tissue[15,17,18]. In squash preparations of these cells we isolated diploid, tetraploid and, rarely, octoploid nuclei in mitosis. After silver staining, the NORs were exclusively dotted in all identifiable SAT-chromosomes of more than 60 metaphase complements. The number of double-dotted silver regions correlated with the number of nucleolar chromatids in diploid as well as in tetra- and octoploid chromosome complements. The homologous double dots corresponded to each other in size as well as in staining intensity.

The data from the two series of investigations resolve the question about the frequency of occurrence of chromatids with active NORs in polyploid nuclei. In restitution as well as in endopolyploid nuclei the occurrence of double-dotted silver regions correlated with the number of nucleolar chromosomes at each ploidy degree. Furthermore, the chromatid silver dots of a complement were uniform in size and staining intensity. Two preliminary conclusions can be drawn, based on the hypothesis brought forward by Schwarzacher et al.[19] that the amount of stainable material is proportional to the size of the dot as such, and that equal sized dots in all chromatid NORs indicate equal amounts of active rRNA genes. The first conclusion is that proportional multiplication of nucleolar chromatids takes place during polyploidization by C-mitosis and endomitosis. The second conclusion is that nucleolus organizing activity of all chromatid rDNA gene sites of a polyploid genome is equal as soon as replication has finished. This interpretation about dosage of rRNA genes and their activity, however, was done by visual examination of size and staining intensity of the Ag-dots in microscopic specimens. Although investigations with *Vicia sativa* show that differences in the amount of rRNA genes up to a factor of three are recognized by Ag-staining[20], these quantitative aspects have to be confirmed by techniques that are more precise.

Our observations are comparable to those of Buys et al.[21] and Howell[2], who studied NOR activity in endoreduplicated metaphase complements of human lymphocytes and fibroblasts by Ag-methods. The results are in agreement; they interpreted quadruple silver dots in diplochromosomes as being due to uniform replication of active NORs. The rate of replication of heteromorphic NORs in different polyploid tissues of the dipteran *Calliphora erythrocephala* was tested with the rRNA filter hybridization method by Belikoff and Beckingham[22]. The authors reported that both genomic NORs were replicated in the course of polyploidization. As a result of the high sensitivity of their method they revealed, however, variations in the extent of cistron replication from the two NORs by individual nuclei. Quantification of the rRNA gene number after polyploidization was done by Oishi et al.[23] as well by 18/25S rRNA filter hybridization. Their experiments indicate a reduction of the rRNA gene number by about one third in maturing female locust *(Locusta migratoria)* fat body (8- to 16-ploid). They attributed this reduction to the reduced replication of rRNA genes within the NORs or to the non-replication in some of the fat body cells. From these results it becomes evident that more detailed analyses combining experiments on the cytological localization of gene sites with gene quantification in tissues of different ploidy degrees and developmental states are necessary to resolve the question of proportional multiplication as well as that of the activity of rRNA genes after polyploidization.

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Complementary replica of freeze-fractured human lymphocyte nuclei

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Summary. Complementary replicas of freeze-fractured human lymphocyte nuclei were obtained, and were studied with the electron microscope. Complementarity was evident in cross-fractured nuclear pore complexes and in particle-free membrane areas. Explanations for the observed structural peculiarities are considered.
Key words. Lymphocytes; nuclear membranes; particle-free areas; nuclear pore complexes; complementary replica; freeze-fracture.

In multicellular organisms the cytoplasm and the nucleoplasm of the cells are separated from each other by the nuclear envelope, which consists of two membranes, the outer and the inner nuclear membrane, and are connected with each other by the pores in the nuclear envelope, the so-called nuclear pore complexes. Although a host of data have been obtained by studying various cell types with quite a few different methods, the functional significance of the nuclear membranes and the nuclear pore complexes is only vaguely known[2,3]. The observations reported here are a by-product of an electron microscopical investigation which was undertaken in order to find qualitative signs of early radiation damage in the membranes of X-irradiated lymphocytes. With doses up to 20 Gy no radiation damage was observed; however, pictures of the nuclear membranes were obtained showing more details than an earlier comparable study did[4], and some of the pictures are now presented in this report.

