genes that may mutate to forms altering both viability and fertility (e.g. events common to mitosis and meiosis).

1 Work supported by the Swiss National Science Foundation, grant 3602079.
2 Correspondence address: P. Hutter, Genetics Department, Universitiy of Geneva, 154bis route de Malagnou, CH-1224 Geneva, Switzerland.
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Proliferation of primordial germ cells of frogs stimulated by a fraction of granules derived from *Rana nigromaculata* embryos at the tail-bud stage

T. Shirane

Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima 730 (Japan)
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Summary. A fraction of heavy granules, obtained from embryos of the frog (*Rana nigromaculata*) at the tail-bud stage by centrifugation at 1700 × g, were shown to stimulate the formation or proliferation of the primordial germ cells not only of the same species but also of a closely related species.
Key words. Primordial germ cells; granule fraction; Anura.

Bounoure [1] proposed that there exists in *Rana temporaria* (Anura, Amphibia) a specifically stained cytoplasm (germ plasm) which is closely associated with the determination of primordial germ cells (PGCs), and which can be traced back from the PGCs in the genital ridges to the subcortical layer at the vegetal pole of eggs at the 2-cell stage. Since then, similar observations have been reported in several species of Anura by other investigators [2, 3]. In addition, Balinsky [4] found, by electron microscopy, that germ plasm contains clusters of particularly dense bodies. This observation has also been confirmed by other investigators [2, 3]. About ten years ago, Wakahara [5, 6] attempted to isolate the germinal determinant in *Rana chensinensis* and *Xenopus laevis*, and he confirmed that a certain fraction obtained by centrifugation was able to stimulate proliferation of the PGCs. However, he did not perform further experiments with respect to this fraction and its effects. Up to now, few researchers have succeeded in isolating the actual germinal determinant(s), even though this seems to be a necessary step for an understanding of their function, and of the mechanism of formation of PGCs.

The germ plasm cannot be detected in all species of Anura during all stages of embryogenesis. In *R. nigromaculata* and *R. brevipoda*, the cells containing the germ plasm (presumptive PGCs or pPGCs) are scarcely detectable after the neurula stage [7], as has also been reported in the case of *R. esculenta* [8]. In the above mentioned two species, it may be relevant that there were PAS-positive granules which were not restricted to a certain type of cell and which showed similar behavior to the pPGCs in terms of distribution in embryos [7]. Although such PAS-positive granules were present in the migrating PGCs, it remains to be determined whether these granules are true organelles, or artifacts formed during the preparation of samples. If they are actual organelles and are related to the formation of PGCs, it is possible that they may be concentrated in a certain fraction of granules prepared by centrifugation and that they may cause the PGCs in larvae into which they are injected to multiply. The pres-

ent report describes the results of an examination of the relationship between the formation of PGCs and the various fractions of granules obtained from embryos at the tail-bud stage.

Materials and methods

Japanese pond frogs, *Rana nigromaculata* Hallowell and *R. rugosa* Schlegel, were used in this experiment. In the case of both species, over 95% of the eggs in each batch developed normally. From the batches, 300 embryos at the tail-bud stage were used for preparing the granule fractions whenever they were required. In preliminary experiments a crude mitochondrial fraction of *R. nigromaculata*, prepared in accordance with the size of PAS-positive granules (1–3 μm in diameter) in this species[7], had been shown to influence the multiplication of PGCs. Therefore in this experiment the crude mitochondrial fraction was divided into two fractions (heavy granules and light granules). The procedure for preparing the granule fractions was as follows: embryos were homogenized in a solution of 0.01 M phosphate buffer (PBS) (pH 7.4) that contained 0.25 M saccharose and 0.2 M KCl in an easy-fitting Teflon homogenizer (at 1000 rpm for 30 s). After the nuclei and unbroken cells had been removed by centrifugation at 1000 × g for 10 min, the supernatant was centrifugated at 1700 × g for 5 min. The pellet was used as a heavy-granule fraction (H-fraction). The supernatant was centrifuged again at 8500 × g for 10 min. The pellet was used as the light-granule fraction (L-fraction). Each pellet was suspended in 100 μl of the same PBS as used for the homogenization of embryos, and 1 μl/embryo of the suspension of granules was injected into the abdomen of tail-bud embryos by micropipette (ca. 100 μm in diameter at the tip). The embryos which were injected with the granules were derived from eggs which had been irradiated with UV light (total dose, ca. 7000 or 9000 erg/mm^2) at the vegetal hemisphere, at the 2-cell stage, in order to suppress the formation of PGCs.

