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Fluorescence replication banding of frog chromosomes

I. Miura[a,*], H. Ohtani[a], M. Nakamura[a] and K. Saitoh[b]

[a]*Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Higashihiroshima 739 (Japan), Fax +81 824 24 0739, e-mail: imiura@ue.ipc.hiroshima-u.ac.jp*
[b]*Department of Bioscience and Biotechnology, Faculty of Engineering, Aomori University, Aomori 030 (Japan)*
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Abstract. To identify individual chromosomes of a frog karyotype by their fluorescence banding patterns, chromosomes were stained with actinomycin D and 4,6-diamidino-2-phenylindole (DAPI) after incorporation of BrdU during the late S-phase. The chromosomes of three *Rana* species which were selected for this study (*R. ridibunda*, *R. lessonae* and *R. japonica*) showed well-defined late replication bands. The fluorescence patterns obtained were the reverse of those produced by a 4Na-EDTA Giemsa-staining technique. Fluorescence patterns of the two water frog species (*R. ridibunda* and *R. lessonae*) were similar to each other, except for the different fluorescence of the centromeric heterochromatin, which gave extremely bright signals in *R. ridibunda* but no signal in *R. lessonae*. Experiments also showed differences between the fluorescence patterns of *R. lessonae* chromosome 13 in the Italian and Luxembourgian populations. These results show that the fluorescence replication banding using actinomycin D and DAPI is very effective in identifying individual frog chromosomes and detecting their structural changes.
Key words. Late replication banding; DAPI; actinomycin D; frogs.

Giemsa replication banding, generally used in chromosome studies in higher vertebrates, has now been applied to amphibians. During the history of amphibian banding, the study of Schempp and Schmid [1] was a major breakthrough. Using a modified fluorescence-plus-Giemsa (FPG) method, they could identify all 13 chromosome pairs in the karyotypes of *Rana esculenta* and *R. temporaria*, based upon the replication patterns of their BrdU-incorporated chromosomes. Since then, modified FPG, original FPG and 4Na-EDTA Giemsa-staining methods have been used for the chromosome analysis of anurans [2–8] and urodelans [9–11]. On the other hand, banding using fluorescent dyes had not yet produced any G- or R-type bands in amphibians, although the technique has been extensively used in their cytogenetic studies. Only Schmid et al. [12], using acridine orange, identified six chromosome pairs of a tetraploid species by the replication patterns. Compared with Giemsa banding, fluorescence banding generally has advantages of (1) the simplicity of the staining process and (2) well-differentiated stainability of sequence-specific heterochromatin associated with the G- or R-type band. In addition, for gene mapping by fluorescence in situ hybridization (FISH), the identification of each chromosome on the basis of its fluorescence pattern is a prerequisite. Thus, to identify anuran chromosomes by their fluorescence replication patterns, we attempted sequential staining with actinomycin D and DAPI after BrdU-incorporation into the chromosomes. The method we applied could produce clear replication patterns and bright adenine and thymine (AT)-rich constitutive heterochromatin, making it possible to identify exactly every chromosome of the complement and detect their structural changes.

* Corresponding author.

Materials and methods

Frogs used. Three species of *Rana* were used in this study. Two of them, *R. ridibunda* from Turkey and *R. lessonae* from Italy and Luxembourg, were offspring of the specimens used in the cross experiments by Kawamura and Nishioka [13]. The third, *R. japonica*, was collected in Wakuya-machi, Toda-gun, Miyagi Prefecture, situated in eastern Japan. Numbers of the specimens used and the metaphases analysed are shown in table 1.

Chromosome preparation and fluorescence replication banding. Mitotic metaphases were obtained by the culture of blood cells [2]. Chromosome spreads were produced by the conventional air-drying method after incorporation of BrdU into the chromosomes for about 4 h of late S-phase of the cells. A 4Na-EDTA Giemsa-staining technique has been described elsewhere [2, 3], as well as the slightly modified C-banding. Sequential staining with actinomycin D (AMD) and 4,6-diamidino-2-phenylindole (DAPI) was performed according to Schweizer [14]. BrdU-treated chromosome slides were immersed in McIlvaine citric acid-Na_2HPO_4 (Mc) buffer (pH 6.9–7.0) for 5–10 min, followed by staining with actinomycin D (0.25 mg/ml in Mc buffer) for 20–

Table 1. Species and number of metaphases.

Species	Locality (country)	Sex	Frog	No. of metaphases	
				AMD-DAPI	4Na-EDTA
R. ridibunda	Turkey	female	1	31	25
			2	25	9
R. lessonae	Italy	female	1	14	10
			2	10	11
	Luxembourg	female	1	11	12
R. japonica	Japan	male	1	10	10
			2	10	9

30 min in the dark. The slides were rinsed with the buffer, stained with DAPI (0.1 μg/ml Mc buffer) for 5 min, and then mounted with the same buffer. They were observed under a transmission-type fluorescence microscope (Nikon) and photographed using Fuji Neopan film (iso32).

