onstrate that the soluble sperm fraction, rather than the added KCl or the act of injection, causes elevation of the fertilization membrane.

Before concluding that the fertilization membrane was triggered by a soluble component of sperm, it is necessary to consider the possibility that the causative agent was injected calcium. Previous injection experiments have indicated that the threshold for obtaining elevation of the fertilization membrane is very dependent on whether or not calcium buffer is added to the injectate[5]. Presumably, when the calcium is unbuffered, the internal calcium concentration is reduced by the natural calcium sequestering system of the cells. When calcium buffer is added, the threshold is 0.2 μM calcium in sea-urchin eggs[5]. The threshold without added buffer has not been measured in sea-urchin eggs, but in frog oocytes it is 0.3 μg of calcium[8]. Taking into consideration the volume ratio between the two preparations, that corresponds to about 300 pg for sea-urchin eggs. The amount of calcium in our soluble sperm fraction injectate was less than 0.1% of this amount (less than 0.2 pg), and is, therefore, unlikely to cause elevation of the fertilization membrane. We tested this point directly, however, by injecting into sea-urchin eggs between 0.2 and 2 pg of unbuffered calcium to which KCl was added to adjust the osmolarity. In this range of calcium, in 17 trials there was no response, and in three trials there was only an unpropagated local response over less than 10% of the egg surface. This demonstrates that the calcium in our soluble sperm fraction is not the trigger for propagation of cortical granule exocytosis.

This conclusion is consistent with two other results. When we reduced the free calcium concentration in the soluble sperm fraction to less than 50 μM by adding EGTA, this soluble sperm fraction was as effective in raising fertilization membranes as was the fraction without EGTA. Also, in the batches of soluble sperm fraction that were ineffective, the calcium concentration was the same as in those that were effective.

Since the soluble sperm fraction caused partial or full elevation of the fertilization membrane in every egg of the first six batches into which it was injected, and control injections of KCl never caused any elevation of the fertilization membrane, we conclude that the soluble sperm fraction contains a trigger for the initiation of cortical granule exocytosis. Although these results do not completely rule out the possibility that the trigger reacts with the egg membrane, they do provide evidence that any putative egg-membrane-mediated reaction is not activated from the external side of the membrane. This conclusion is based on our experiments demonstrating activity when the sperm factor is injected directly into the cytoplasm and on Hiramoto's experiments[6] demonstrating that the sperm factor is not membrane-permeable.

Increased cytosolic calcium is responsible for other important egg-activation processes in addition to cortical granule exocytosis. One of these is an increase in intracellular pH, leading to cytoskeletal reorganization, increased protein synthesis, and initiation of DNA synthesis[9]. Another is the activation of NAD kinase, leading to the regulation of sugar metabolism[9]. Thus, fertilization-membrane formation can be regarded as an assay for the more general processes of cytosolic calcium increase and egg activation. A likely source of internal calcium is the tubular reticulum recently described[10].

The idea that cortical granule exocytosis and egg activation are triggered by a component of sperm that enters the egg cytoplasm was suggested more than 70 years ago[11,12]. In other models for triggering, the effect of the spermatozoon on the external surface of the egg membrane was emphasized[13,14]. In this paper we have demonstrated that injection alone can trigger cortical granule exocytosis in sea-urchin eggs. If this is also true for mammals, the soluble component of sperm that triggers exocytosis, leading to a block to sperm entry, might be useful as an agent to prevent fertilization.

Acknowlegment. We thank Dr Mitzi Canessa for determining cation concentrations of the sperm fractions by atomic absorption.

