only is kinase specific activity virtually identical in both envelope and matrix but the main endogenous substrates as judged by two-dimensional gel electrophoresis appear to be the same and labelled to a similar extent. One of the spots labelled in both envelope and matrix corresponds to lamin B, the phosphorylation of which has been reported [35].

TABLE IV

INCORPORATION OF ^{32}P FROM [γ-^{32}P]ATP INTO ENVELOPE AND MATRIX

	Incorporation of radioactivity (dpm/100 μg protein)
Envelope	$4\,6 \cdot 10^4$
Matrix	$4\,2\ 10^4$

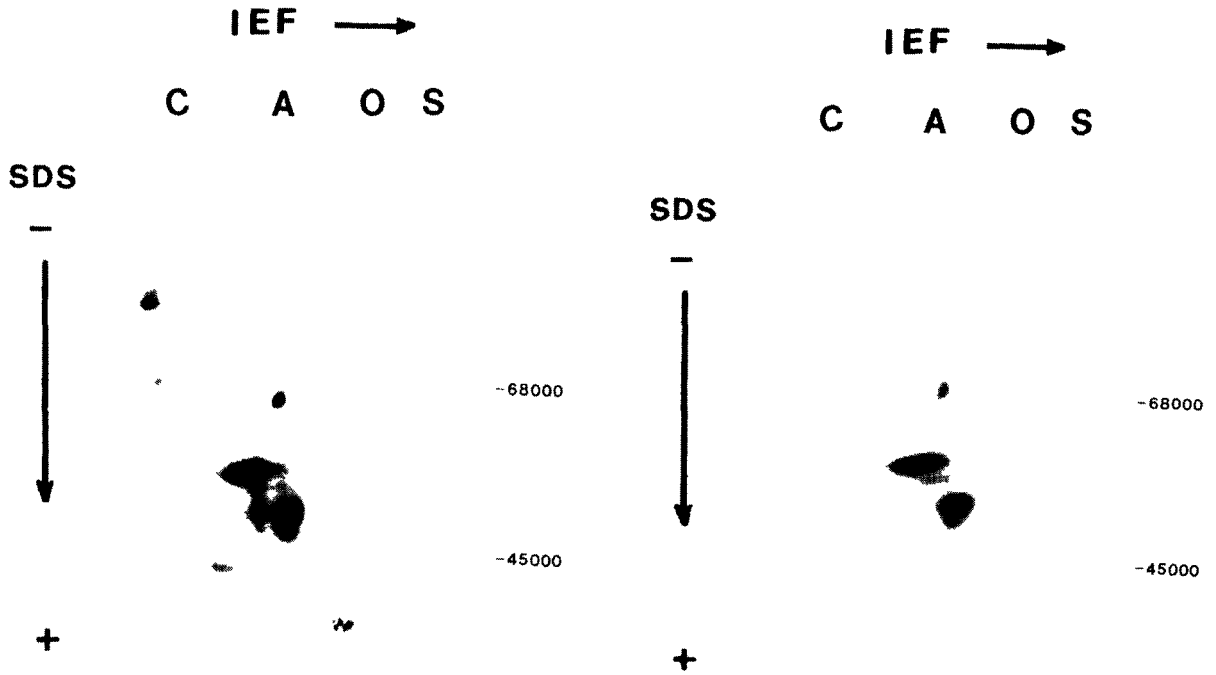

Fig 4 Protein kinase activity in nuclear envelope and matrix 200 μg of (A) nuclear envelopes and (B) nuclear matrix were incubated with 2 μCi of [γ-^{32}P]ATP (see Materials and Methods) and subjected to two-dimensional electrophoresis The gels were stained with Coomassie brilliant blue, dried and autoradiographed Standards were as for Fig 3.

Discussion

Since the first report of the isolation from nuclei of a residual structure termed the nuclear matrix [36], numerous methods have been reported which yield from nuclei structures which appear on electron microscopic examination to be similar to those obtained by Berezney and Coffey (for review see Refs. 7, 37–39).

As currently isolated the matrix has three electron microscopically identifiable structural domains, namely: the surrounding pore complex-lamina, residual nucleoli and the fibrogranular matrix in the interior. The bulk of matrix material is assumed by most authors to derive from the latter [2]. A number of authors have suggested that the stability of the internal matrix may depend on its association with pore complexes and the lamina (for review see Ref. 2), but more recent evidence [11] indicates that the matrix is stabilized by intermolecular disulphide bonds. Nucleolar and pore complex-lamina proteins are considered to be minor matrix components and most functions ascribed to the matrix are usually assumed to be associated with the internal fibrogranular material [2]. Both inner and outer nuclear membranes are generally assumed to be removed by detergents during matrix isolation.

Our results show the extensive presence of envelope proteins and glycoproteins in the matrix although the bulk of envelope lipid is extracted during isolation. The latter is in agreement with results from other laboratories [8–10]. ^{125}I-labelling of nuclear surface proteins clearly shows that most outer nuclear envelope-associated proteins survive detergent extraction. Over half of the label originally present in envelope protein remains associated with the matrix.

Kawasaki and Yamashina [40] found all nuclear mannose could be accounted for by the nuclear envelope. Consistent with this, nuclear binding of concanavalin A occurs exclusively along the cisternal surfaces of the inner and outer nuclear membranes [32,41,42]. In the absence of glycolipids in the envelope [26] the concanavalin A-binding must be exclusively due to nuclear membrane glycoproteins. In accordance with this, gel electrophoretograms of nuclear membrane proteins show a large variety of concanavalin A-binding fractions [14,32,43]. Our results confirm the envelope localization of nuclear concanavalin A-binding glycoproteins. These envelope glycoproteins are recovered almost quantitatively when the matrix is isolated from nuclei. As the amount of internal matrix material recovered in a particular isolation can vary considerably [11], those matrix preparations where the yield of total nuclear protein recovered in the matrix is low (for example Allen et al. [37] recovered 3.5% of nuclear protein in the matrix and Berezney et al. [1] 4.6%) may well consist to a considerable extent of envelope derived proteins and glycoproteins.

The presence of protein kinase(s) in the matrix of almost identical activity and directed against identical endogenous substrates to those found in the envelope, illustrates the current problem of the validity of siting functions ascribed to the matrix, in the interior of the nucleus. Kinase activity and kinase substrates have been observed in both the envelope and the matrix [34,37,38,44]. Nuclear envelope phosphoproteins with molecular weights similar to those identified here have been found by others [34,44].

Functions and properties assigned to the matrix may therefore derive from the presence of envelope. For example, initiation of replication of DNA has been shown to be both an envelope [45] and matrix associated event [1]. The association of this function with the matrix may be due to envelope components and does not provide, in itself, any proof that replication points are scattered in all cells throughout the nucleus associated with the internal fibrous network, rather than confined to the peripheral envelope components. A similar point may be made about the association of active genes with the matrix [46], the presence of hormone binding sites [47] and a variety of other functions.

We have shown that as currently isolated the insoluble nuclear matrix contains not only pore lamina components but also nuclear membrane proteins and glycoproteins. The preparation of matrices using nonionic detergents results therefore, in the precipitation of membrane and other nuclear surface proteins onto the matrix rather than their selective removal. Although solubilized internal matrix material may be obtained substantially free of envelope components [11], a new approach to nuclear membrane removal from ma-

trix preparations will be needed before an insoluble matrix in its natural structure becomes available. Until such a separation is achieved, speculations as to the functions of the matrix as currently isolated will not easily be experimentally confirmed.

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