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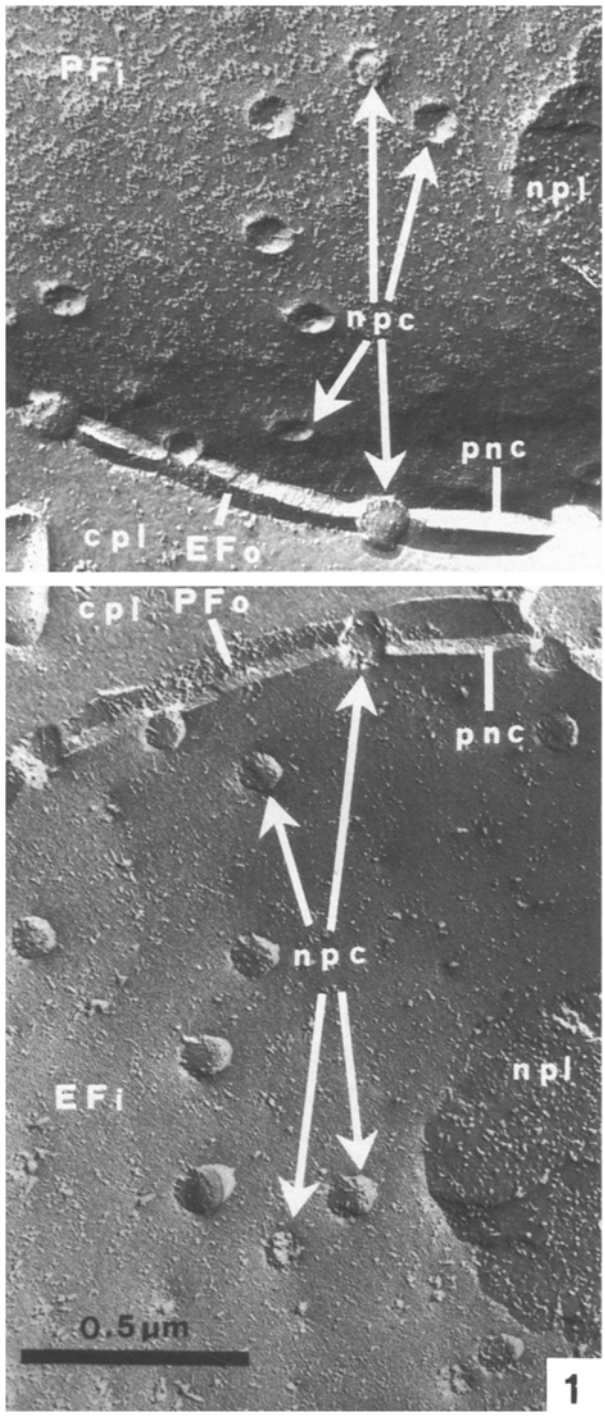

Figure 1. Complementary replicas (above and below) showing a portion of a freeze-fractured human blood lymphocyte. Complementary structures coincide when the figure is folded along the horizontal axis between the two pictures. Departures from the expected symmetry were thought to be due to the variable tilt among the replicas relatively to the beam of the electron microscope which could not be corrected for. In complementary replicas of cell nuclei all four half membrane leaflets of the two nuclear membranes constituting the nuclear envelope can be seen: the protoplasmic (PFi) and the exoplasmic face (EFo) of the inner and the outer nuclear membrane, respectively (upper picture), and the exoplasmic (EFi) and the protoplasmic face (PFo) of the inner and the outer nuclear membrane, respectively (lower picture). Inner and outer membrane enclose the perinuclear cisterna (pnc). Pores in the nuclear envelope, so-called nuclear pore complexes (npc, in fig. 1 only four were labeled with arrows), provide the connection between cytoplasm (cpl) and nucleoplasm (npl).

