ISOLATION AND CHARACTERIZATION OF NUCLEAR ENVELOPES AND THEIR BIOSYNTHESIS DURING THE CELL CYCLE

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Summary

Nuclear envelopes isolated from CHO cells were characterized by morphological and physicochemical methods and the biosynthesis of their protein constituents was examined at different stages of the cell cycle. Electrophoresis on SDS-polyacrylamide gels resolved the nuclear envelope into 47 polypeptide bands. Nine of them contained aminosugars. The banding pattern did not change noticeably. Only 5 bands showed a more pronounced fluctuation of the incorporation rate during the cell cycle. The results suggest that the nuclear envelope proteins are not synthesized de novo during mitosis but that the envelope is probably reassembled from preexisting membranes.

Previous studies on nuclear envelopes have either been done using exclusively morphological methods (1, 2) or - if biochemically oriented - have been restricted to only one stage of the cell cycle (G_o) because the nuclear envelopes had been isolated from non-proliferating cells such as rat liver cells (3) or bird erythrocytes (4). This communication reports the isolation of nuclear envelopes from synchronized cultures of CHO (Chinese hamster, ovarian line) cells, their characterization by morphological and physicochemical techniques and an analysis of the biosynthesis of their protein components at various stages of the cell cycle.

Methods

Cells: CHO cells were grown in suspension or as monolayer cultures in modified (5) Ham F-10 medium (GIBCO) supplemented with 10 % calf serum and 5 % fetal calf serum.

Synchronization and double labelling: The double label experiments were performed in order to relate amino acid incorporation into the nuclear envelope at any stage of the cell cycle to the incorporation throughout the cycle. In addition it facilitated comparisons between different experiments. Cells in suspension culture were grown for 80 hours in medium containing 2 µCi of L-[4,5-^{3}H]-leucine (6 Ci/mmol; Schwarz/Mann) per ml of medium yielding labelled cells in stationary phase (G_o; 5, 6). In order to prepare G_1-, S- and G_2-cells, a prelabelled stationary culture was diluted with an equal amount

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of fresh medium with 2 µCi ^{3}H-leucine/ml and incubated for another 3 (G_1) to 13 (G_2) hours. Mitotic cells were prepared by synchronizing monolayer cultures with colcemide (GIBCO); 7, 8). For pulse labelling the prelabelled culture was washed once in leucine-free medium at 37 °C and divided in two equal parts. One aliquot was resuspended in ^{3}H-leucine-containing medium, the other in leucine-poor, conditioned medium (8) containing 1 µCi of L-[U-^{14}C]-leucine (300 mCi/mmol; Schwarz/Mann) per ml of medium and per 10^6 cells for 30 minutes (for G_o-, G_1-, S- and G_2-cells) or 45 minutes for mitotic cells (i.e. until the nuclear envelope was reconstructed). Subsequently the two parts were combined and after a chase of 15 minutes with excess cold leucine the nuclear envelopes were isolated.

Isolation of nuclei and nuclear membranes: Nuclei were isolated according to Schildkraut and Maio (9) with the modification that the protease inhibitors phenylmethane sulfonyl fluoride (0.1 mM; Sigma) and sodium tetrathionate (1 mM; Fluka) were added to the buffer. The nuclear envelopes were prepared as described by Kay et al. (10) using 20 µg DNase I (bovine pancreas; Sigma) per 10^6 nuclei suspended in 1 ml of buffer.