Results and discussion

Table 1 shows the results obtained in two series of experiments with *R. nigromaculata*. The mean numbers of PGCs in the larvae that received no special treatment (the control) were somewhat different in the two series (41 in series 1 and 62 in series 2), but the reason for this difference is unknown. The overall effect of UV irradiation in both series was not very great because the dose of UV light was low (7000 erg/mm^2). The mean number of PGCs in the larvae derived from UV-irradiated eggs and not injected with anything (UV control) was 17 in series 1 and 30 in series 2. The deviation of the number of PGCs in the UV-irradiated controls from that in the controls was statistically significant ($p < 0.01$, t-test).

In the injected groups, it was obvious that the H-fraction increased the number of PGCs in both series (26 cells in series 1 and 46 in series 2). The PGCs of larvae in these groups appeared to assemble predominantly within a range of about 100 μm along the length of the presumptive genital ridges (ca. 350 μm in total length). The deviation of the average number of PGCs from that in the UV-irradiated controls was statistically significant ($p < 0.01$, t-test). The increment in the number of PGCs in the groups injected with the H-fraction corresponded to 22.0% (9 cells) and 25.8% (16 cells) of the mean number of PGCs in each control group, respectively. In contrast, the groups injected with the L-fraction showed little increase in the numbers of PGCs. Figure 1 shows the distribution of PGCs in each experimental group in series 1. The results reflect the mean number of PGCs in the experimental groups. The rate of appearance of embryos with high levels of PGCs is elevated only in the group injected with the H-fraction.

Table 1. The number of primordial germ cells (PGCs) in embryos of *Rana nigromaculata* at stage 25*, which were raised from UV-irradiated eggs and injected with the granule fractions derived from the tail-bud embryos of the same species.

Animals	Injection	Series 1 No. of embryos	Series 1 Mean no. of PGCs (SE)	Series 2 No. of embryos	Series 2 Mean no. of PGCs (SE)
Normally developed	None (control)	8	41 ± 3	7	62 ± 2
UV-irradiated	Heavy granules	16	26 ± 1	9	46 ± 2
UV-irradiated	Light granules	14	18 ± 1	10	31 ± 1
UV-irradiated	None (UV-control)	10	17 ± 2	7	30 ± 2

*The stage corresponds to the developmental stages reported for *Rana pipiens* by Shumway ('40).

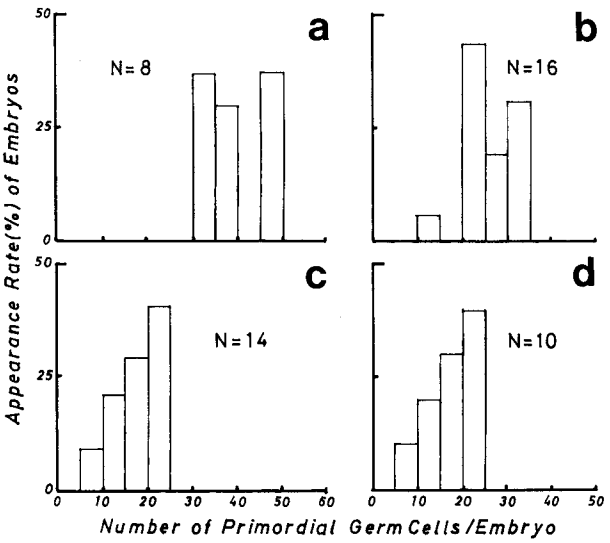

Figure 1. Histograms indicating the distribution of *Rana nigromaculata* embryos in each range (at five-cell intervals) with increasing numbers of PGCs in the experimental groups in series 1. *a* Normally developing group. *b* Group of embryos that were derived from UV-irradiated eggs and were injected with the heavy-granule fraction. *c* Group of embryos that were derived from UV-irradiated eggs and were injected with the light-granule fraction. *d* Group of embryos that were derived from UV-irradiated eggs and not injected with anything (UV-control).

