Discussion. The clonal insulin-producing cell line, RINm5F, does not display a normal secretory response to glucose, but responds with a significant release of insulin after stimulation by other secretagogues including high concentrations of K^{+}[2,4]. In view of the defective secretory response to glucose this tumor cell line might be used as a model for the pancreatic β-cells in non-insulin dependent diabetics.

In attempts to further characterize possible changes in the ultrastructural morphology associated with secretion, virus-like particles were identified in the RINm5F cells. When investigating RINm5F cells also from other laboratories similar virus-like particles were identified. These latter findings suggest that the virus-like particles identified in our RINm5F cells are not simply due to an infective contamination.

The virus-like particles could not be identified in samples of the original transplantable tumor, obtained from three different sources. The fact that it is not possible, at least by morphological means, to identify virus-like particles in the original tumor may indicate that these were induced in the RINm5F cell line at some later stage. Whether the virus-like particles were induced by processing the original tumor cells[13] through the athymic nude mouse or by the cloning procedure[12] will be the subject of further investigations.

Previous studies have shown that multiple injections of streptozotocin into mice resulted in a syndrome characterized by diabetes mellitus, insulitis and the induction of endogenous C type virus in pancreatic β-cells[14]. Although more detailed investigations are needed, preliminary characterization strongly indicates that the virus-like particles expressed in RINm5F cells are C type viruses.

We have no evidence, so far, that the functions of the RINm5F cells are affected by the presence of the viruses but all investigators using this line should be aware of their possible existence. Furthermore, from the present study it is obvious that this cell line is not a suitable candidate for use in investigations of the cellular effects of viral infections[15].

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2 Gylfe, E., Andersson, T., Rorsman, P., Arbrahamsson, H., Arkhammar, P., Hellman, P., Hellman, B., Oie, H.K., and Gazdar, A.F., Biosci. Rep. *3* (1983) 927.
3 Halban, P.A., Praz, G.A., and Wollheim, C.B., Biochem. J. *212* (1983) 439.
4 Praz, G.A., Halban, P.A., Wollheim, C.B., Blondel, B., Strauss, A.J., and Renold, A.E., Biochem. J. *210* (1983) 345.
5 Rorsman, P., Berggren, P.-O., Gylfe, E., and Hellman, B., Biosci. Rep. *3* (1983) 939.
6 Wollheim, C.B., and Pozzan, T., J. biol. Chem. *259* (1984) 2262.
7 Abrahamsson, H., Berggren, P.-O., and Hellman, B., Am. J. Physiol. *247* (1984) E719.
8 Di Virgilio, F., Pozzan, T., Wollheim, C.B., Vicentini, L.M., and Meldolesi, J., J. biol. Chem. *261* (1986) 32.
9 Hoftiezer, V., Berggren, P.-O., and Hellman, B., Biomed. biochim. Acta *44* (1985) 77.
10 Hoftiezer, V., Berggren, P.-O., and Hellman, B., Cancer Lett. *27* (1985) 7.
11 Hoftiezer, V., Berggren, P.-O., and Hellman, B., Cancer Lett. *29* (1985) 15.
12 Gazdar, A.F., Chick, W.L., Oie, H.K., Sims, H.L., King, D.L., Weir, G.C., and Lauris, V., Proc. natn. Acad. Sci. USA *77* (1980) 3519.
13 Chick, W.L., Warren, S., Chute, R.N., Like, A.A., Lauris, V., and Kitchen, K.C., Proc. natn. Acad. Sci. USA *74* (1977) 628.
14 Appel, M.C., Rossini, A.A., Williams, R.M., and Like, A.A., Diabetologia *15* (1978) 327.
15 Maratos-Flier, E., Goodman, M.J., Fields, B.N., and Kahn, C.R., Endocrinology *116* (1985) 2430.

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Multiplied nucleolus organizer regions (NORs) in polyploid nuclei of *Vicia faba* revealed by ammoniacal silver staining

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Summary. The number of chromatids with transcriptionally active NORs in 4-, 8-, and 16-ploid restitution nuclei, as well as in endopolyploid nuclei with ploidy degrees of 4 and 8, were investigated by Ag-staining. In the genomes studied, the frequency of Ag-dots was equal to the number of chromatid NORs.

Key words. Vicia faba; endopolyploidy; nucleolus organizer region; silver staining; restitution nucleus.

The technique of ammoniacal silver staining is a standard method for staining chromosomal sites of rDNA (nucleolus organizer regions)[1,2], which are actively involved in rRNA transcription during the preceding interphase[3]. Chromosomal sites of rRNA activity can be recognized by dark brown silver precipitates. In late metaphase, when both chromatids of a nucleolar chromosome are separated, both are silver-dotted in the NOR. These double dots give evidence for rRNA gene activity of the parental codogen DNA-strand in one chromatid and of the just-replicated filial codogen DNA-strand in the other chromatid. Thus, the method offers the means of measuring the number of chromatids with transcriptionally active NORs in individual cells. This possibility complemented the investigation in the genome of *Vicia faba* to study proportional multiplication and transcriptional activity of chromatid NORs during somatic polyploidization. We first examined 4-, 8- and 16-ploid restitution nuclei of root tip meristems after colchicine treatment, and then endopolyploid nuclei with ploidy degrees of 4 and 8 of differentiated epidermis cells in the shoot axis. From the frequency and size of multiple chromatid Ag-dots in all types of nuclei investigated we conclude that all of the genomic NORs are proportionally multiplied and transcriptionally active during the course of somatic polyploidization.

