On the Identity of Nuclear Membrane and Non-Histone Nuclear Proteins†

Robert C. Jackson‡

ABSTRACT: The fate of plasma and nuclear membrane polypeptides in preparations of acidic chromosomal protein from chicken erythrocytes has been investigated. It is shown that detergent extraction procedures (Nonidet P-40, Triton X-100, and saponin), commonly employed in the preparation of acidic chromosomal protein, cannot be relied upon to remove plasma and nuclear membrane polypeptides. These polypeptides persist in nuclear and chromatin preparations and subsequently fractionate as acidic chromosomal protein. In fact, the polypeptides in a preparation of erythrocyte acidic chromosomal protein are shown by gel electrophoresis in dodecyl sulfate to be almost identical to those in a preparation of erythrocyte nuclear membrane. The implication of these results for the preparation of acidic chromosomal protein is discussed.

Evidence has been presented that acidic chromosomal proteins are responsible for the control of gene expression (see MacGillivray and Rickwood, 1974; Gilmour et al., 1975 for recent reviews). For example, Gilmour and his colleagues (Gilmour et al., 1975; Gilmour and Paul, 1975) have demonstrated that acidic chromosomal proteins can stimulate the synthesis of specific mRNA transcripts from reconstituted chromatin. Furthermore, antibodies prepared against acidic chromosomal proteins have been shown to complex with the polytene chromosomes of *Drosophila melanogaster* salivary glands (Silver and Elgin, 1976). Nevertheless, the fraction of the acidic protein, which is responsible for the control of chromosomal functions, remains unknown. Indeed, since these proteins are operationally defined by the procedures used in their preparation, it is possible that a large fraction of the protein is not derived from chromatin at all.

During the course of studies on the nuclear membrane of the chicken erythrocyte, it was discovered that the sodium dodecyl sulfate gel electrophoresis pattern of the nuclear membrane (Jackson, 1976) was very similar to patterns of avian non-histone chromosomal protein published by others (Shelton and Neelin, 1971; Shelton et al., 1972; Shelton, 1973; Elgin and Bonner, 1970; Vidali et al., 1973; Ruiz-Carrillo et al., 1974; Sanders, 1974). This observation prompted the investigation of the origin of non-histone chromosomal protein which is reported here.

Experimental Procedures

Procedures for the collection and washing of erythrocytes, as well as for the preparation of ghosts, plasma membrane, nuclear membrane, and Waring blender nuclei, have been described (Jackson, 1975, 1976). Detergent nuclei were prepared by the method of O'Brien (1964), using 0.1% (v/v) Nonidet P-40 (Shell), 0.1% (v/v) Triton X-100 (New England Nuclear), or 1.0% (v/v) Triton X-100. Briefly, 1 ml of packed cells were resuspended in 9 ml of Tris[1]-Ringer's buffer, pH 7.5, and enough 10% Nonidet P-40 or 10% Triton X-100 was added to bring the suspension to the desired concentration of detergent. In some experiments, as described in the text, the Tris-Ringer's buffer contained PMSF[1] (0.1 mM) and sodium tetrathionate (1 mg/ml) as protease inhibitors. The suspensions were incubated on ice for 10 min with occasional agitation, at which time the nuclei were collected by centrifugation (5000*g* for 5 min). The nuclei were extracted twice more with the appropriate detergent in buffer and washed twice with 20-ml volumes of ice-cold Tris-Ringer's. Saponin nuclei were prepared (via extraction with 0.3% saponin (Fisher Scientific Inc.)) as described by Vidali et al. (1973). The final nuclear pellets were resuspended in 2 ml of ice-cold 70% glycerol. An aliquot (200 μl) of each suspension was removed for treatment with DNase I (Worthington, grade DPFF) and the rest was stored at approximately −20 °C. Protein, lipid and DNA analyses were later performed on these samples. The 200-μl aliquot of nuclear suspension was diluted with 2 ml of 0.1 mM $MgCl_2$, containing 0.1 mM PMSF and 1 mg/ml of sodium tetrathionate (K & K Laboratories), and vortexed vigorously. A 50-μl aliquot of a 1 mg/ml solution of DNase I and 8 ml of 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, with 0.1 mM PMSF, and 1 mg/ml of sodium tetrathionate were added, and the mixture was incubated at 30 °C for 15 min. The hydrolysis was stopped by immersing the samples in an ice bath. Aliquots of the digested nuclei were analyzed for membrane polypeptides via disc gel electrophoresis in sodium dodecyl sulfate (Jackson, 1975).

