Chromomycin staining of vertebrate chromosomes: enhancement of banding patterns by NaOH

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Abstract

A simple technique is described for staining vertebrate chromosomes with the GC-base pair specific DNA fluorochrome, chromomycin A_3. Addition of NaOH crystals to a glycerol mounting medium resulted in good differentiation of chromosome bands and eliminated the need for counterstaining.

Introduction

Since Caspersson and colleagues (1968, 1969) first demonstrated that chromosomes could be differentiated *via* fluorescent stains, the use of fluorochromes to reveal banding patterns on metaphase chromosomes has become invaluable to cytogenetic analysis (Schweizer, 1981). This is due largely to the simplicity of fluorochrome staining and the general reproducibility of results.

Most of the literature regarding fluorescent staining of chromosomes has dealt with AT-base pair specific DNA stains such as quinacrine, Hoechst 33258, and DAPI (Schweizer, 1981; Amemiya and Gold, 1986). Much less has been documented regarding GC-base pair specific fluorochromes such as chromomycin A_3 (CMA) and mithramycin (Schmid, 1982; Amemiya and Gold, 1986).

Early attempts to use CMA met with a variety of problems, primarily photobleaching or fading (Schweizer, 1976, 1977). Methods developed to reduce rapid fading included employment of glycerol instead of saturated sucrose or buffer as the mounting medium (Sahar and Latt, 1978, 1980), and the use of nonfluorescent counterstains such as distamycin or methyl green (Sahar and Latt, 1978, 1980; Schweizer, 1981).

Although counterstaining substantially increases the staining contrast, hence facilitating good CMA banding (Sahar and Latt, 1978, 1980; Schweizer, 1981), distamycin is very expensive and difficult to obtain (Donlon and Magenis, 1983) and methyl green is relatively unstable (Sahar and Latt, 1980) and lacks consistency.

While attempting to determine factors which would enhance CMA staining of vertebrate chromosomes, we discovered that addition of sodium hydroxide (NaOH) crystals to a glycerol mounting medium produced striking chromomycin banding, even in the absence of counterstains. The bands produced were essentially of the same quality as those produced by counterstaining. Herein, we describe the protocol for employing this technique.

Materials and methods

Organisms used in this study included several species of fish, a bufonid toad, two species of ranid frogs, a colubrid snake, a caiman (Crocodilidae), a vespertilionid bat, and humans. Chromosome preparations were made either directly from tissues (spleen, kidney, gill or bone marrow) or from fibroblast or lymphocyte cultures. Fresh or aged (up to 3 months) preparations were used.

The staining protocol required three solutions. (1) A buffer (modified McIlvaine's) consisting of 9.6 g citric acid, 14.2 g Na_2HPO_4, and 0.2 g $MgCl_2.6H_2O$ which were added to distilled H_2O (500 ml total volume), and brought to pH 7.0 with NaOH. This was a stable solution and could be kept at room temperature for more than 6 months. (2) A staining solution composed of 1—2 mg chromomycin A_3 (Sigma) in 10.0 ml buffer (see above). This solution should be stored in the refrigerator at 4°C and will keep for *ca* 2 months. It should be noted that staining cannot be performed immediately since CMA usually takes 1—2 days to completely dissolve in the buffer. (3) A mounting medium consisting of 0.1—0.2 g NaOH crystals in 10.0 ml glycerol. The crystals were dissolved in glycerol by either microwaving, double boiling, or gentle heating at 50—60°C overnight. The mounting medium was stable and could be kept at room temperature for more than 6 months.

The staining procedure was as follows. The slide was rinsed briefly (*ca* 10—15 sec) with buffer solution. Most of the buffer was then removed by flicking the slide a few times. Two to three drops of stain solution were applied to the surface of the slide using a Pasteur pipette. A number 1 coverslip (preferably 24 x 50 or 24 x 60 mm) was floated on the staining solution. The slide was then allowed to stand for 2—10 min in the dark at room temperature.

The coverslip was removed by briefly washing the slide with tap water; excess liquid was removed by flicking the slide as before. The preparation was mounted using the mounting medium described above. After removing residual mounting medium with tap water, the slide was blotted dry and examined.

A Zeiss Universal research microscope equipped with epifluorescence (XBO 75 W/AC lamp) and fitted with an Olympus OM2 35 mm camera was used. All photomicrographs were taken using x63 Planapochromat or x100 Neofluar objectives on either Kodak panatomic-X FX402 (ASA 100—200) or technical pan 2415 (ASA 125—400) film developed in Diafine (Acufine) or D19 (Kodak). Exposure times ranged from 10 to 90 sec depending on the overall fluorescence of the specimen.