Material and methods. Plants: Seeds of *Vicia faba* L. var minuta 'Kleine Thüringer', 2n = 12, were purchased locally.

Root tip meristem: Seeds were germinated in a desiccator at 19 ± 1 °C for 80 h. To increase the number of metaphases roots were immersed in a well-aerated colchicine bath, of concentration 0.05%, for 2 h. To induce restitution nuclei, roots were immersed in a colchicine bath, concentration 0.005%, to grow for 20, 40, 80 and 100 h respectively, as described by Deka and Sen[4]. Subsequently, primary root tips of 5 mm in length were harvested.

Fig. 1. Silver-stained diploid metaphase complement of *Vicia faba* root tip meristem. Two pairs of chromosomes are double-dotted. The silver precipitates are uniform in size and staining intensity and confined to the NORs of both chromatids (× 1200).

Epidermis and subepidermis cells of the shoot axis: Bean plants were cultivated in a greenhouse for 5–6 weeks at 20 °C. The shoot axis of actively growing plants was transversally hurt by a cut with a scalpel in young internodia. A wedge-shaped piece of regeneration tissue was isolated from the lip of the wound 4, 6 and 8 days after wounding.

Chromosome spreading: Root tips and regeneration tissues of the shoot axis were fixed in 1:3 glacial acetic acid – 99.9% ethanol at 4 °C for 30 min. Specimens were passed through a descending ethanol series (70, 50, 30 and 15%, 5 min each) and washed in distilled water for 5 min. For enzymatic maceration we modified the formula of Hizume et al.[5]: 4% cellulase (Onozuka R-10; Serva), 4% macerocym (R-10; Serva) in distilled water, pH adjusted to 4.0 with HCl, incubation at 37 °C. The treatment lasted for 30 to 60 min depending on the consistency of the material. After rinsing the partial digest with distilled water a squash preparation was made using the dry ice method.

Staining: 50 µl of 50% $AgNO_3$-solution, prepared as previously described by Burger and Knälmann[6], was layered on the squash preparation and covered with a nylon slip (mesh size 150 µm), which had been immersed in the same solution as recommended by Kodama et al.[7]. The specimens were then placed in a moisture chamber and incubated for 20–30 min at 50 °C. Rinsing the slides thoroughly under running twice-distilled water terminated the staining and removed the nylon slip. The slides were bathed for a further 30 min in twice-distilled water and then dried by passing them through an ascending ethanol series before mounting in Euparal.

Results and discussion. After Ag-staining of chromosomal spreads, active rRNA gene sites can be identified by dark brown silver precipitates that contrast well with the yellowish stained metaphase chromosomes. As soon as sister chromatids can be distinguished in advanced mitosis, both carry their own precipitates. Figure 1 shows such a typical double-dotted chromosome pair. The silver dots are located in the secondary constriction (SC) of the satellite (SAT)-chromosome pair[5,8–12], which is known to be the single gene site of 18/25S rDNA in this species[13]. The Ag-dots studied in well-spread metaphase complements are always uniform in size and staining intensity. This finding confirms observations we made by using a Giemsa staining technique[14], but it is contrary to that of Mehra et al.[12], who described the two Ag-stained NORs as heteromorphic.

To elucidate the question of proportional multiplication and transcriptional activity of chromatid NORs in polyploid nuclei, we did not look for the moderately polyploid nuclei in the primary root of *V. faba*[15], particularly because these single nuclei are in interphase stages[16]; instead, we induced restitution nuclei by colchicine treatment. During a permanent treatment up to 100 h, polyploidization took place in geometric progression of 2n to 4n to 8n to 16n (Fig. 2), as already described by Deka and Sen[4]. After silver staining, silver precipitates over chromosomes were recognizable in the metaphase complements of each ploidy degree. In more than 50 metaphase stages with a ploidy degree of 4n and in at least 30 metaphase stages with ploidy degrees of 8 or 16n, we counted 4, 8 and 16 stained regions, respectively. The number correlated with the number of multiplied SAT-chromosomes. In the tetraploid metaphases we could localize the four silver precipitates in the SC of the four SAT-chromosomes. In nuclei of the higher ploidy degrees the morphological discrimination of the stained regions was not possible in all dotted chromosomes of a metaphase complement, because some of the chromosomes were always clumped or the chromatids were insufficiently separated. However, the silver-stained regions that could be identified were exclusively the SCs of SAT-chromosomes. Moreover, all SAT-chromosomes that could be discriminated were stained; the precipitates were exclusively confined to the SCs. Considering the count values as well as the localizations, we conclude that all nucleolar constrictions in the polyploid genomes under investigation were silver-dotted. In all of the polyploid metaphase complements studied, nucleolar sister chromatids which were well-spread were double-dotted; sometimes the two precipitates formed dumb-bells. The dots were equal in size and in staining intensity as visualized by light microscopy.