1 Schuel, H., Gamete Res. *1* (1978) 299.
2 Vacquier, V.D., Devl Biol. *43* (1975) 62.
3 Zucker, R.S., and Steinhardt, R.A., Biochim. biophys. Acta *541* (1978) 459.
4 Baker, P.F., Knight, D.E., and Whitaker, M.J., Proc. R. Soc. Lond. *B 207* (1980) 149.
5 Hamaguchi, Y., and Hiramoto, Y., Exp. Cell Res. *134* (1981) 171.
6 Hiramoto, Y., Exp. Cell Res. *27* (1962) 416.
7 Patton, J., and Reeder, W., Analyt. Chem. *28* (1956) 1026.
8 Hollinger, T.G., and Schuetz, A.W., J. Cell Biol. *71* (1976) 395.
9 Epel, D., in: Ions, Cell Proliferation, and Cancer, p.327. Academic Press, New York 1982.
10 Poenie, M., Patton, C., and Epel, D., J. Cell Biol. *95* (1982) 161a.
11 Robertson, T.B., Arch. Entwicklungsmech. *35* (1912) 64.
12 Loeb, J., in: Artificial Parthenogenesis and Fertilization. University Press, Chicago 1913.
13 Lillie, R.S., and Baskervill, M.L., Am. J. Physiol. *61* (1922) 272.
14 Mazia, D., Schatten, G., and Steinhardt, R., Proc. natn. Acad. Sci. USA *72* (1975) 4469.

0014-4754/85/081068-03$1.50 + 0.20/0

Nucleolus DNA synthesis in *Vicia faba* root-tip meristems

R.E. Rowland

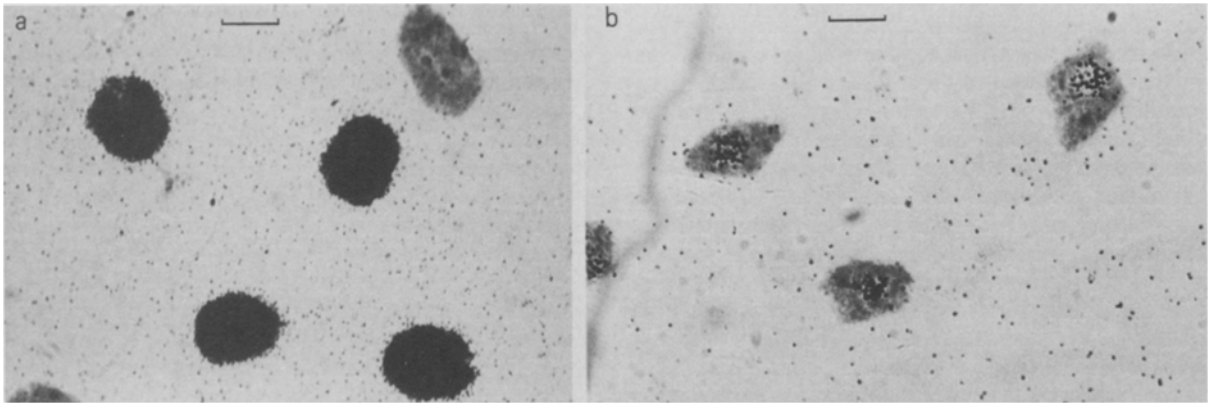
Botany and Zoology Department, Massey University, Palmerston North (New Zealand), 6 July 1984

Summary. Autoradiographs of *Vicia faba* lateral root-tip meristems were prepared following a 30 min pulse with ^{3}H-thymidine. 1/3 of all interphase nuclei in the meristem are labeled, most with a uniform distribution of silver grains. 2–5% of labeled nuclei show specific nucleolus DNA labeling.

Key words. Vicia faba; nucleolus; amplification; ^{3}H-thymidine; ribosomal DNA.

The existence of developmentally regulated gene amplification is well known. The nucleolar rDNA of the *Xenopus* oocyte nucleus is some 1400–2000-fold more abundant than the rDNA of a somatic nucleus[1]. DNA puffs have been studied extensively in *Rhynchosciara*[2-4]. More recent studies of insect chorion multigene families have revealed similar results: in *Drosophila* only 1–3 copies of one gene set are present in germ and somatic cells, but they are amplified up to 25 copies in the ovarian follicle cells producing chorion proteins[5]. Amplification of actin gene sequences has been discovered during chick myogenesis[6]. In addition, recent studies of cultured animal and human cells have related resistance of the antitumor drug methotrexate to spontaneous gene amplification[7].