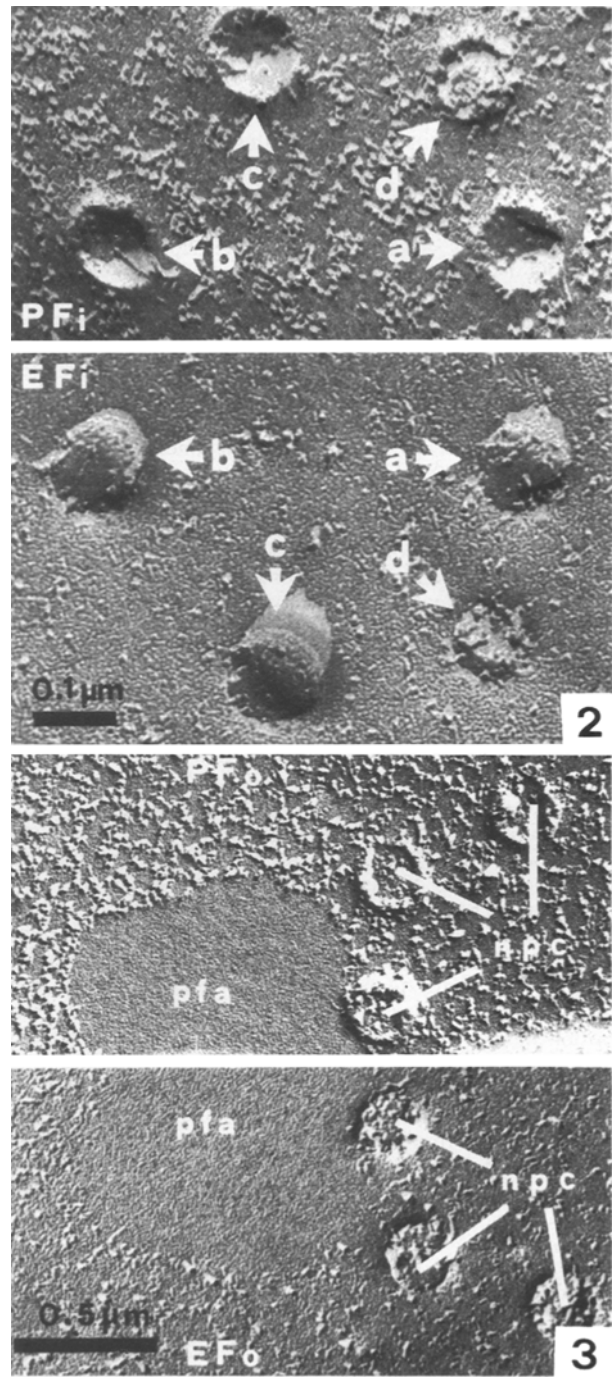

Figure 2. Enlarged views of corresponding areas of the nucleus shown in figure 1, displaying the protoplasmic (PFi) and the exoplasmic face (EFi) of the inner nuclear membrane. Numerous membrane particles (not labeled) with diameters around 10 nm and four cross-fractured nuclear pore complexes (arrows a–d) can be seen. Membrane pits complementary to the membrane particles are difficult to see. However, the fracture path through the nuclear pore complexes shows complementarity independently of the cross-fracture level, either peripherally (arrows a–c), or more centrally (arrow d).

Figure 3. Complementary replicas showing a portion of another freeze-fractured human blood lymphocyte nucleus. On freeze-fractured nuclear membranes areas completely free of membrane particles, so-called particle-free areas, were occasionally observed. The pictures of this figure illustrate the complementarity of the particle-free areas (pfa, above and below) on the corresponding half membrane leaflets (PFo, protoplasmic, and EFo, exoplasmic face of the outer nuclear membrane). npc, nuclear pore complexes.