As previously described (Jackson, 1975), erythrocyte ghosts were prepared from 1 ml of packed cells by hypotonic lysis with 20 ml of 10 mM Tris-HCl, pH 7.5, 1.0 mM $MgCl_2$. The ghosts were extracted with 10 ml of ice-cold 1% Triton X-100 in Tris-Ringer's for 10 min and centrifuged at 5000*g* for 5 min. Sodium dodecyl sulfate was added to the supernatant to a final concentration of 2% (w/v) and the proteins were analyzed via gel electrophoresis in sodium dodecyl sulfate. In some instances, Triton X-100 was removed, as described by Fukuda and Osawa (1973) or as described by Holloway (1973), and the polypeptides were concentrated by lyophylization prior to gel electrophoresis in sodium dodecyl sulfate. All three methods gave identical results. The "nuclear" pellet was digested with DNase I, as described above, and analyzed via gel electrophoresis in sodium dodecyl sulfate.

Chromatin and non-histone nuclear protein were prepared by the method of Wilson and Spelsberg (1973). Briefly, nuclei prepared by Waring blender procedure were homogenized with

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received June 10, 1976.* This research was supported by Grant HL-08893 from the National Institutes of Health, and Grant GB-36837 from the National Science Foundation to Dr. Guido Guidotti.

‡ Current address: Department of Cell Biology, The Rockefeller University, New York, N.Y. 10021.

[1] Abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

TABLE I: Composition of Chicken Erythrocyte Nuclei.

Sample	Phospholipid[a] (mg/mg of DNA)	Protein[a] (mg/mg of DNA)
0.1% Nonidet P-40 nuclei	0.115 ± 0.006	1.38 ± 0.07
0.1% Triton X-100 nuclei	0.122 ± 0.001	1.49 ± 0.02
1.0% Triton X-100 nuclei	0.048 ± 0.004	1.32 ± 0.24
Saponin nuclei	0.281 ± 0.014	1.43 ± 0.10
Erythrocyte ghosts	0.282 ± 0.010	1.86 ± 0.09
Waring blender nuclei	0.030 ± 0.002	1.17 ± 0.10

[a] Average of at least two determinations ± average deviation of mean.

a motor-driven Teflon pestle homogenizer in 0.5 M sucrose, 0.2% Triton X-100. After centrifugation (10 000*g* for 10 min), the pellet was washed by repeated resuspension (with the Teflon-pestle homogenizer) and centrifugation in (a) 80 mM NaCl, 20 mM EDTA, pH 6.3 (three times); (b) 0.35 M NaCl; (c) 1.5 mM sodium citrate, pH 7.0, 1.5 mM NaCl (three times). The final pellet of chromatin was resuspended in 1.5 mM NaCl, 1.5 mM sodium citrate, pH 7.0, at a concentration of 2.25 mg of protein/ml. An aliquot of the chromatin (2 ml) was extracted twice with ice-cold 0.2 M H_2SO_4 in a total volume of 30 ml for 30 min. After centrifugation (2000*g* for 10 min), the pellet was resuspended in 6 ml of 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1 M Tris-HCl, pH 7.0, DNase I (75 μl of a 1.0 mg/ml solution) was added, and the suspension was incubated at 30 °C for 30 min. Digestion was stopped with 30 ml of ice-cold $HClO_4$ (0.4 M) and the suspension was centrifuged at 2000*g* for 10 min. The pellet was washed with 5 ml of 0.4 M $HClO_4$ and dissolved in 1% dodecyl sulfate for analysis by dodecyl sulfate gel electrophoresis. Histones were prepared from the combined H_2SO_4 extracts, as described by Ruiz-Carrillo et al. (1974).