The present study reports the presence of specific nucleolus



Autoradiograph of root-tip cells pulsed with ^{3}H-thymidine for 30 min: *a* Nuclei with a uniform distribution of silver grains, *b* nuclei with specific localization of silver grains over nucleoli. Bar = 10 μm.

DNA labeling of *Vicia faba* interphase nuclei in root-tip meristems. Increase in the number of ribosomes is greater in the root-tip meristems of higher plants than in differentiated regions[8] and the genes for rDNA are known to be located at the NOR (nucleolus organizer region) of the large *V.faba* M chromosome[9]. It is postulated that this specific nucleolus DNA labeling is related to the rapid increase in ribosome production in the root-tip.

Material and methods. Vicia faba var. Nagasaya Soramame beans were germinated in vermiculite and the young seedlings suspended by perspex plates in an aerated water bath at 22 °C. The roots of 10-day-old seedlings were immersed in a beaker of (6-^{3}H)-thymidine solution (25C/mM; 3.3 μC/ml) for a 30-min 'pulse'. The seedlings were then rinsed thoroughly in distilled water and then transferred back to the water bath for a 'chase' period. Some lateral roots were excised immediately, pretreated with 0.05% colchicine for 2 h and fixed. Further roots were pretreated and fixed at each 20-min interval up to a chase of 20 h. Root-tip meristems were stained and prepared by the Feulgen squash technique. Coverslips were removed with liquid nitrogen and the slides air dried. Slides were then dipped in Ilford L4 nuclear gel emulsion (diluted with distilled water, 1:1) by standard procedures and exposed for 3–5 weeks. The autoradiographs were developed with Microphen for 5 min, fixed, washed in several changes of distilled water, air dried and mounted in Depex.

Results. All autoradiographs prepared following a 30-min ^{3}H-thymidine pulse showed label distributed over ⅓ of all interphase nuclei. Most labeled nuclei have a uniform distribution of silver grains (fig. a) although a few show specific regions with label, e.g. the heterochromatic chromocenters. Nuclei of particular interest were those showing specific nucleolus labeling. Between 2% and 5% of labeled interphase nuclei show silver grains located exclusively over the nucleoli (fig. b). The number of specifically nucleolus-labeled nuclei ranges from 80 to 200 cells per meristem from an average total of 4000 labeled nuclei per meristem. Nuclei showing such a labeling pattern occur in groups on the slide indicating a common location in the meristematic tissue in vivo at the time of replication.

Pulse-chase experiments were conducted to determine the time during interphase when specific nucleolus labeling occurred. Experiments designed to follow the label into nucleolus organizing regions of metaphase chromosomes, however, have so far proved negative. No NOR labeling was found in c-metaphase chromosomes despite exhaustive searching of autoradiographs. Even after chasing root tips for up to 20 h to monitor all of S phase there was no evidence of these cells having entered mitosis. High concentration of label was found over the nucleoli of specifically nucleolus-labeled nuclei in all root tips chased although the frequency of such labeled nuclei declined after chasing for 6–8 h. The localization in the root tip and fate of these cells is not known. In contrast, labeled heterochromatic regions in interphase nuclei were clearly localized in c-metaphase chromosomes after an 8–9 h chase. A control slide treated with DNAase showed no silver grains over the nuclei.