Evaluation of the double label experiments: The nuclear envelopes were dissolved in SDS, reduced with 2-mercaptoethanol, fractionated on SDS-polyacrylamide slab gels and stained with Coomassie Brilliant Blue (12). Gels were cut into 0.5 mm thick slices and dissolved in NCS (Amersham-Searle; 13). The radioactivity was counted in a Packard Tri-Carb scintillation counter. ^{3}H-cpm were corrected for overlapping ^{14}C-cpm and the ^{14}C-cpm were normalized by multiplying by the fraction "total ^{3}H-cpm/total ^{14}C-cpm". The difference between normalized ^{14}C-cpm and corrected ^{3}H-cpm was calculated for each gel slice and expressed as percentage of the total number of corrected ^{3}H-cpm. This term is referred to as "partial specific synthesis" (PSS) and used as a measure for newly synthetized membrane protein per total membrane protein during a given interval in the cell cycle.

$$PSS = \frac{(^{14}\text{C-cpm}_{\text{norm.}} - {}^{3}\text{H-cpm}_{\text{corr.}}) \times 100}{\text{total } {}^{3}\text{H-cpm}_{\text{corr.}}}$$

The differences of PSS between subsequent steps in the cell cycle were added up (see legend to Table 1) for each of the 32 major bands and the mean PSS was calculated (0.41). Bands with a sum of differences in PSS of at least 50 % higher than 0.41 were considered as proteins with relatively high variations in their biosynthetic rate during the cell cycle.

Glycoproteins: Aminosugar-containing proteins were identified by electrophoresis of D-[6-^{3}H]-glucosamine (12 Ci/mmol; Amersham)-labelled membranes followed by autoradiography of the fixed gel (14).

Miscellaneous procedures: Electronmicroscopy was performed on OsO_4/glutaraldehyde-fixed and uranylactate-stained thin sections. The efficiency of the DNase treatment was evaluated by its ability to digest the DNA of ^{14}C-thymidine labelled cells. The density of the isolated nuclear envelopes was determined by isopycnic centrifugation in a linear metrizamide gradient (11) in phosphate buffered saline.

Results.

Electronmicrographs of isolated nuclei and nuclear membranes showed well preserved double membranes and pore complexes. Ribosomes were still attached

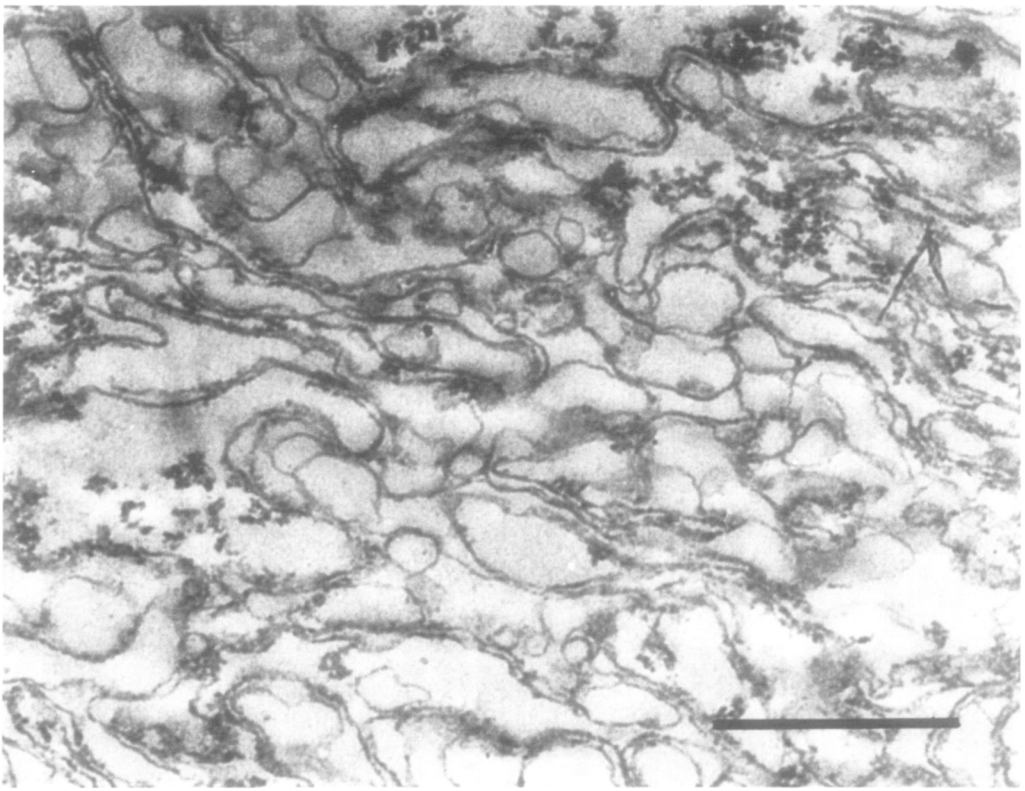

Figure 1: Electronmicrograph of isolated nuclear membranes. Horizontal bar = 1 μm