Protein was determined by the method of Lowry et al. (1951), as described by Bailey (1967). The assay was standardized with bovine serum albumin (Sigma, fraction V), $E_{279nm}^{1\%} = 6.7$ (Foster and Yang, 1954). Lipids were extracted as described by Hajra et al. (1968) except that H_2SO_4 was substituted for phosphoric acid. Lipid phosphate was assayed by the method of Ames (1966). DNA was determined by the diphenylamine method of Burton (1956).

Results

Removal of Phospholipid. The ability of nonionic detergents to remove the phospholipid and protein components of cellular membranes was investigated by preparing chicken erythrocyte nuclei in the presence of the detergents Nonidet P-40, Triton X-100, and saponin, as described under Methods. For comparison, chicken erythrocyte ghosts, which contain both their plasma and nuclear membranes, were prepared by hypotonic lysis. Nuclei free of plasma membrane contamination were prepared by Waring blender homogenization as previously described (Jackson, 1976). Table I compares the relative compositions of these various nuclei. It is clear that both Triton X-100 and Nonidet P-40 are capable of extracting the majority of the membrane phospholipid. At a concentration of 0.1%, both detergents remove approximately 57% of the lipid phosphate. At a concentration of 1%, Triton X-100 removes 83% of the lipid phosphate. Although it clearly lysed the cells, saponin (0.3%, w/v) failed to remove any phospholipid. This is not surprising, since saponin is a heterogeneous mixture of sapogenin glycosides some of which, e.g., digitonin, form specific complexes with cholesterol (Bladon, 1958). This ability to specifically bind cholesterol probably explains why the low concentration of saponin used in the nuclear preparation procedure lysed the cells without removing significant amounts of phospholipid.

Nuclei prepared by the Waring blender procedure have a very low phospholipid to DNA ratio which reflects the loss of the plasma membrane and a fraction of the outer nuclear membrane (Jackson, 1976; Zentgraf et al., 1969). The second column in Table I demonstrates that, even though Nonidet P-40 or Triton X-100 extraction removes a large fraction of the membrane phospholipid, it apparently fails to remove the membrane protein. In spite of the fact that saponin removes no membrane phospholipid, the mass ratio of protein to DNA of the saponin-prepared nuclei is the same as that of the Nonidet P-40 and Triton X-100 nuclei. Erythrocyte ghosts have a somewhat higher protein to DNA ratio, but this is apparently due primarily to the removal of histone during the detergent extraction procedures (see Figure 2). Nuclei prepared by the Waring blender procedure had a lower protein to DNA ratio than the detergent nuclei. These results suggest that nuclei prepared by the detergent methods commonly employed have a higher protein to DNA ratio than expected. This finding is consistent with the proposition that detergent nuclei are contaminated with non-nuclear protein.

Removal of Plasma Membrane. To establish further that the detergent extractions had failed to remove the membrane polypeptides, the nuclear preparations were treated with DNase I and subjected to sodium dodecyl sulfate gel electrophoresis along with a sample of chicken erythrocyte plasma membrane, prepared as previously described (Jackson, 1976).

Although it has been shown that proteolysis can alter the sodium dodecyl sulfate gel electrophoresis pattern of chicken erythrocyte plasma membrane polypeptides (Jackson, 1975, 1976), protease inhibitors were deliberately not used in order to follow precisely the procedures of the other investigators. For sake of comparison, protease inhibitors were also excluded from the ghost and plasma membrane preparations. The results (Figure 1) reveal that detergent-prepared nuclei (0.1% Nonidet P-40 (gel C), 0.1% Triton X-100 (gel D), 1.0% Triton X-100 (gel E), and saponin (gel F)), as well as the ghost preparation (gel B), contain polypeptide components which are not found in plasma membrane-free nuclei (gel G). These additional components clearly derive from plasma membrane (gel A).

