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The isolation of the envelopes of rat liver nuclei

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SUMMARY

1. A strict morphological definition of the nuclear envelope is established, and a procedure for the isolation of envelope fragments consistent with this definition is described.

2. The composition of the isolated envelope (% by weight) is: protein 64%; lipid 23%; DNA 8%; RNA 5%.

3. The nuclei and envelopes contained little or no detectable succinoxidase, 5'-nucleotidase or glucose-6-phosphatase activity.

4. It is demonstrated that the application of techniques reducing the envelope DNA content to significantly less than 8% leads to a loss of morphological integrity of the system.

5. It is concluded that DNA is probably an essential structural component of the nuclear envelope.

Available information concerning the nuclear envelope of mammalian liver suggests no characteristic functional property whereby isolated preparations of the envelope can be recognised as such. However, electron microscopy reveals the following characteristic morphological features: (a) The envelope is a double-membrane system; (b) under certain conditions of negative staining, some 20–30% of the surface can be seen to be occupied by "pore complexes"[2]. The latter are complicated structures comprising an octagonally symmetric annulus[3], a "central spot"[4], and occupying the space between these possibly a diaphragm[5] or struts[6]. It follows that any preparation of nuclear envelope is satisfactory if and only if it is a double membrane, and 20–30% of its surface consists of pore complexes with the above-mentioned ultrastructural features. Only when this criterion has been satisfied can consideration reasonably be given to the "purity" of the preparation; this may be represented, for example, as the minimum attainable DNA/lipid ratio (assuming most of the nuclear lipid to be associated with the envelope). Material of

membranous appearance, prepared from nuclei and having a low DNA content, does not constitute a satisfactory nuclear envelope preparation unless it satisfies the criterion of morphological integrity.

Nuclei were isolated by a modification of the methods of Widnell and Tata[7] and Sporn *et al.*[8]. Three male albino rats (150–200 g) were killed by a blow on the head, and the livers excised as quickly as possible and placed in ice-cold homogenising buffer (0.32 M sucrose–0.03 M sodium phosphate buffer, pH 6.1–3 mM $MgCl_2$). After the liver had been minced with scissors for 1–2 min, the fluid was decanted and replaced by 80–90 ml fresh homogenising buffer. The suspension was homogenised in a Potter–Elvehjem homogeniser with a 0.5 mm clearance, the pestle being rotated at 3000 rev./min. Five to six up-down movements of an average of 20–30 sec each were used, care being taken to ensure that the temperature did not rise above 5°. The homogenate was filtered through eight thicknesses of cheesecloth and centrifuged at 800 X *g* for 10 min. The supernatant was carefully decanted and the pellet resuspended in 4 vol. of dense sucrose medium (2.3 M sucrose–0.03 M sodium phosphate buffer, pH 6.1–3mM $MgCl_2$). This suspension was layered over 10 ml of the same dense sucrose medium in 35-ml capacity tubes and centrifuged at 60 000 X *g* for 60 min. The pellet of nuclei was resuspended in 5 ml homogenising buffer and centrifuged at 800 X *g* for 10 min; this washing was repeated 2–3 times.

Electron and phase contrast microscopy of the nuclei showed no contamination other than traces of collagen. Succinoxidase, assayed by the method of King[9], had 3% of the activity of the total liver homogenate. 5′-Nucleotidase, assayed by the method of Emmelot and Bos[10], and glucose-6-phosphatase, assayed by the method of Swanson[11], had no detectable activity. It was concluded that microsomal, mitochondrial and plasma membrane contamination of the nuclei was minimal.