to the outer side of the outer nuclear membrane. Some nuclear material was still visible after DNase treatment, but no cytoplasmatic contaminants were discernible (Figure 1). The nuclear membrane preparation reduced 10 nanomoles of cytochrome c per minute and per mg of protein, which was in good agreement with low contamination values for the mitochondrial enzyme succinate- cytochrome c reductase (15) reported in the literature (16). 98% of the ^{14}C-labelled thymidine was solubilized by the DNase treatment yielding membranes with a buoyant density of 1.235 gr/cm^3.

Electrophoresis on SDS-polyacrylamide gels resolved the nuclear envelopes in 32 major and about 15 weakly stained polypeptide bands. The number and the relative intensity of the bands did not change noticeably during the cell cycle (Figure 2). Nine bands with apparent molecular weights of 25 K, 36 K, 48 K, 56 K, 68-72 K (double band), 100 K, 120 K, 145 K and 165 K Daltons contained substantial amounts of aminosugars.

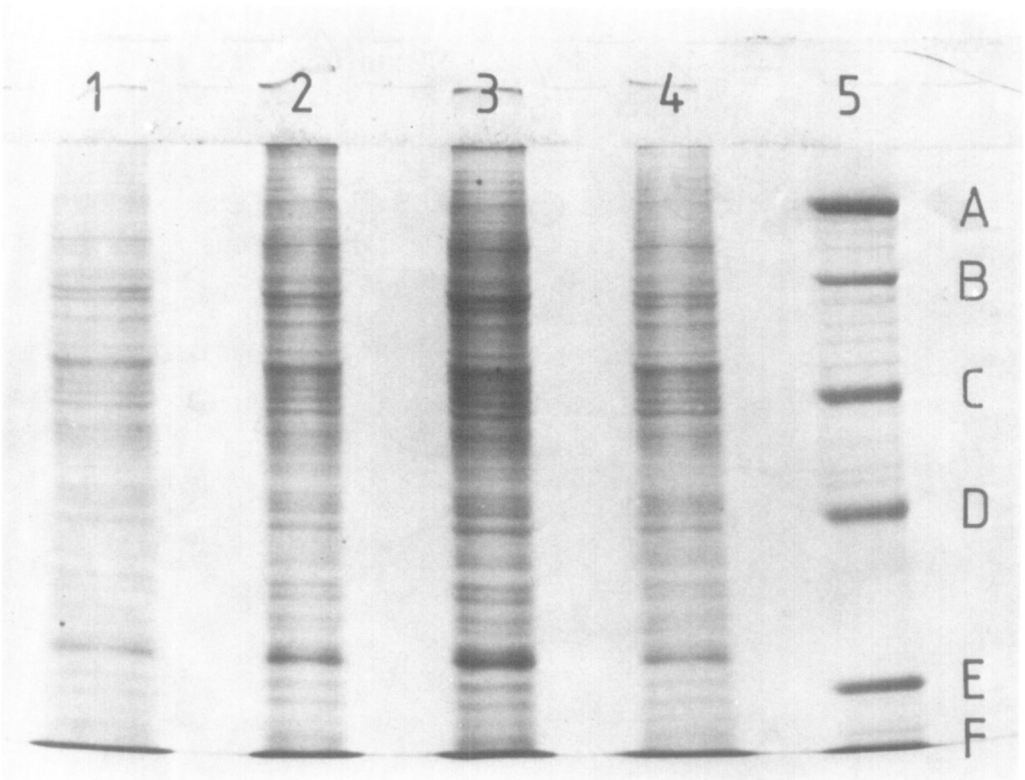

Figure 2: Polyacrylamide gel of nuclear envelopes at different stages in the cell cycle and protein markers:
1) G_o-phase (stationary culture)
2) mid G_1-phase (3 hours after dilution of the cell culture)
3) late G_1-/early S-phase (6½ hours after dilution)