Results

Constitutive heterochromatin. Immediately after ultraviolet (UV) irradiation of the chromosome slides stained with actinomycin D and DAPI, a strong fluorescence was observed in the centromeric region of the

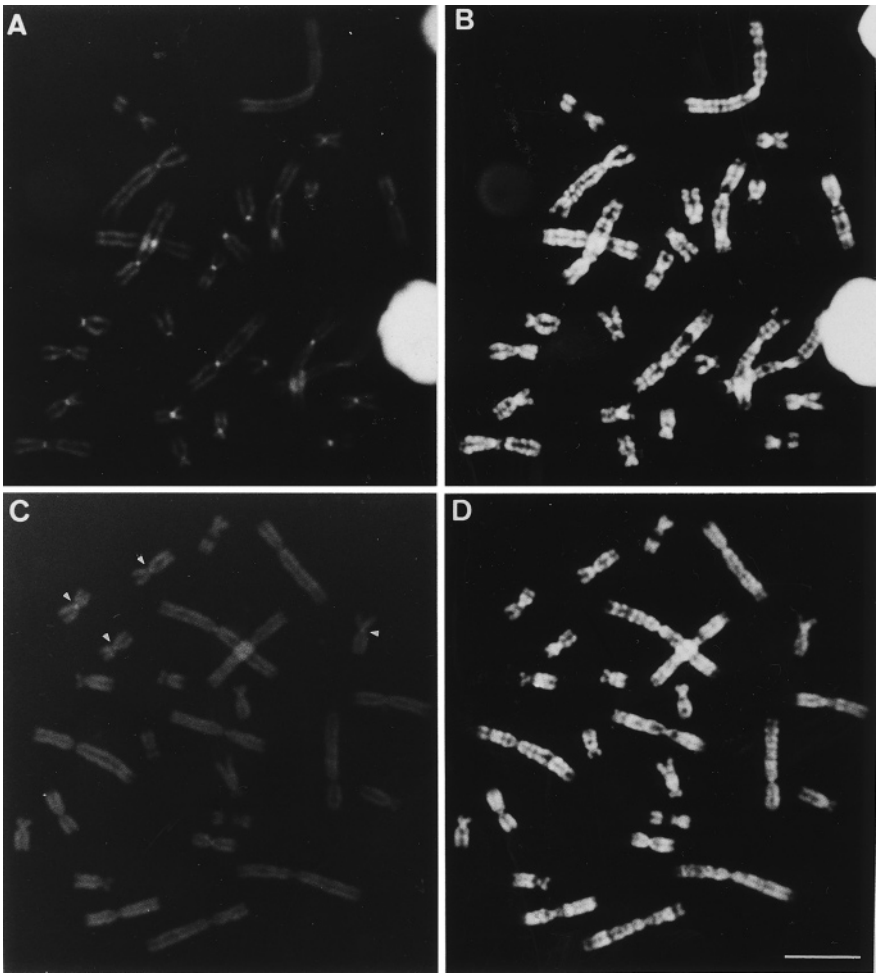

Figure 1. The actinomycin D-DAPI-stained metaphase plates of *Rana ridibunda* and *R. lessonae*. Immediately after UV irradiation, the centromeric heterochromatin of *R. ridibunda* chromosomes emitted strong fluorescence (*A*), while no such differentiation was observed in the chromosomes of *R. lessonae* from Luxembourg (*C*). About 2 min later, late replication patterns appeared all along the chromosome axes in both species (*B, D*). Arrowheads indicate the centromeric heterochromatin regions of the *R. lessonae* chromosomes 6 and 10, which are slightly brighter than the euchromatic regions. Bar = 10 μm.