Homogenisation, passage through a hypodermic needle, freeze–thawing and shearing in a Waring blender failed to lyse the nuclei; these observations are consistent with those of Kashnig and Kaspar[12]. Osmotic shock had no effect if the magnesium concentration was greater than 0.2 mM. The nuclei were disrupted by sonication after suspension at a concentration of about 5 mg protein/ml in 5 ml 0.02 M Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$. (The concentration of nuclei in the suspension is not critical). 2 X 20 sec sonication at 1 μm amplitude, 21 kHz, in a M.S.E. ultrasonicator with a 0.5 cm probe was used at 0°. The suspension was then diluted with an equal volume of ice-cold water and centrifuged at 3000 X *g* for 10 min; intact nuclei, nucleoli, collagen and dense chromatin were pelleted. The supernatant was centrifuged at 30 000 X *g* for 30 min; the pellet of crude nuclear envelopes was rinsed with and resuspended in distilled water. Electron microscopy, using 2.0% ammonium molybdate at pH 7.0 (ref. 13), revealed on the envelope fragments pore complexes of the expected structure and frequency. There was little membrane vesicle formation (see Fig. 1).

The envelopes were purified by layering on a 5-ml sorbitol gradient, 1.0–5.0 M in distilled water, and centrifuging for 16 h at 25 000 rev./min (50 000 X *g* average) in the SW 39 rotor of a Spinco L centrifuge at 0–5°. The envelopes were recovered from a narrow band with specific gravity 1.27. Centrifugation for up to 64 h did not alter the position of this band; it therefore represented the equilibrium position of the envelopes on the gradient. There was no pellet, but much dissolved material remained in the upper part of the gradient. The envelopes, after 24 h dialysis against water, showed the same morphological integrity as the crude envelopes.

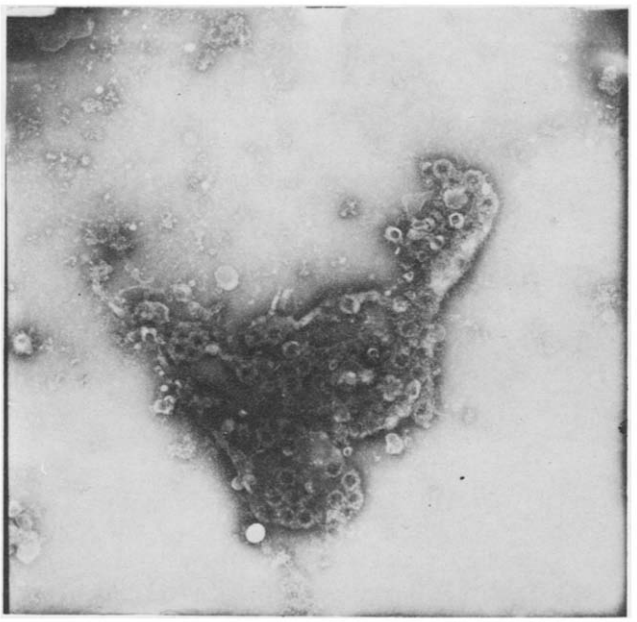

Fig. 1. Nuclear envelope fragment. Fragment of nuclear envelope prepared by the procedure described in the text. Negatively stained with 2% ammonium molybdate, pH 7.2. Magnification, x 25 000 diameters.

The most significant features of this preparation procedure are: (a) The absence of K^+ from all media; (b) low ionic strength throughout; (c) No DNAase treatment of the envelopes. Any variation in these three criteria leads to loss of morphological integrity. However, K^+, high ionic strength and DNAase have been employed by other groups in the isolation of nuclear envelopes. Berezney *et al.*[14], Kashnig and Kaspar[12] and Franke *et al.*[15] all used high ionic strength media; the latter two groups used high K^+ concentrations, and the first used a prolonged exposure to DNAase. Attempts to repeat these procedures in this laboratory have yielded unsatisfactory results. The method of Kashnig and Kaspar[12] gives a tangled mass of membranous vesicles essentially free of pore complexes. The method of Franke *et al.*[15] gives highly vesicularised material with few pore complexes, most of which lack the characteristic structural features. The method of Berezney *et al.*[14] gives envelopes in which pores are visible, but in general the annulus and central spot are lacking. All these envelope preparations are of low DNA content.