4) late S-/G_2-phase (13 hours after dilution)
5) protein markers: heavy meromyosin (A; courtesy of Dr.B.Jockusch, Basel), β-galactosidase, monomer (B), BSA (C), ovalbumin (D), carbonic anhydrase (E), lysozyme (F).

The incorporation of ^{14}C-leucine during the pulse labelling was a linear function of time (8). The partial specific synthesis values (PSS) of the 32 major bands changed only slightly during the cell cycle and were lowest after mitosis (Figure 3). Only 5 bands showed a considerable variation (Table 1 and Figure 3). Band Nr. I had its highest synthetic rate during interphase and its lowest between metaphase and G_1. Nr. II was a faint double band. Its PSS was highest in G_o and decreased continuously during the cell cycle and reached a minimum between metaphase and G_1. Band Nr.III was heavily stained and had its highest PSS from early G_1 to early S and the lowest one in G_2. Band Nr.IV was

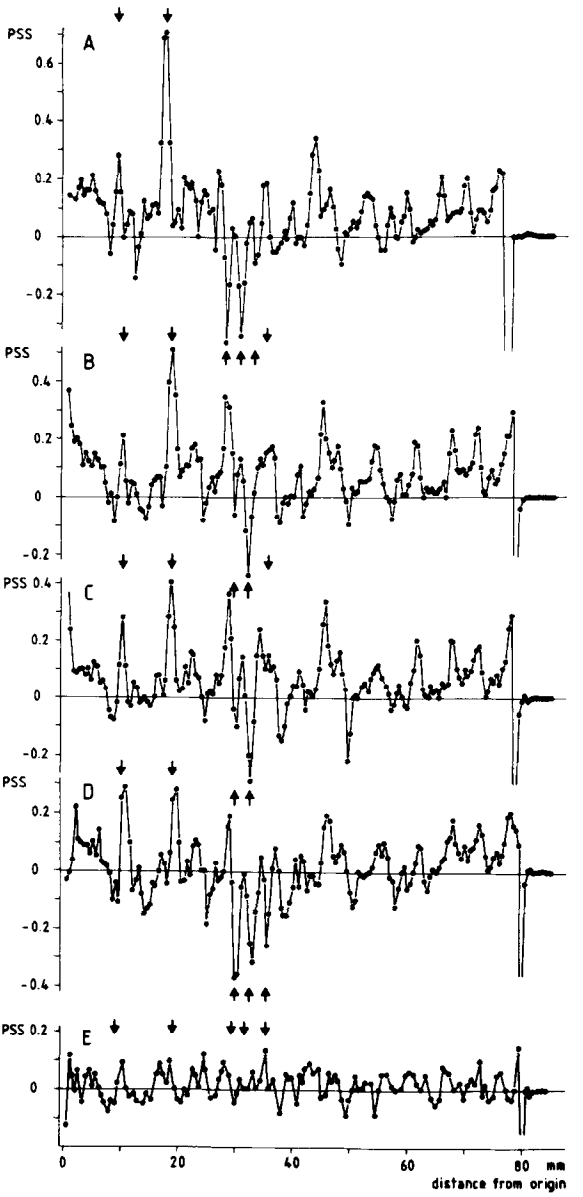

Figure 3: PSS values of nuclear envelopes in G_o (A), mid G_1 (B), late G_1/early S (C), late S/G_2 (D) and metaphase/early G_1 (E). The five arrows on each curve indicate the positions of the five bands with relatively high changes in biosynthesis during the cell cycle (corresponding to bands I to V in Table I).