Chromomycin A_3 has an absorption maximum of 430 nm and a fluorescence maximum of 570 nm (Jensen *et al.*, 1977). The microscope used was equipped with the following two filter systems: (1) a BP 495 exciter filter, FT 510 chromatic beam splitter and LP 530 barrier filter; and (2) a UG-1 exciter filter, FT 420 chromatic beam splitter and LP 395 barrier filter. The first was used for fluorochromes such as quinacrine and acridine orange; the second was used for shorter wavelength fluorochromes such as DAPI and Hoechst 33258. Surprisingly, both filter systems worked equally well for chromomycin fluorescence when the mounting medium described was used.

Results and discussion

Results representative of the method are shown in Figures 1—3. CMA can differentiate three distinct chromosomal components on vertebrate chromosomes: heterochromatic C-bands (Sahar and Latt, 1980; Figure 1), nucleolus organizer regions (NORs) (Schmid, 1982; Amemiya and Gold, 1986; Figure 2) and euchromatic R-bands (Sahar and Latt, 1978; Figure 3).

Table 1 lists the type(s) of chromatin bands differentiated by CMA in the taxa examined in this study. The patterns observed were in close agreement with those from other accounts of chromomycin staining of vertebrate chromosomes (Sahar and Latt, 1978, 1980; Schweizer, 1981; Schmid, 1982; Amemiya and Gold, 1986).

The key factor appears to be the addition of NaOH crystals to the glycerol mounting medium. The effect of NaOH on the differentiation and contrast of CMA chromosome bands is clearly seen by comparing Figures 3 and 4. The CMA R-bands are better defined and more pronounced when NaOH is used in the mounting medium (Figure 3) than when it is not used (Figure 4). The same results, *i.e.* enhancement of CMA banding using NaOH in the mounting medium, were observed regardless of whether the CMA-bright regions were R-, C-, or NOR-bands. This enhancing effect of NaOH on CMA banding was virtually the same as that observed when CMA stained preparations were counterstained with distamycin or methyl green.

Subsequent to the finding that NaOH induced good differential CMA banding, efforts were made to determine the optimal NaOH/glycerol concentration and the effects of other bases. Replacing NaOH in the mounting medium with the bases Na_2SO_3, KOH, $NaHCO_3$ and THAM (tris hydroxymethyl aminomethane) was not as effective as NaOH in producing good contrast and differentiation of CMA chromosome bands. The optimal NaOH concentration was found to be between 0.01 and 0.02 g per ml of glycerol. Lower NaOH concentrations resulted in poor contrast and marginal band differentiation;

Table 1 Taxa examined and the types of CMA bands differentiated

Taxon	R-bands	C-bands	NOR-bands
Homo sapiens	+	−	−
Pipestrellus abramus (Bat)	+/−	+	−
Caiman crocodylus (Caiman)	−	+	−
Hypsiglena torquata (Snake)	−	+	+
Rana catesbiana (Frog)	−	+	+
Rana pipiens (Frog)	−	+	+
Bufo marinus (Toad)	−	+	+
Fish (>40 species)	−	−	+

Figure 1 CMA stained chromosomes of *Pipestrellus abramus* (vespertilionid bat) mounted in NaOH supplemented glycerol. x 1,400.

