Differential Staining of Cultured Bone Marrow Cell Chromosomes of *Rana pipiens*

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Chromosome banding has been widely utilized to identify homologous chromosomes more precisely, and also to analyze genetic rearrangements apropos evolutionary speciation and medical genetics. Q-banding employs alkylating agents, such as quinacrine hydrochloride and quinacrine mustard (Caspersson *et al.* 1968, Caspersson *et al.* 1969, Caspersson *et al.* 1970 a, b, Miller *et al.* 1971). C-banding which identifies heterochromatic DNA has been reported (Arrighi and Hsu 1971, Sumner 1972, Gallagher *et al.* 1973, Ruiz and Becak 1976). G-banding produces distinct bands along the entire chromosome with various post-fixation treatments followed by Giemsa staining. G-banding has been elicited through post-fixation treatment with alkaline-saline (Drets and Shaw 1971, Schnedl 1971), acid-saline (Gagne *et al.* 1971, Sumner *et al.* 1971), Giemsa 9 staining (Patil *et al.*, 1971), pronase (Dutrillaux *et al.* 1971), trypsin (Wang and Federoff 1972, Seabright 1972, Sun *et al.* 1974), urea (Shiraishi and Yosida 1972), potassium permanganate (Utakoji 1972, Matsukuma and Utakoji 1976), and caesium chloride (Meisner *et al.* 1973). R-banded chromosomes, which show a pattern the opposite of G-banding, have also been described (Dutrillaux and Lejeune 1971, Verma and Lubs 1975, 1976). F-banding has been produced through the use of a post-fixation treatment together with the standard Feulgen reaction (Rodman and Tahiliani 1973, Rodman 1974). Pre-fixation treatment of chromosomes with agents such as ethidium bromide have also induced chromosome cross-banding (Hsu *et al.* 1973). Most of the above banding studies involved mammalian chromosomes, with emphasis on those of humans.

Banding of amphibian chromosomes has also been reported. Utilizing the squash method, epithelial cell chromosomes of *Pleurodeles waltlii* were stained differentially with Giemsa 9 (Labrousse *et al.* 1972). Q-banding has also been utilized to study the tail cell chromosomes of *P. waltlii* (Bailly 1972, 1976). Giemsa 9 and Q-banding patterns of *P. waltlii* chromosomes have been compared (Bailly *et al.* 1973). Other banding studies involve members of the genus *Triturus*. C-banding patterns of squash preparations of *T. vulgaris meridionalis* and *T. italicus* have been reported (Nardi *et al.* 1973). C-banding patterns have also been described for *T. cristatus* and *T. marmoratus* (Mancino *et al.* 1973). G-, C-, and Q-banding patterns were reported for *T. alpestris apuanus*, *T. cristatus carnifex*, *T. marmoratus marmoratus*, *T. italicus*, and *T. vulgaris meridionalis* (Ragghianti *et al.* 1973). This latter work involved the production of G-bands according to

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Drets and Shaw (1971), Dutrillaux (1971), Sumner *et al.* (1971), and Schnedl (1971). C-bands have also been reported on the lampbrush chromosomes of *T. marmoratus* (Batistoni *et al.* 1974). The chromosomes of *T. cristatus* have been studied with C- and G-banding (Rudak and Callan 1976). The above amphibian studies involved members of the order Urodela.

Although earlier work on banding in Amphibia has been largely confined to the members of the order Urodela, more recently studies have been reported for banding in the Anura. G-banding of certain select chromosomes has been reported for chromosomes of cultured cells of *Xenopus muelleri* (Stock and Mengden 1975). Extensive work on C-banded chromosomes has been reported for numerous species of Anura. C-banding has been demonstrated for the diploid and tetraploid species of *Odontophrynus americanus* and *O. cultripes* (Ruiz and Becak 1976). Extensive studies in 22 species of bufonids and hylids utilizing Q- and C-banding methods have delineated the distribution and quantity of constitutive heterochromatin and nucleolus organizer regions (Schmid 1978a). However, in euchromatic regions and metaphase chromosomes neither G- nor Q- bands have been found (Schmid 1978). Data have been presented on the constitutive heterochromatin of *Rana ridibunda* (Raicu and Geormăneanu 1977), and on constitutive heterochromatin and nucleolus organizer regions in 12 species of the families Ranidae, Microhylidae, and Rhacophoridae (Schmid 1978b) with demonstrations of centric heterochromatin, and telomeric and interstitial C-bands. However, Q- and G-bands have not been demonstrated in metaphase chromosomes (Raicu and Geormăneanu 1977, Schmid 1978b).

Materials and methods

Specimens of the North American leopard frog, *Rana pipiens*, were obtained from Champlain Biological Service, Glen Gardner, New Jersey. The medium, medium additives, and balanced salt solutions were purchased from Grand Island Biological Supply Company, Grand Island, New York.