Inclusion of protease inhibitors in the detergent extraction procedure does not qualitatively affect this conclusion.

To confirm that detergent extraction does not remove the bulk of the plasma membrane protein, the polypeptide composition of a 1.0% Triton X-100 extract of erythrocyte ghosts was analyzed by gel electrophoresis in sodium dodecyl sulfate. Ghosts, prepared from 1 ml of packed cells, were extracted with 10 ml of 1.0% Triton X-100, as described under Methods. Dodecyl sulfate gel electrophoresis of the extracted polypeptides (Figure 2, gel B) confirms that 1% Triton X-100 is incapable of removing the majority of the plasma membrane polypeptides (Figure 2, gel A). Although some of the extracted polypeptides comigrate with membrane polypeptides, the majority of the extracted material, apparently, does not derive from membrane at all; rather, it comigrates with histone (Figure 2, gel D). In contrast, the nuclear pellet (gel C) contains polypeptides which comigrate with all the plasma membrane polypeptides.

Origin of Non-Histone Nuclear Protein. The origin of erythrocyte non-histone nuclear protein was investigated by

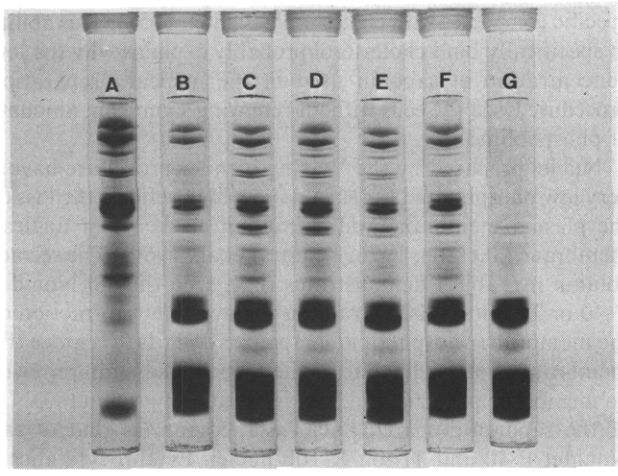

FIGURE 1: Comparison of chicken erythrocyte plasma membrane and nuclei prepared in the absence of protease inhibitors. Chicken erythrocyte nuclei were prepared by extraction with nonionic detergent in the absence of protease inhibitors, as described under Methods. Plasma membrane, ghosts, and nuclei free of plasma membrane contamination were also prepared in the absence of protease inhibitors, as previously described (Jackson, 1975, 1976). The nuclei and ghosts were treated with bovine pancreatic DNase I in the presence of protease inhibitors and subjected to gel electrophoresis in sodium dodecyl sulfate. Gel A, plasma membrane (ca. 25 μg); gel B, erythrocyte ghosts (ca. 30 μg); gel C, nuclei prepared via extraction with 0.1% (v/v) Nonidet P-40 (ca. 30 μg); gel D, nuclei prepared via extraction with 0.1% Triton X-100 (ca. 30 μg); gel E, nuclei prepared via extraction with 1.0% (v/v) Triton X-100 (ca. 30 μg); gel F, nuclei prepared via extraction with 0.3% (w/v) saponin (ca. 30 μg); gel G, plasma membrane-free nuclei prepared via the Waring blender technique (ca. 50 μg).