Table 2. The number of primordial germ cells (PGCs) in embryos of *Rana rugosa* at stage 25, which were raised from UV-irradiated eggs and injected with the heavy-granule fraction derived from tail-bud embryos of the same species (H-fraction R) and of *R. nigromaculata* (H-fraction N).

Animals	Injection	Series 1 No. of embryos	Mean no. of PGCs (SE)	Series 2 No. of embryos	Mean no. of PGCs (SE)
Normally developed	None (control)	12	41 ± 3	16	42 ± 2
UV-irradiated	H-fraction N	15	10 ± 2	16	8 ± 1
UV-irradiated	H-fraction R	12	2 ± 1	–	–
UV-irradiated	None (UV-control)	15	2 ± 1	12	2 ± 1

R. rugosa is another species in which the pPGCs can hardly be detected after the neurula stage. The appearance of PAS-positive granules was confirmed in this species, although their diameter (ca. 0.5 μm) was much less than that in the preceding species (unpublished data). In the present experiments, this species was used mainly for an examination of the effects of the H-fraction from *R. nigromaculata* (H-fraction N) on the formation of PGCs in a different species. Only the H-fraction from *R. rugosa* (H-fraction R) was prepared in this case. Table 2 summarizes the results in terms of the numbers of PGCs. It shows that the groups of H-fraction N caused the PGCs of *R. rugosa* to proliferate. The deviation in the average numbers of PGCs in the group injected with the H-fraction N from that in the UV-irradiated control group was statistically significant ($p < 0.01$, t-test). In contrast, the H-fraction R had no effect on the proliferation of PGCs in the larvae of the same species (series 1 and data not shown). Figure 2 shows the distribution of PGCs in each group in series 1. There is a similar shift in the numbers of PGCS the group injected with H-fraction N as that shown in figure 1.

From the above results, it is clear that some of the heavy granules in embryos of *R. nigromaculata* at the tail-bud stage are involved in the proliferation of PGCs, not only in the same species but also in a closely related species. Although it remains to be investigated whether or not the active granule is identical to the PAS-positive granule, the PAS-positive granule is a valid candidate as the factor that determines proliferation of PGCs, as judged from the different sizes of PAS-positive granules in the species examined.

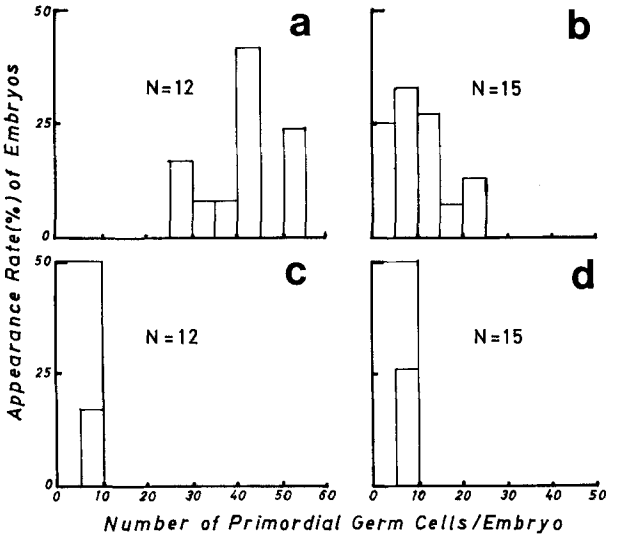

Figure 2. The histograms indicate the distribution of *R. rugosa* embryos in each range (at five-cell intervals) with increasing numbers of PGCs in the experimental groups in series 1. *a* Normally developing group. *b* Group of embryos that were derived from UV-irradiated eggs and were injected with the heavy-granule fraction from *R. nigromaculata*. *c* Group of embryos that were derived from UV-irradiated eggs and were injected with the heavy granule-fraction from the same species. *d* Group of embryos that were derived from UV-irradiated eggs and were not injected with anything (UV-control).

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