Bone marrow cultures were established according to the method of Beckert and Doyle (1967). The frogs were sacrificed by pithing or decerebration, and then bathed in 70% ethanol, thimerosal tincture, 1:1000 (Eli Lilly & Co., Indianapolis, Indiana), and 70% ethanol, respectively. The dissections were carried out in a hood utilizing aseptic technique. The femurs were isolated and scraped clean of all adhering muscle; rinsed in 70% ethanol, and followed by immersion for 30 sec in Earle's or Hanks' Balanced Salt Solution (isotonic EBSS or HBSS). The ends of the femurs were removed and 5 ml of medium were forced through the medullary canal to remove the marrow. This suspension was incubated at 25°C for 2–3 days.

The medium of Doyle and Beckert (1970) was modified slightly. Medium 199, with HEPES buffer, was utilized since there was a better maintenance of optimal pH (7.2–7.3). The composition of the medium is as follows:

	<i>Quantity (ml)</i>
Medium 199 with 25 mM HEPES buffer, glutamine, and Hanks' salts (1 ×)	20.0
Sterile glass distilled water	10.5
Fetal calf serum	8.0
Bovine embryo extract (50%)	3.5
Penicillin-streptomycin (5000 μ /ml–5000 mcg/ml)	1.0
L-Glutamine (200 mM)	0.5
Fungizone (250 mcg/ml)	0.5

Four hours prior to harvesting, each culture received 0.5 ml of colcemid (10 mcg/ml) to yield a final concentration of 1 mcg/ml colcemid. The cultures were re-incubated for 4 hours. At the end of this period cultures were centrifuged for 800 rpm in a Sorvall GLC-1 centrifuge for 6 min. The supernatant was removed and the pellet re-suspended in 4 ml isotonic HESS or EBSS for a brief washing. This was then recentrifuged. The pellet was re-suspended in 4 ml of hypotonic HBSS or EBSS (5:1 dilution) for 10 min. Following centrifugation, the pellet was re-suspended in 4 ml of freshly prepared fixative (3:1, methanol-acetic acid) for 10 min. This suspension was then centrifuged and re-suspended in fresh fixative for 20–30 min. At the end of this period the cells were again centrifuged and the pellet re-suspended in about 0.5 ml of the remaining volume. One drop of the fixed cell suspension was dropped onto precleaned, cold, wet (distilled water) slides from a height of about 4–5 inches. Slides were either air-dried or flame-dried, depending on the specific banding method studied.

The following banding methods were utilized in this investigation:

G-banding: Schnedl (1971), and modifications, such that the time of 0.07 N NaOH treatment was varied between 60–120 sec, and incubation in Sørensen buffer (pH 6.8) was varied from 1–2 days; Drets and Shaw (1971), and modifications, such that the time of 0.07 N NaOH treatment was varied between 30–90 sec, and incubation in 12×SSC (at 65°C) was varied from 1–3 days; Utakoji (1972), and modified so that KMnO₄ (10 mM) treatment was varied between 20–40 min; Shiraishi and Yosida (1972), and modifications, such that the 8 M urea treatment was varied between 10–40 min, and staining time was varied from 5–20 min; Sun *et al.* (1974); Wang and Federoff (1972), and modifications, such that the time of trypsin treatment was varied between 15 sec–15 min; Patil *et al.* (1971), and modifications, such that the time of staining in Giemsa 9 was varied between 1–5 min, and pH of stain varied between pH 9.0–12.0; Sumner *et al.* (1971), and modified so that the time of incubation in 2×SSC (at 60°C) was varied between 15–120 min.

C-banding: Sumner (1972); Gallagher *et al.* (1973).

F-banding: Rodman (1974); Rodman and Tahiliani (1973), and modifications, such that the time in 0.14 M Na₂HPO₄ was varied between 5–20 min, and time in 12×SSC (at 20°C) was varied from 1–3 days.

Photomicrographs were taken with a Zeiss Photo-microscope using a green filter (No. 58) and Kodak High Contrast Copy Film. All prints were made on Agfaveart Rapidoprint Contrast 4 paper.

Results

Most of the methods employed yielded similar results in terms of chromosome appearance, i.e., uniformly spaced light and dark regions of a beadlike character.

The method of Schnedl (1971) yielded optimal results when chromosomes were exposed to 0.07 N NaOH for 90 sec, incubated for 24 hr in Sørensen buffer (pH 6.8), and stained in Giemsa for 40 min. Since dotted regions correspond across both sister chromatids, there is a suggestion of banding in some of the chromosomes (Fig. 1).

The best results were obtained with the method of Rodman and Tahiliani (1973) when chromosomes were treated with 0.14 M Na_2HPO_4 for 15 min, followed by incubation in $12\times\text{SSC}$ at 20°C for 2 days (Fig. 2). Once again note the prominent F-banded appearance of the chromosomes. C-banding of centromeric heterochromatin was elicited with this method using another modification which involved a 10 min incubation in Na_2HPO_4 followed by 2 days in $12\times\text{SSC}$.

The method of Patil *et al.* (1971) yielded optimal results when chromosomes were stained for 5 min in pH 9.0. The chromosomes showed a granulated appearance (Fig. 3).

The procedure of Wang and Federoff (1972) produced maximal results when chromosomes were exposed to 0.1% trypsin for 1 min. Fig. 4 illustrates the typically bloated trypsin-treated chromosomes, with a suggestion of striated regions.