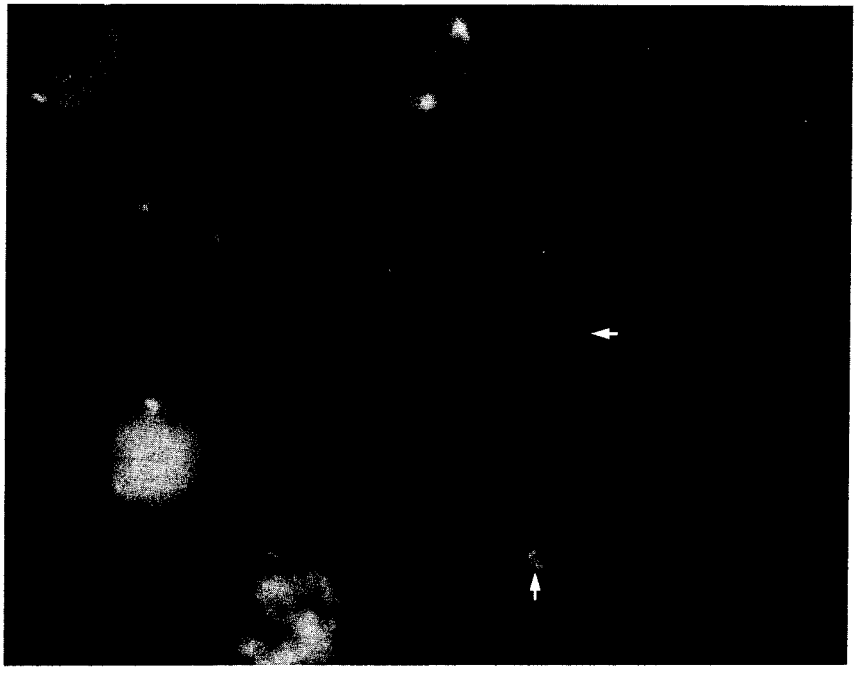

Figure 2 CMA stained chromosomes of *Notropis venustus* (cyprinid fish) mounted in NaOH supplemented glycerol. The chromosomal NORs are highly conspicuous and indicated by arrowheads. x 2,200.

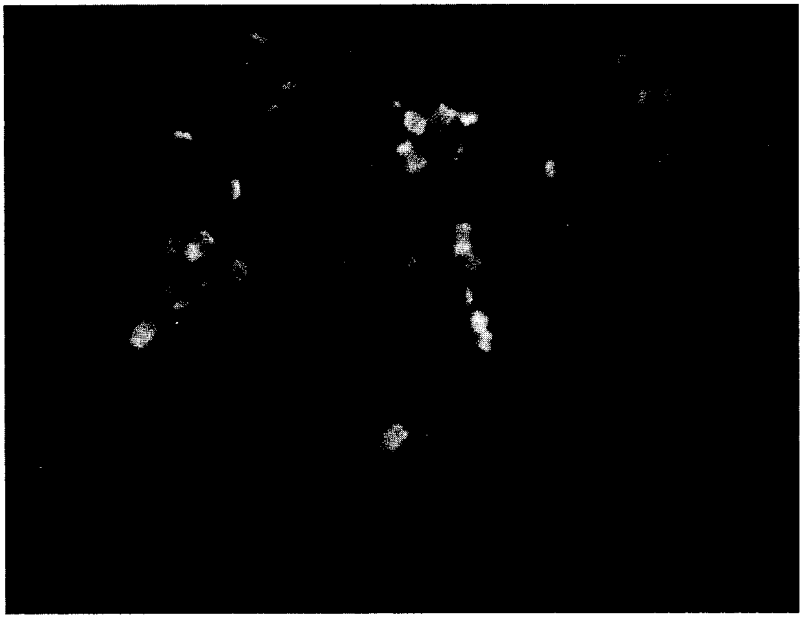

Figure 3 CMA stained human chromosomes mounted in NaOH supplemented glycerol. x 1,600.

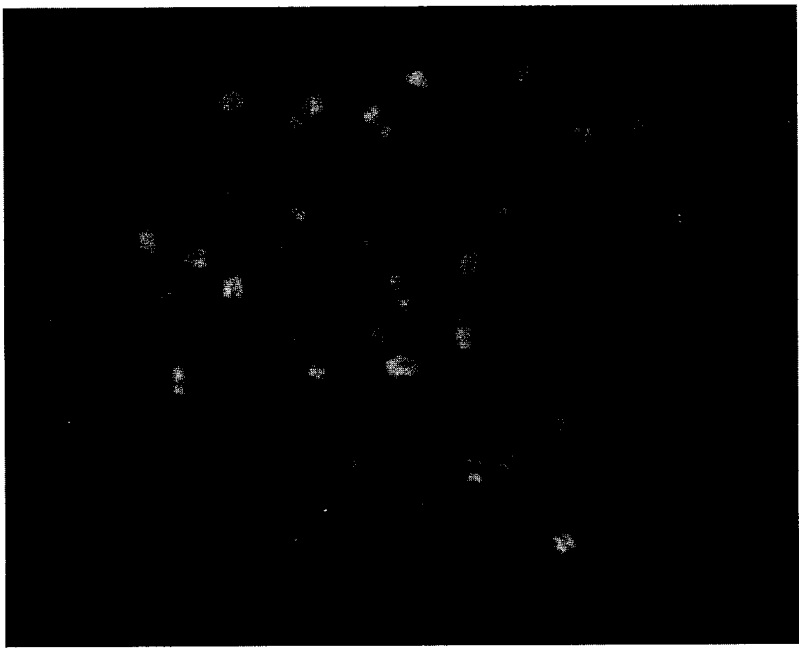

Figure 4 CMA stained human chromosomes mounted in glycerol without NaOH. Note the reduced differentiation of bands as compared to Figure 3. x 1,400.

higher concentrations resulted in increased photobleaching. Finally, it should be noted that the NaOH enhancing effect was also observed when mithramycin was used instead of CMA. The physicochemical bases for our observations are unknown and warrant further investigation.

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