14. Studies on Amphibian Chromosomes by the High Resolution Banding. I

Technique for the High Resolution R-banding to Frogs^{†)}

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Since the amphibian species are the important material in the cytogenetic analysis, many studies have been referred to the literatures concerning the karyotypes by the conventional staining method. After the band analysis in *Leptodactylus ocellatus* by Bianchi *et al.* (1972), some authors have analysed the bands of the anuran chromosomes. Because of the strong condensation of the amphibian metaphase chromosomes, only the techniques for the specific staining of the constitutive heterochromatin and of the nucleolus organizer regions have been reported. In order to obtain the multiple bands of the anuran chromosome, Schempp *et al.* (1981) used the Brdu-hoechst-Giemsa technique, by which the identification of the sex chromosome of *Rana esculenta* and also making of the multiple banding patterns came to make it possible. However, there are some difficulties to show the multiple bands on the small chromosomes and also to get the multiple bands reproducibly. To resolve these problems, we put forward a new high resolution R-banding method (Heng 1984). The present paper deals with the banding technique, with special regard to the high resolution R-banding karyotypes of *Rana nigromaculata* and *R. japonica japonica*.

Material and methods. Animals: *Rana nigromaculata* captured from the suburb of Chendu, China and *Rana japonica japonica* from the E-mei mountains (southwestern China) were used in the present study.

Cell synchronization and Brdu-labeling: Peripheral lymphocytes obtained from the heart of the frog were cultured at 26°C in RPMI-1640 medium supplemented with 20% fetal bovine serum and PHA. The pH of the medium was 7.2. After 68 hr, the cells were partially synchronized by the treatment with thymidine (0.5–0.8 mg/ml). Twenty hours after the thymidine treatment, the cells were washed two times with Ringer's medium to release the treatment (Ringer's medium is consisted of solution-A 95 ml + solution-B 5 ml. The solution-A is composed of NaCl 6.0 g, KCl 0.075 g, CaCl₂·6 H₂O 0.10 g and the distilled water 950.0 ml. The solution-B is consisted of NaHCO₃ 0.10 g, glucose 1.0 g, and the distilled water 50.0 ml). Then, the cells were cultured in RPMI-1640 medium supplemented with Brdu (10 µg/ml) and incubated for the last 7 hr.

Slide preparation: The cultures were treated with colcemid (0.03 µm/ml) for 60 min, followed by the conventional hypotonic treatment with KCl (0.4%) for 30–40 min at 26°C and the 3:1 methanol-acetic acid fixation. After 20 min, the cells were washed twice with the fixative solution and stored overnight at

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4°C. The following day, the cells were washed three times with the fresh fixative and the cell suspension was dropped onto the slides moistened with little water at 4°C, and dried in air.

Slide stain: One day later, the slides were exposed to ultraviolet light at 254 nm for 15 min in a distance of 6 cm. At the same time, the slides were incubated in $1 \times$ SSC solution at 35°–40°C. Finally, the slides were stained for 7–9 min with 3% Giemsa solution buffered at pH 6.8. After the air-drying for few hours, the slides were used for the observations.

Results and discussion. The high resolution R-banding of chromosomes in the frog was obtained by the synchronization of cells and the Brdu incorporation in the early stage of the DNA synthesis. The Brdu incorporated segments showed the pale blue colour resulting from the substitution of Brdu. They were distinguished from dark Giemsa segments, where the thymidine are existed. The cell synchronization is important to get more number of cells at the prometaphase stage, in which more band information are provided. The figures (Fig. 1, A and

Fig. 1. Prometaphase chromosomes of *Rana japonica japonica* by the R-banding technique. A: *R. japonica japonica* (female). B: *R. japonica japonica* (male).

B) showed the high resolution R-banding chromosomes of frog by our technique. We can accurately identify all chromosomes and compare the karyotypes between two related *Rana species* (*R. japonica japonica* and *R. nigromaculata*) (Fig. 2).

Camargo and Cervenka (1982) have studied the high resolution chromosome analysis due to the Brdu-incorporation in the human chromosomes. They found that the incorporation of Brdu in the early DNA synthesis resulted to the multiple bands similar to R-bandings. Since our procedure was the same as that used by Camargo, the present bands obtained in the frogs were the high resolution R-banding. The important fact is that the Brdu-replicating bands are dynamic.

Therefore, the use of the standardized time of the Brdu incorporation is a key point in the present technique. Our experiment satisfies the requirement by using the technique of the synchronization and letting the Brdu incorporation at the same stage of the cell cycle. Burkholder (1979) studied the mechanism of

Fig. 2. High resolution R-banding karyotypes of *Rana japonica japonica* and *R. nigromaculata*. A: *R. japonica japonica* (male). B: *R. nigromaculata* (male).

the differential staining of the Brdu-substituted, unsubstituted DNA of chromosomes, and found that the Brdu-substituted DNA was partially photolysed by exposure to the normal daylight during the harvesting procedure. The degraded DNA was subsequently solubilized and extracted from the slides during the phosphate buffer treatment. The decrease in the amount of DNA in the Brdu-substituted chromosome regions resulted in a decrease in an amount of Giemsa dye binding, thereby leading to the production of pale staining in these regions. Our new staining method can also be explained by the same way.

It is generally assumed that the sex chromosomes of the lower vertebrates have still maintained an initial stage of the differentiation (Ohno 1967). In the high resolution R-banding in *R. japonica japonica*, the sex chromosomes were not identified in either sexes. An interesting result, however, was obtained in *R. nigromaculata* that there were two unpaired chromosomes in the male specimens after the high resolution R-banding, although any heteromorphic chromosomes could not be found after the conventional staining. The details of this problem will be reported elsewhere.

Summary. By using the high resolution R-banding technique, the clear multiple bands in the amphibian chromosomes are successfully obtained. This R-band is one form of the Brdu-replicating bands, which is useful to identify all chromosomes and compare the chromosomes of different species of Amphibia. The karyotypes of two *Rana* species, *R. nigromaculata* and *R. japonica japonica* are shown by the present technique.

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