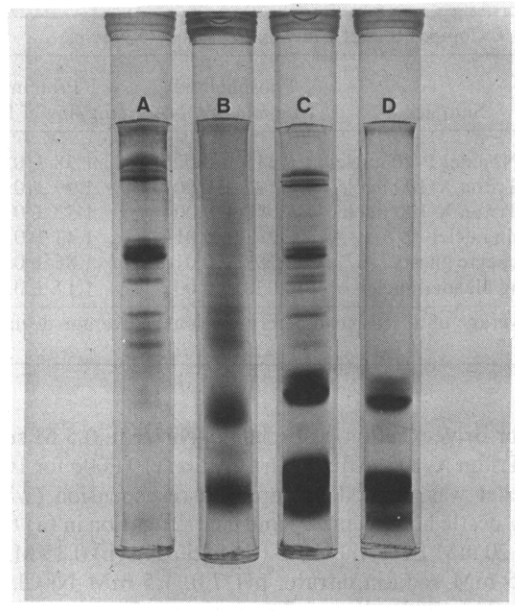

FIGURE 2: Triton X-100 extraction of erythrocyte ghosts. Chicken erythrocyte ghosts from 1 ml of packed cells were extracted with 10 ml of 1.0% (v/v) Triton X-100, containing 0.1 mM PMSF and 1 mg/ml of sodium tetrathionate, as described under Methods. After centrifugation (5000g for 5 min), the supernatant and nuclear pellet were analyzed via gel electrophoresis in sodium dodecyl sulfate. Gel A, erythrocyte plasma membrane (ca. 15 μg); gel B, supernatant fraction containing proteins released by the Triton X-100 extraction procedure (ca. 20 μg); gel C, "Nuclear" pellet fraction (ca. 30 μg); gel D, chicken erythrocyte histone (ca. 20 μg), prepared as described by Ruiz-Carrillo et al. (1974).

comparing the dodecyl sulfate gel electrophoresis patterns of non-histone nuclear protein and nuclear membrane (Figure 3). Chromatin (Gel F) and non-histone chromosomal protein (Gel A) were prepared by the method of Wilson and Spelsberg (Wilson and Spelsberg, 1973). This particular procedure was chosen since it included a 0.2% Triton X-100 extraction step designed to remove nuclear membrane. The 0.2% Triton X-100 extract (Gel C) was also examined by dodecyl sulfate gel electrophoresis. As shown in Figure 3, nuclear membrane (Gel B) and non-histone chromosomal protein (Gel A) have virtually identical polypeptide compositions. The only major difference between the two gel patterns is the large increase in the amount of protein in bands X and Y in the non-histone chromosomal protein gel. These bands comigrate with chicken erythrocyte histones (Gel D) H2 and H3 (band X), and histone H4 (band Y), and, probably, represent unextracted histone. Evidence in support of this view has been presented by Ruiz-Carrillo et al. (1974); however, Elgin and Bonner (1970) and MacGillivray et al. (1972) have evidence that the low-molecular-weight polypeptides observed in acidic chromosomal protein preparations from non-erythroid tissues have amino acid compositions incompatible with histone. Gels E and F demonstrate that, despite the Triton X-100 extraction, chromatin and the plasma membrane-free nuclei, from which the chromatin was prepared, have indistinguishable gel patterns. Bands N1 and N2 are just barely visible in both gels E and F. The Triton X-100 extract contained very little protein; in fact, the protein of gel C was extracted from 3.85 mg of nuclei (expressed as DNA), while the protein of gel A was prepared from 0.164 mg of nuclei. Furthermore, it is clear that the Triton X-100 extract contains primarily histones with little membrane protein.