Discussion. Autoradiography is a useful technique for cell cycle studies. The fate of ^{3}H-thymidine incorporated into actively synthesizing DNA can be followed. The clustering of cells undergoing an identical nuclear event, namely the specific synthesis of nucleolus DNA, indicates some zone of synchrony within the *V.faba* root-tip meristem. Furthermore, there is no evidence of such specifically labeled cells entering mitosis. The observation could very well be a visualization of the genetic machinery undergoing amplification, possibly of rDNA genes, necessary to sustain the rapid production of ribosomes for cell growth and expansion. This conjecture is the most reasonable explanation. There is considerable evidence to indicate that RNA synthesis is greatest in meristematic and early elongating tissue of higher plants and that during this time the number of ribosomes formed is sufficient to sustain subsequent expansion growth[8,10,11]. The current cytological study supports these data. Gene amplification is now known to be a widespread phenomenon in many developing tissues[12] and it would not be surprising to detect its occurrence in root-tip meristems, especially of rDNA genes. Indeed, in view of the low percentage of the *V.faba* genome which codes for rRNA it would be surprising if rDNA could be labeled by a 30-min pulse of ^{3}H-thymidine at 3.3 μCi/ml without amplification. Burger and Scheuermann[9] showed rDNA genes to be located at the NOR in *V.faba* chromosomes by in situ hybridization and ultrastructural studies in various organisms indicate that the rDNA is located specifically within the FC (fibrillar center) of the nucleolus[13,14]. Pulse labeling in the present study clearly shows localization of high intensity DNA synthesis within the nucleolus. It is not located over perinucleolar heterochromatin known to be associated with the nucleolus in *V.faba*[15]. A similar feature was observed by Innocenti and Avanzi[16] in their studies on the differentiation of metaxylem in *Allium cepa* root tips.

Further steps in the program are 1) to determine precisely where in the root-tip meristem specific nucleolus labeling is occurring and 2) to determine whether the labeling represents amplification of rDNA genes. Attempts to establish conclusive proof of the latter (even though it is likely) have so far posed technical difficulties.

1 Evans, D., and Birnstiel, M.L., Biochim. biophys. Acta *166* (1968) 274.
2 Meneghini, R., Armelin, H.A., Balsamo, J., and Lara, F.J.S., J. Cell Biol. *49* (1971) 913.

3 Armelin, H. A., and Marques, N., Biochemistry *20* (1972) 3672.
4 Stocker, A. J., and Pavan, C., Chromosoma *45* (1974) 295.
5 Spradling, A. C., and Mahowald, A. P., Proc. natn. Acad. Sci. USA *77* (1980) 1096.
6 Chisholm, R., Trends Biochem. Sci. *7* (1982) 161.
7 Johnston, R. N., Beverley, S. M., and Schimke, R. T., Proc. natn. Acad. Sci. USA *80* (1983) 3711.
8 Chaly, N. M., and Setterfield, G., Can. J. Bot. *53* (1975) 200.
9 Burger, E-C, and Scheuermann, W., Cytobiologie *9* (1974) 23.
10 Wright, S. T. C., J. exp. Bot. *12* (1961) 303.
11 Rizzo, P. J., and Cherry, J. H., Pl. Physiol. *55* (1975) 574.
12 Schimke, R. T. (ed.), Gene Amplification. Cold Spring Harbour Lab. (1982).
13 Goessens, G., Int. Rev. Cytol. *87* (1984) 107.
14 Jordan, E. G., and Cullis, C. A. (eds), The Nucleolus. Cambridge Univ. Press (1982).
15 Rowland, R. E., J. theor. appl. Genet. *60* (1981) 275.
16 Innocenti, A. M., and Avanzi, S., Caryologia *24* (1971) 283.

0014-4754/85/081070-03$1.50 + 0.20/0

Male tarsal sex-comb teeth pattern in the *Drosophila bipectinata* complex

S. Crossley and I. Taylor*

Department of Psychology, Monash University, Clayton, Victoria 3168 (Australia), 6 August 1984

Summary. Species within the *D. bipectinata* species complex pose problems experimentally because of their morphological similarity. By measuring the number of teeth in each row of the male tarsal sex-comb, an unknown specimen can be allocated to a hybrid or parental group by means of a discriminant function. This paper describes which rows of the sex-comb are the best discriminators for particular comparisons.
Key words. Sex-comb pattern; discriminant analysis; *Drosophila* sibling species; hybrids.