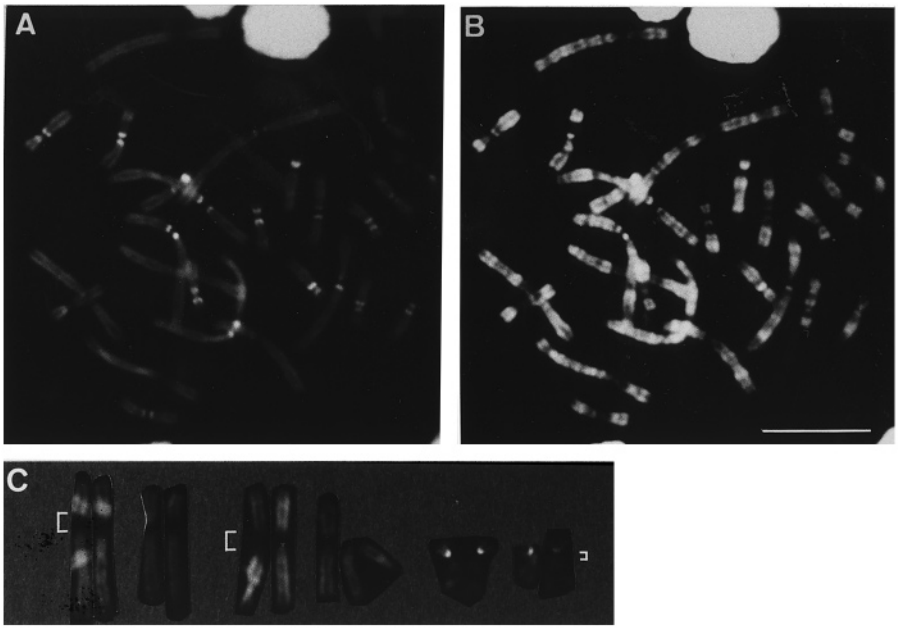

Figure 2. The AMD-DAPI-stained metaphase plates of *R. japonica*. The pericentromeric heterochromatin emitted strong fluorescence (*A*), except for that of chromosomes 3, 5 and of the long arm of chromosome 10, indicated by hooks (*C*). C-banded chromosomes 3, 5 and 10 (lower row) are shown for comparison with the AMD-DAPI-stained ones (upper row). The late replication bands were will defined (*B*). Bar = 10 μm.

chromosomes in *R. ridibunda* and the pericentromeric region of those in *R. japonica*. However, no such fluorescent regions were detected in *R. lessonae* (figs 1 and 2). Close comparison of the fluorescence-banded and C-banded metaphases indicated that the bright regions were heterochromatic. The results of the *R. ridibunda* and *R. lessonae* chromosomes obtained in this study were consistent with those of Heppich et al. [15], who used actinomycin D and the fluorescent dye Hoechst 33258. The present observations, however, verified that the centromeric regions of chromosomes 6 and 10 of *R. lessonae* were slightly brighter than the euchromatic regions (fig. 1). As shown in figure 2A and C, three pairs of *R. japonica* chromosomes showed specific fluorescing differentiation; the pericentromeric regions of both arms of chromosomes 3 and 5 and that of the long arm of chromosome 10 were completely lacking in fluorescence.

Fluorescence late replication pattern. About 2 min after UV irradiation, light and dark banding patterns appeared, first along the chromosome axes, until well-defined bands were apparent in all three species (figs 1–3). This contrast lasted for about 5 min and thereafter faded away gradually. Thirteen pairs of chromosomes from these three species could be identified on the basis of their characteristic late replication patterns. These patterns were the reverse of those produced by a 4Na-EDTA Giemsa method that was used to densely stain a BrdU-incorporated region (fig. 3). The characteristics of the fluorescing patterns were as follows: (1) The region near the centromere replicated early and fluoresced brightly, which made it easy to identify the centromeric position. In *R. ridibunda*, the centromeric position in each chromosome was detected more easily by the especially bright constitutive heterochromatin. Since the 4Na-EDTA method did not stain the paracentromeric regions, it was not suitable for identification of centromere position. (2) The two water frog species (*R. ridibunda* and *R. lessonae*) showed similar banding patterns, but except for chromosome 13 their karyotypes were distinguishable by the differentially stained centromeric heterochromatin. The centromeric region of chromosome 13 of *R. lessonae* from Italy was quite brightly fluorescent, whereas its long arm pericentromeric region was completely dark. This pattern was not observed in *R. lessonae* from Luxembourg or *R. ridibunda* (fig. 3).

Discussion

Applied to higher vertebrates, the DAPI staining highlights G-type (Q-type) banding patterns and bright AT-rich heterochromatin. The staining differentiation can be improved by counterstaining with AMD [14, 16, 17]. In addition, BrdU-incorporated chromosome regions are quenched by double staining with Hoechst 33258, which is a fluorescent dye that exhibits a binding and fluorescence specificity similar to DAPI, and AMD [18]. In amphibians both DAPI and AMD-DAPI highlight AT-rich heterochromatin, but neither of them gives any