Discussion

Non-ionic detergents have often been used in the preparation of nuclei which are assumed to be free of cytoplasmic and membraneous contaminants (Shelton and Neelin, 1971; Shelton, 1973; Vidali et al., 1973; Ruiz-Carrillo et al., 1974; Sanders, 1974; O'Brien, 1964; Wilson and Spelsberg, 1973; Tata et al., 1972; Blobel and Potter, 1966). This assumption stems from the observation that nuclei, which have been treated with these detergents, appear to have lost their cytoplasmic and outer nuclear membranes, as determined by electron microscopy; however, recent evidence indicates that nonionic detergents may not remove plasma and nuclear membrane proteins. Walmsley and Davies (1975) have observed that chicken erythrocyte nuclei, prepared with Triton X-100, contain a halo of proteinaceous material, presumably derived from the plasma and nuclear membranes. This material is visible in thin sections stained with phosphotungstic acid, but is obscured in sections stained by the normal uranyl acetate–lead citrate procedure. Likewise, Kartenbeck et al. (1973) have shown that Triton X-100 washes of liver nuclei do not remove the outer nuclear membrane completely. Recently, Aaronson and Blobel (1974, 1975) have demonstrated that the nuclear pore complex remains associated with the nucleus after treatment with Triton X-100.

Most investigators of the non-histone chromosomal protein of avian erythrocytes have not discussed the possibility of membrane contamination. Elgin and Bonner's (1970) nuclear preparation consisted of hypotonically lysed erythrocytes comparable to the ghost preparation used in this investigation. Allfrey and his co-workers have used nuclei prepared by the saponin method (Vidali et al., 1973) and, more recently, by a method comparable to the Waring blender method used in this work (Ruiz-Carrillo et al., 1974). Shelton, who has prepared

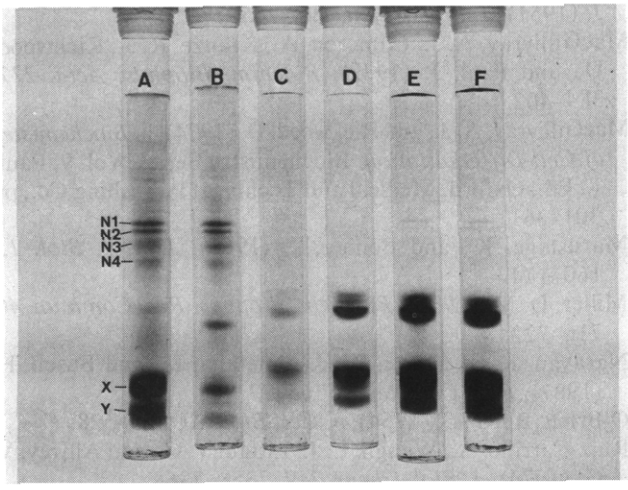

FIGURE 3: Comparison of chicken erythrocyte non-histone nuclear protein with nuclear membrane via gel electrophoresis in sodium dodecyl sulfate. Chicken erythrocyte chromatin and non-histone nuclear protein were prepared from plasma membrane-free nuclei as described by Wilson and Spelsberg (1973). Gel A, non-histone nuclear protein (ca. 40 μg); gel B, erythrocyte nuclear membrane (ca. 15 μg) prepared as previously described (Jackson, 1976); gel C, 0.2% (v/v) Triton X-100 extract of plasma membrane-free nuclei (ca. 5 μg); gel D, chicken erythrocyte histone (ca. 20 μg); gel E, plasma membrane-free erythrocyte nuclei (ca. 50 μg); gel F, chicken erythrocyte chromatin (ca. 50 μg).

nuclei by the saponin method (Shelton and Neelin, 1971; Shelton et al., 1972), has determined that a large fraction of the nuclear acidic protein in his preparations derived from plasma membrane contamination (Shelton et al., 1972). Shelton did not, however, investigate the origin of the residual acidic protein of plasma membrane-free nuclei, which he prepared by a method comparable to the Waring blender method used in this study. Harlow et al. (1972) and Harlow and Wells (1975) have also suggested that a large fraction of avian erythrocyte nuclear acidic protein is derived from membrane.