The approximate composition of the envelopes obtained from the sorbitol gradient was as follows: 8% by weight DNA (assayed by the method of Giles and Myers[16]); 5% RNA (assayed according to Schneider[17]); 23% by weight lipid (measured as phosphate by a slight modification of the method of Bartlett[18]). The remaining 64%, after enzymatic removal of the nucleic acids and extraction with chloroform–methanol (2:1, v/v) to remove lipids, was protein. The most significant difference between this composition and that of envelopes prepared by other groups is the relatively high DNA content, which may account

for the relatively high apparent density.

The pore complexes were rapidly destroyed by treatment with KCl ($\geqslant$ 0.01 M) or NaCl ($\geqslant$ 0.25 M), or by exposure to any high ionic strength medium irrespective of the ions used. The most significant fact to emerge from these observations is the extreme sensitivity of the envelope structure to K^+. Concommitant with the destruction of the pore complexes was the release of outer and inner nuclear membrane material in the form of minute single-membrane vesicles, of diameter generally less than 100 nm. Inner and outer membrane could not, of course, be distinguished in this highly fragmented form. At the same time, there was a reduction of around 40% in the DNA content of the remaining solid material (*i.e.* the material which could be recovered by centrifugation at 30 000 X *g* for 30 min). It was concluded either (1) that DNA is an essential structural component of the nuclear envelope, being probably associated with the pore complexes, so that removal of DNA from the system causes morphological destruction; or (2) that high ionic strength and K^+ treatments destroy the envelope structure directly, and in the process release any attached DNA.

To distinguish between these possiblities, the envelopes from the sorbitol gradient were treated with 10 μg pancreatic DNAase/mg protein in the presence of 5 mM Tris–HCl, pH 7.5, containing $1 \cdot 10^{-4}$ M $MgCl_2$, at room temperature for 10–15 min. This treatment destroyed the pore complexes and led to the formation of minute single-membrane vesicles from the remainder of the envelope. DNAase inactivated with $1 \cdot 10^{-4}$ M EDTA, or the products of DNAase digestion of chromatin, had no effect on the morphology of the envelope. The DNAase was shown to be essentially protease and lipase free by the following procedure. Erythrocyte membranes, prepared by the method of Maddy[19], were suspended in 5 mM Tris–HCl–$1 \cdot 10^{-4}$ M $MgCl_2$, pH 7.5, containing 0, 5, 10 or 20 μg pancreatic DNAase/mg protein. After incubation for 1 h at room temperature and centrifugation at 40 000 X *g* for 30 min, the absorbance in all supernatants was the same. This demonstrated that the enzyme was not catalysing the release of protein or peptides or scattering material, from a nucleic-acid-free membrane. It is concluded that removal of DNA leads to morphological disruption of the whole envelope, which implies that DNA is an essential structural component of the system.

The destruction of morphological integrity by K^+, high ionic strength or DNAase treatment results in material which does not meet the primary criterion for a nuclear envelope preparation cited at the beginning of this report. It is reasonable to interpret all these effects as involving primarily the removal of DNA, and postulating that the pore complexes are stable structures only in the presence of DNA. When the pore complexes are destabilised by the DNA removal, the two membranous components of the envelope separate and fragment. Thus DNA is, on this interpretation, required to maintain the morphological organisation of the whole envelope system; it cannot be said to stabilise either one or the other of the component membranes.

The finding that nuclear envelopes are associated with significant amounts of DNA is consistent with the results of electron microscopic studies which have revealed apparent structural linkages between envelopes and chromatin fibrils in a range of mammalian and avian cells[20–22]. It is possible that this association has significance in DNA replication[23,24].

More detailed investigations of the nucleic acid components of the nuclear envelope, and of the ionic sensitivity of the morphology of the system, are in preparation.

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