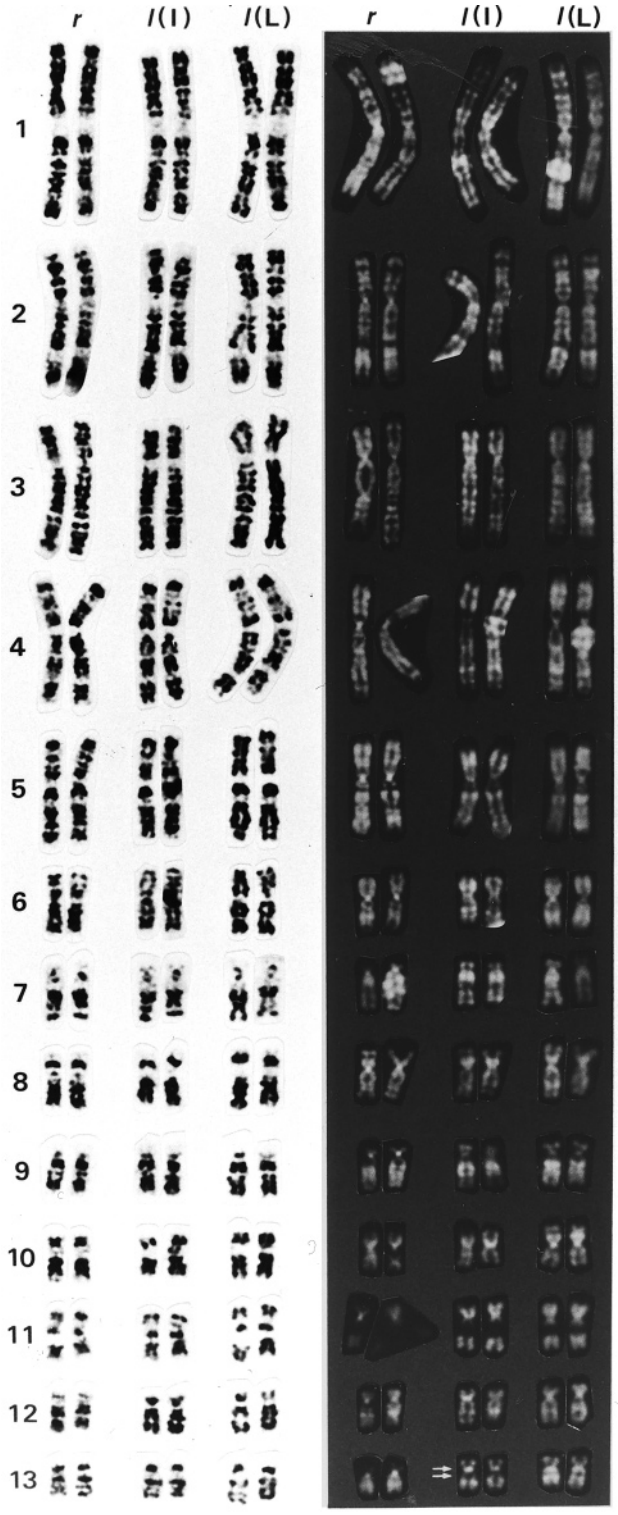

Figure 3. Two different patterns of late replication in *R. ridibunda* and *R. lessonae* from Italy and Luxembourg. The AMD-DAPI patterns were the reverse of the 4Na-EDTA Giemsa patterns in both species. A slight difference in banding pattern of chromosome 13 was found between the two *R. lessonae* populations. Arrows indicate the bands specific for chromosome 13 of the Italian *R. lessonae*. Abbreviations: $r = R.$ *ridibunda*; $l(I) = R.$ *lessonae* from Italy; $l(L) = R.$ *lessonae* from Luxembourg.

G-type (Q-type) band [19–22]. As far as we know, this is the first attempt to produce DAPI fluorescence replication patterns after BrdU incorporation into amphibian chromosomes.

The present study demonstrated that the AMD-DAPI sequential staining could produce well-defined replication bands in the three *Rana* species; while the BrdU-incorporating regions were dark, the nonincorporating regions were bright. Thus it became apparent that although no replication banding patterns could be produced by DAPI staining alone (data not shown), counterstaining with AMD was very effective in producing replication bands in the frog chromosomes. The patterns obtained in this study were the reverse of those produced by the 4Na-EDTA Giemsa method used in staining of the BrdU-incorporating regions. This shows that when BrdU was incorporated into the chromosomes during the late S-phase, the AMD-DAPI bands to R-bands and the 4Na-EDTA Giemsa bands to G-bands (Q-bands) corresponded, respectively. Sequential use of these two banding methods makes it possible to analyse both G-band positive and negative regions of frog chromosomes, thus allowing detection of minute chromosomal rearrangements that occurred during the evolution of frog chromosomes. For example, the structural differences in chromosome 13 between the two populations of *R. lessonae*, which were undetectable by the Giemsa method alone, could be shown using AMD-DAPI sequential staining (fig. 3).

FISH has now been extensively used for physical gene mapping. Exact identification of every chromosome of a given karyotype is indispensable for a successful FISH experiment. Sequential staining with AMD and DAPI, which we report here, will clearly be useful for FISH gene mapping in frogs.

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