The evidence presented here confirms and extends the findings of Shelton (1972), Harlow et al. (1972), and Harlow and Wells (1975): nonionic detergents do not entirely remove erythrocyte plasma membrane polypeptides; consequently, non-histone nuclear protein, prepared from detergent nuclei, is heavily contaminated with plasma membrane polypeptides. Furthermore, a direct comparison of non-histone nuclear proteins prepared from plasma membrane-free nuclei with nuclear membrane proteins reveals that the major polypeptide components of these two preparations are identical (Figure 3). One possible interpretation of this result is that, in this cell, virtually all non-histone nuclear protein derives from the nuclear membrane. However, it should be noted that, by definition, these two sets of proteins are not mutually exclusive. In fact, subfractionation of the nuclear membrane via NaOH extraction (Jackson, 1976) indicates that the two highest molecular weight nuclear membrane bands (bands N1 and N2 in Figure 3) probably are not deeply embedded in the phospholipid bilayer. Consequently, it is possible that these particular polypeptides are associated both with chromatin and with the nuclear membrane. In fact, a proteinaceous structure which appears to be associated with both chromatin and the nuclear envelope has been known for many years—the dense lamella (Fawcett, 1966). Although the bulk of the dense lamella is apposed to the inner surface of the inner nuclear membrane, projections of the lamella may extend into the nucleoplasm, which, in turn, may explain reports of a proteinaceous intranuclear matrix (Berezney and Coffey, 1974; Zbarsky et al., 1962; Narayan et al., 1967). In light of this information, a more reasonable interpretation of the results is that the nuclear membrane preparation consists of a class of proteins which are deeply embedded in the phospholipid bilayer and another class of proteins which are merely associated with the bilayer. This latter class of proteins may comprise the dense lamella. In any case, both classes of proteins are prominent in non-histone nuclear protein preparations.

Another possibility, which cannot be entirely eliminated at this time, is that the association of the band N1 and N2 polypeptides with the nuclear membrane is an artifact arising from protein migration during the DNase I digestion or the 2 M NaCl extraction steps of the nuclear membrane preparation. However, this hypothesis seems unlikely, based upon the composition, buoyant density, and electron microscopic appearance of the nuclear membrane fraction (Jackson, 1976).

These results suggest that the origin of non-histone chromosomal protein, prepared from active tissues like liver or kidney, should be reevaluated. Since nonionic detergents apparently do not solubilize membrane proteins as readily as phospholipids,[2] contamination by cellular membranes must be reevaluated. In this regard, Tata et al. (1972) have demonstrated that the sheared chromatin preparation of Marushige and Bonner (1966), commonly used in the preparation of non-histone chromosomal protein, contains substantial amounts of phospholipid. As a solution to this problem, Tata et al. advocated the use of chromatin prepared from nuclei washed with 1% Triton X-100. Unfortunately, the data presented above indicate that such a solution only masks the contaminating membrane protein. The bulk of the contaminating membrane in chromatin, prepared from whole tissue, is microsomal (Tata et al., 1972), while in chromatin, prepared from isolated nuclei, nuclear membrane is probably the major membrane contaminant.

The actual fraction of the nuclear acidic protein, which derives from the nuclear membrane, will, of course, vary from tissue to tissue, depending upon the surface area of the nucleus and its acidic protein content. Furthermore, the diversity of procedures used in the preparation of acidic nuclear protein necessarily complicates any estimate of the contamination by nuclear membrane protein. While those procedures which quantitatively extract all the acidic protein from chromatin will definitely coextract nuclear membrane proteins, milder, nonquantitative procedures may not (see MacGillivray and Rickwood, 1974, for a review of the various procedures used in the preparation of acidic nuclear protein).

Acknowledgments

I thank Dr. Guido Guidotti for his generous support and advice.

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[2] The fraction of membrane protein solubilized will undoubtedly vary, depending upon the source of the membrane and the exact conditions of the extraction procedure (Kirkpatrick et al., 1974). The inclusion of divalent cations in the extraction buffers has been reported to be especially detrimental to complete membrane solubilization (Miller 1970; Yu et al., 1973). On the other hand, divalent cations are required for the maintenance of morphologically intact nuclei. This dilemma may explain why the detergent extraction procedures commonly employed in the preparation of detergent nuclei remove so little membrane protein.

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