Materials and methods. Several blood samples were taken from two healthy human donors. The fresh blood was treated with heparin (50 UPS-units/ml), diluted with two parts of Hanks's Balanced Salt solution (HBS)[5] at pH 7.40, and layered over Ficoll-Paque solution[6]. The subsequent centrifugation (400 × g during 20 min at 20 °C) yielded a rather pure lymphocyte fraction, as was monitored by blood counts. After washing the lymphocyte fraction with HBS, another centrifugation (150 × g during 30 min at room temperature) gave a lymphocyte pellet which was resuspended in cold 0.30 glycerol-HBS. From now on, up to the storage of the frozen specimens, the preparation took place in a refrigerator (1–4 °C). The pellet obtained from one more centrifugation (250 g during 20 min) was resuspended in a small amount of 0.30 glycerol-HBS. The lymphocyte-rich cell suspension was distributed among several grid-sandwiches each consisting of two golden electron microscope grids with matching bars. Then each grid-sandwich was placed between two copper discs, frozen in melting Freon 22, and stored in liquid nitrogen until freeze-fracturing. For freeze-fracturing the sandwiches were mounted on a liquid nitrogen-cooled complementary replica device[7]. Then the replica device was mounted on the cold stage (−150 °C) of a Balzers freeze-etch unit (BAF 300), and as soon as a vacuum of 5×10^{-7} mbar was reached, the platinum-carbon evaporation began, and the replica device was opened. After shadowing (3 nm platinum-carbon) and coating (20 nm carbon) the grid-sandwiches which had been split apart were cleaned in 0.14 sodium hypochlorite solution and in distilled water. Pictures of the complementary replicas were taken with a Hitachi electron microscope (HU-11E-1).

Results and discussion. Frozen biological membranes tend to split along their hydrophobic mid-plane during the freeze-fracturing process, yielding two half membrane leaflets[8]. By using a complementary replica device both half membrane leaflets are preserved and can be studied with the electron microscope[7]. Since the nuclear envelope consists of two membranes, namely the outer and the inner nuclear membrane, four half membrane leaflets can be seen on complementary replicas (fig. 1): The protoplasmic (PFo) and the exoplasmic face (EFo) of the outer, and the protoplasmic (PFi) and the exoplasmic face (EFi) of the inner nuclear membrane, respectively (nomenclature of Branton et al.[9]). The two membranes have a varying distance from each other and enclose the perinuclear cisterna (pnc). Nuclear pore complexes can be seen on either face (npc).

Ideally the pictures obtained from complementary replicas should show perfect complementarity. Two limitations ought to be mentioned here, though: 1) Some pictures obtained from complementary replicas were slightly distorted (fig. 1, upper picture), whereas others were less so (fig. 1, lower picture). The distortions were thought to be due to a tilt of the replicas relatively to the beam of the electron microscope which could not be corrected for. 2) The membrane pits corresponding to the membrane particles on the complementary half membrane leaflet are difficult to see within the grains of the replica background (cf. fig. 2). Aside from these two limitations, complementarity was evident with respect to both the nuclear pore complexes and the nuclear membranes: A) The fracture path through the nuclear pore complexes showed complementarity independently of the cross-fracture level at which the nuclear pore complexes were fractured, either peripherally (fig. 2, arrows a–c), or more centrally (fig. 2, arrow d). It may be of interest to point out that the peripheral cross-fracture (a term based on the interpretation proposed by Severs and Jordan[10]) of the nuclear pore complex yields a dome-shaped plug on the exoplasmic and a complementary depression on the protoplasmic face, respectively. One is tempted to assume a dome-shaped boundary between the nuclear pore complexes and the adjacent nucleo- or cytoplasm which guides the fracture. The true nature of this boundary remains to be shown, however. B) The particle-free areas which were occasionally observed on the nuclear membranes showed complementarity among complementary replicas (fig. 3, pfa). It is known from studies of artificial as well as of biomembranes that the lipids of these systems can undergo reversible fluid-solid transition when the temperature is dropped below a critical level (see Wunderlich et al.[11], and references contained therein). As a consequence, chilling of cellular membranes can result in the exclusion of the membrane particles from the frozen lipid domains yielding particle-free membrane areas, like the ones observed in the investigation reported here.

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