In a second series of investigations we studied polyploid nuclei of another type. These were isolated from epidermis and subepidermis cells of the peripheral shoot axis of *V. faba* greenhouse plants. During the process of differentiation, nuclei of these cells undergo somatic polyploidization by endomitosis. If mitosis is subsequently stimulated by wounding cells, the level of endopolyploidization can be discriminated in the regeneration

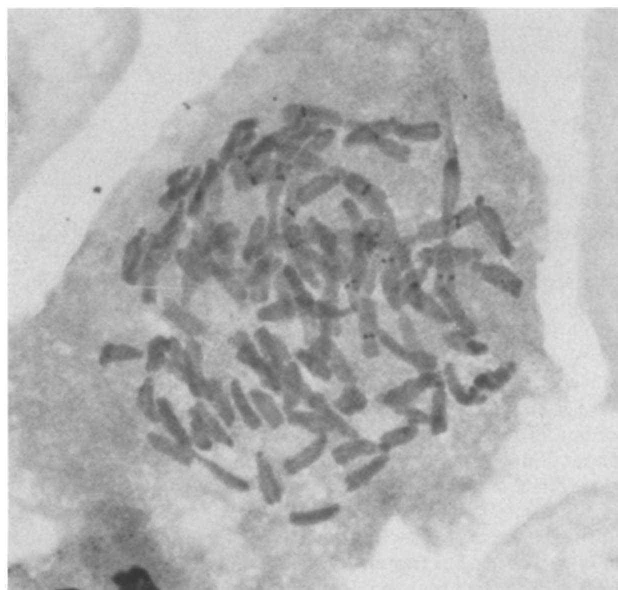

Fig. 2. Silver-stained 16-ploid metaphase complement of *Vicia faba* root tip meristem after 80 h colchicine treatment (0.005%). Eight pairs of chromosomes are double-dotted or the dots form confluent blocks (× 600).

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.

1 Goodpasture, C., and Bloom, S.E., Chromosoma *53* (1975) 37.
2 Howell, W.M., The Cell Nucleus, vol. 11, p. 89. Ed. H. Busch. Academic Press, New York 1982.
3 Miller, D.A., Dev, V.G., Tantravahi, R., and Miller, O.J., Expl. Cell Res. *101* (1976) 235.
4 Deka, P.C., and Sen, S.K., Can. J. Genet. Cytol. *15* (1973) 863.
5 Hizume, M., Sato, S., and Tanaka, A., Stain Technol. *55* (1980) 87.
6 Burger, E.-Ch., and Knälmann, M., Pl. Syst. Evol. *142* (1983) 117.
7 Kodama, Y., Yoshida, M.C., and Sasaki, M., Jap. J. hum. Genet. *25* (1980) 229.
8 Schubert, I., Anastassova-Kristeva, M., and Rieger, R., Expl. Cell Res. *120* (1979) 433.
9 Schubert, I., and Rieger, R., Biol. Zbl. *99* (1980) 65.
10 Hizume, M., Tanaka, A., and Miyata, Y., Bot. Mag. Tokyo *95* (1982) 81.
11 Friebe, B., Microsc. Acta *87* (1983) 49.
12 Mehra, R.C., Breskus, S., and Butler, M.G., Can. J. Genet. Cytol. *27* (1985) 255.
13 Knälmann, M., und Burger, E.-Ch., Chromosoma *61* (1977) 177.
14 Burger, E.-Ch., und Scheuermann, W., Cytobiologie *9* (1974) 23.
15 Fenzl, E., and Tschermak-Woess, E., Österr. Bot. Z. *101* (1954) 140.
16 Scheuermann, W., Cytobiologie *17* (1978) 232.
17 Resch, A., Chromosoma *5* (1952) 296.
18 Linnert, G. (Ed.), Cytogenetisches Praktikum. G. Fischer Verlag, Stuttgart/New York 1977.
19 Schwarzacher, H.G., Mikelsaar, A.-V., and Schnedl, W., Cytogenet. Cell Genet. *20* (1978) 24.
20 Burger, E.-Ch., and Knälmann, M., Eur. J. Cell Biol. *21* (1980) 313.
21 Buys, C.H.C.M., Osinga, J., and Anders, G.J.P.A., Mech. Ageing Dev. *11* (1979) 55.
22 Belikoff, E.J., and Beckingham, K., Genetics *111* (1985) 325.
23 Oishi, M., Locke, J., and Wyatt, G.R., Can. J. Biochem. Cell Biol. *63* (1985) 1064.

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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.