The *Drosophila bipectinata* complex is classified in the *ananassae*-subgroup of the *D. melanogaster* species group[1]. The complex includes four species, *D. bipectinata, D. malerkotliana, D. parabipectinata,* and *D. pseudoananassae*. The four species and a majority of their hybrids can be distinguished from one another by the number of teeth in the male tarsal sex-comb[2]. The only other differences reported between the species are in the coloration of the abdominal tergites in males; in *bipectinata* and some populations of *pseudoananassae* and *malerkotliana,* male abdomens are light, while in *parabipectinata* and other populations of *pseudoananassae* and *malerkotliana,* male abdomens are black. Male genitalia are similar in all four species[3,4]. Females, of the four species, are similar in appearance to one another.

Teeth in the sex-comb are arranged in rows, and the presence or absence of teeth in rows, as well as the total number of teeth in a row, vary between right and left legs of the same individual, between individuals, and between species. This study examines the pattern of the sex-combs and uses discriminant analysis to test which rows best distinguish the species and their hybrids. The question is whether sex-comb pattern or total sex-comb teeth number is the best character to use in situations where studies involving the species and their hybrids require some means of distinguishing them.

20 male flies from each of the four species and 12 different hybrids (six crosses and six reciprocal crosses) were measured. Hybrid males were either obtained from vials which housed five virgin females of one species and five virgin males of another species, or from virgin females which had been observed to mate with a foreign species in another study concerned with behavior. The location and number of teeth making up the sex-combs were recorded for the right foretarsus for all males. In addition, the pattern of teeth on the left foretarsus was recorded for the *bipectinata, malerkotliana,* and *parabipectinata* males. Magnification used for examination was ×100.

Figure 1 illustrates the sex-comb pattern characteristic of each species. Teeth which make up the sex-comb occur on the first and second tarsal segment of each front leg in all species. In addition, the front foretarsi of *malerkotliana* also bear teeth on tarsal segment three. In *bipectinata* and *parabipectinata* teeth in rows 1 and 2 are comb-like and project horizontally. In *malerkotliana* and *pseudoananassae,* teeth in the same row may overlap one another. Teeth arrangement is, therefore, less comb-like in these two species than in *bipectinata* and *parabipectinata.*

There were no significant differences in row teeth number between sex-combs on the right and left sides of the body (table 1). Individual differences between right and left sides rarely exceeded one.

Table 1 gives the number of teeth per row in the sex-combs of all the species and species hybrids together with the standard errors. Linear discriminant-function analyses were used to classify the species and their hybrids. The discriminating variables were the teeth number in each of the rows 1–5. Any row for which no teeth were present for both of the groups compared was omitted. Linear discriminant procedures require equality of covariance matrices. In our study, these matrices were equal except in comparisons which included *malerkotliana.* However, Colgan[5] argues that this requirement does not influence results when sample sizes are, as in our case, equal and large. We assume, therefore, that the above analysis is appropriate for all the groups compared.

Figure 2 shows the distribution of the discriminant scores for the six species comparisons. 100% correct classification was achieved for all pairs of species compared except for *bipectinata* and *parabipectinata* which were classified with an accuracy of 95%.

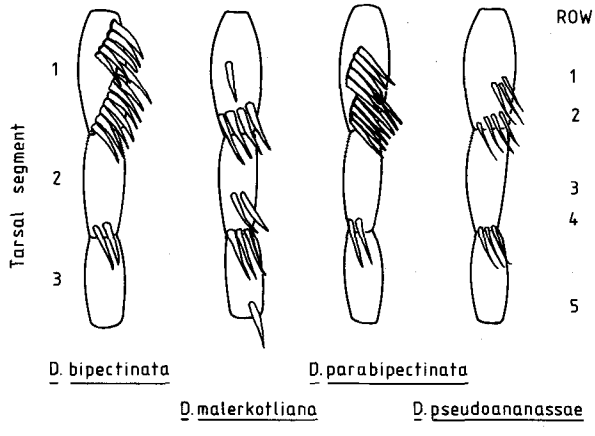


Figure 1. Male tarsal segments 1, 2 and 3 bearing sex-combs.