The most promising results using 8 M urea (Shiraishi and Yosida 1972) were produced when treatment was for 10 min, followed by staining in Giemsa for 15 min. Definite banding patterns were found in many of the chromosomes (Fig. 5).

All other methods of banding and modifications, as presented in the Materials and methods section, produced unsatisfactory results.

There was no difference in banding patterns between male and female sexes.

Discussion

This work, utilizing several common banding methods (G-, F-, and C-banding) and their modifications, is the first report of banding of the chromosomes of *Rana pipiens*. Similar staining characteristics of the chromosomes have been elicited with diverse methods with the production of differential chromosome staining of a beadlike character. However, the methods utilized have not allowed satisfactory differentiation of different chromosomes within the complement.

Although extensive work has been reported for C-banding in various anuran families (Ruiz and Becak 1976, Raicu and Geormăneanu 1977, Schmid 1978a, 1978b),

Figs. 1–5. 1, G-banding, Schnedl, 1971 (modified). Chromosomes were treated for 90 sec in 0.07 N NaOH, incubated for 24 hr in Sørensen buffer (pH 6.8), and stained with Giemsa for 40 min. (Bar=0.5 μm). 2, F-banding, Rodman and Tahiliani, 1973 (modified). Chromosomes were treated for 15 min in 0.14 M Na_2HPO_4 , followed by incubation in $12\times\text{SSC}$ at 20°C for 2 days. (Bar=0.5 μm). 3, G-banding, Patil *et al.* 1971 (modified). Chromosomes were stained for 5 min at pH 9.0. (Bar=0.5 μm). 4, Trypsin method of Wang and Federoff, 1972 (modified). Chromosomes were treated for 1 min in 0.1% trypsin (Bar=0.5 μm). 5, G-banding, Shiraishi and Yosida, 1972 (modified). Chromosomes were treated for 10 min in 8 M urea, followed by staining with Giemsa for 15 min. (Bar=0.5 μm).

only the chromosomes of *Xenopus muelleri* have been G-banded (Stock and Menden 1975), and the latter G-banding was only of certain chromosomes and not the entire complement. It has been suggested that the extreme contraction of anuran metaphase chromosomes may be the explanation for the lack of the characteristic Q- and G-bands, (Schmid 1978a, 1978b). This is supported by the fact that the mean DNA content per μm of chromosome length of metaphase chromosomes is 1.5–3.0 times greater in bufonids, hylids, and other anuran species (Schmid 1978a). However, perhaps it is very significant that a major difference between members of the Urodela (which are more readily banded) and the Anura (which apparently are less easily banded) exists in terms of nuclear DNA content, with nuclear DNA of Anura being markedly less than that found in Urodela. Studies on several members of the family Ranidae have shown a DNA content of 6.6–15.8 pg/nucleus, while the DNA content of the members of the family Salamandridae (order Urodela) is 28.0–98.0 pg/nucleus (Chiarelli and Capanna 1973). It is conceivable that both nuclear DNA content and DNA content/chromosome length ratios may explain banding difficulties in anurans. Nevertheless, this inference does not seem to hold for *Xenopus muelleri* (family: Pipidae) which has a comparable DNA content as Ranidae, but yet can be banded satisfactorily.

There have been a number of theories or explanations proposed on the mechanisms involved in chromosome banding. One explanation of banding proposed was an accentuation of a pre-existing distribution of DNA and associated chromatin components (Rodman and Tahiliani 1973). A bimodal mechanism has been proposed which states that positive bands are due to precipitation of a selective group of chromatin proteins with a condensation of their associated DNA, while negative bands are due to a loss or displacement of protein with concomitant relaxation and stretching of DNA (Rodman 1974). It has also been suggested that the specific non-histones of positive regions render such regions more resistant to dispersion than interband regions (Burkholder 1975). Sumner and Evans (1973) reported that Giemsa is attached to DNA molecules at two separate points which are brought into close proximity by the folding of DNA. The spatial arrangement of dye-binding sites in the chromosomes is influenced by non-histone proteins, and the positive bands are due to binding of the dye in regions with specific non-histone proteins. It has been shown that an uneven extraction of acid-insoluble non-histone protein occurs during various G-banding procedures, such that a correspondence was observed between dansyl-Cl fluorescent-stained regions obtained after Giemsa pre-treatments and normal G-banded chromosomes (Matsukuma and Utakoji 1976). It is suggested that perhaps there is a variation in different non-histone proteins along the chromosomes so that banding is produced differentially. Further work must be undertaken to elucidate whether such an intrachromosomal variation of non-histone molecular species does indeed exist along the chromosomes.

It is concluded that perhaps in the chromosomes of *Rana pipiens* various physical and chemical reasons exist for the phenomenon of the beaded differential chromosome staining elicited. Further studies are being undertaken to clarify whether or not different modifications of present banding methods or different methods may produce more distinctive banding patterns in *Rana pipiens* and other Anura.

Abstract

Differential chromosome staining has been obtained for the chromosomes of cultured bone marrow cells of *Rana pipiens*. Although a wide variety of methods were investigated, only certain banding methods and modifications thereof gave promising results of particular significance for further investigation.

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