a glycoprotein and showed generally very low PSS-values with a maximum between metaphase and G_1. Nr. V was a strong band and had its highest PSS from early G_1 to early S and the lowest one in G_2 (Table I). At any of the tested stages of the cell cycle the fraction "^{14}C-cpm / ^{3}H-cpm of the nuclear

Table I : Polypeptides with relatively high changes in biosynthesis during the cell cycle

Number	Molecular weight	PSS_{G_0}	PSS_M	PSS_{G_1}	PSS_S	PSS_{G_2}	$\Sigma\lvert\Delta PSS\rvert$	%above average
I	190 000	0.28	-0.05	0.20	0.28	0.24	0.74	180
II	130 000	0.70	0.10	0.50	0.40	0.24	1.04	253
III	80 000	-0.37	-0.03	-0.06	-0.10	-0.37	0.99	241
IV	72 000	-0.35	0.00	-0.28	-0.30	-0.30	0.67	163
V.	65 000	-0.07	0.15	0.15	0.15	-0.25	1.02	248

In order to evaluate the degree to which the biosynthesis of a particular band varied during the cell cycle, the changes of the biosynthetic activities between subsequent phases of the cell cycle of a given band were added up (second last column). In the last column total variabilities over the cell cycle of the 32 bands analyzed were averaged and expressed in percent of the average. 27 of the 32 bands had variabilities below 50% and are therefore not entered into the table. The absolute differences of the following PSS values were taken and added up: G_1-G_0, G_1-M, S-G_1, G_2-S and M-G_2.

membranes per ^{14}C-cpm / ^{3}H-cpm of the non-nuclear membranes" of the same preparation (8) was close to 1 and did not differ more than 6% from experiment to experiment.

When protein synthesis was suppressed to 0.4% of its normal value by the addition of cycloheximide (80 µg/ml, added in G_2 or in metaphase), the number of cells entering mitosis was not affected and the normal timecourse of mitosis was not disturbed. When lipid synthesis was suppressed by the use of delipidated serum (17) and the addition of avidin (1 unit/ml) to the medium, reconstruction of the nuclear envelopes could still be observed under the light microscope; however, cytokinesis did not take place and karyokinesis was observed in only 10% of the total cell population (resulting in cells with either one large or two small nuclei) although viability was 99% as determined by uptake of Trypan Blue.

Discussion.

Since it was our intention to examine possible differences in the biogenesis of all possible nuclear envelope proteins, we chose mild purification procedures in order to minimize loss of peripheral proteins.

The banding pattern of isolated nuclear envelopes of CHO cells was similar to the one reported for rat liver (3) in having the major part of the proteins concentrated at positions corresponding to apparent molecular weights of 53 K and 64-78 K. One band with an apparent molecular weight of 30 K had the same R_f-value as the lysine-rich histone H1 (isolated from rat liver; courtesy of Dr.M.Noll,Basel), which represents a substantial portion of the

intranuclear protein. Assuming that rabbit and hamster ribosomes are very similar, an interference of the ribosomal proteins with the banding pattern of nuclear envelopes seems unlikely: Rabbit ribosomes consist of 71 different proteins in equimolar amounts with molecular weights between 8 K and 58 K Daltons (18). We detected in this range only 15 bands and they varied in relative intensity.

The double label experiments and the experiments with inhibited protein and lipid synthesis lead us to the conclusion that the biogenesis of the nuclear envelope is quite evenly distributed in the cell cycle and that the nuclear envelope is reassembled from preformed or persisting components after mitosis and not synthesized de novo. The break-down products could either persist in the cytoplasm to be reused in the assembly of the nuclear envelopes of the daughter cells or they could become part of the endomembrane system and partitipate in its normal turnover. Elements of the endoplasmatic reticulum would in this case be the most probable basic building blocks of the new envelope. Whether these elements are integrated in "free" molecular or in micellar form - as proposed for the blue green alga Acetabularia (19) - or as vesicles, will remain an interesting question for future work.

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