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SILVER STAINING FOR NUCLEOLAR ORGANIZING REGIONS OF VERTEBRATE CHROMOSOMES

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ABSTRACT. Refinements to a simple, one-step silver staining technique for nucleolar organizing regions are described. These include fixation of silver stained material with sodium thiosulfate and standardization of silver development conditions for different groups of vertebrates. The central advantages to the method are that it is rapid, reliable, simple, and inexpensive. Additional benefits include (i) consistent and uniform silver staining of nucleolar organizing regions, (ii) few reduced silver deposits elsewhere on the chromosomes or on the slides, (iii) generally unaltered chromosome morphology after silver treatment, and (iv) relative permanence of Permounted preparations. The method works equally well on chromosomes made from cell cultures and from solid tissues of live specimens.

Silver staining to detect the chromosomal location(s) of nucleolar organizing regions (NORs) has been widely used since the seminal techniques were developed by Howell *et al.* (1975) and Goodpasture and Bloom (1975). The deposition of metallic silver at a NOR site apparently occurs via the reduction (development) of ionic silver by nonhistone nuclear protein(s) associated with NORs that were active at the preceding interphase (Howell *et al.* 1975, Goodpasture and Bloom 1975, Miller *et al.* 1976a,b, Howell 1977, Schwarzacher *et al.* 1978, Buys and Osinga 1980). Reduced silver also is found occasionally at or near centromeric heterochromatin or at the kinetochore itself (Howell and Denton 1974, Denton *et al.* 1975, Ved Brat *et al.* 1979, Ruiz *et al.* 1981).

Present technological problems with NOR silver staining include (i) standardization of silver development, (ii) uneveness of silver deposition, (iii) short shelf-life of reagents (*e.g.*, ammoniacal silver), and (iv) incomplete fixation. Howell and Black (1980) recently solved most of these problems for staining human NORs by using a protective colloidal solution to control silver development and deposition. In this note, we describe further refinements to their method and demonstrate the general applicability of the technique to different vertebrate groups.

MATERIALS AND METHODS

Chromosome Source and Preparation. Metaphase chromosomes of the vertebrates used in this study were obtained either from cell culture (human, bat, turtle) or directly from live specimens (fish). Preparation of chromosomes from cell cultures essentially followed conventional techniques, *viz.*, incubation with mitotic inhibitor, hypotonic treatment, fixation, and air drying of fixed cells which had been dropped onto cold, wet slides. Preparation of chromosomes from kidney tissue of live fish essentially followed the solid tissue method of Kligerman and Bloom (1977). All slides, regardless of how prepared, were stored desiccated at 37 C until use.

Silver Staining. The staining solutions used follow Howell and Black (1980) and include (i) a colloidal developer containing 2 g powdered gelatin USP in 100 ml deionized water and 1 ml pure (91.5%) formic acid, and (ii) an aqueous silver

nitrate solution (50% w/v). Our modifications include a fixing solution of sodium thiosulfate (5% w/v), and a counterstaining solution of 4% Giemsa (Fisher) in 10^{-2} M sodium phosphate buffer, pH 6.8–7.0.

For staining, one part of the colloidal developer and two parts of the aqueous silver nitrate are pipetted directly onto the surface of the slide, mixed, and covered with a coverslip. The slide is then placed on a slide warmer preset and stabilized at 40–45 C (fish, turtle) or 45–50 C (human, bat). At these temperatures for the species examined, good NOR differentiation usually occurs in 6–8 minutes, or when the staining solution has turned a deep golden-brown color. The slide is then removed from the slide warmer, rinsed briefly in distilled water to remove the coverslip, and fixed 4–5 minutes in 5% sodium thiosulfate. Following another brief distilled water rinse, the slides are then counterstained (30–45 sec for human and bat, 60–75 sec for fish and turtle), rinsed in distilled water, air dried, cleared in xylene for 10 minutes, and mounted in Permount. As with most NOR silver techniques, the staining reaction may be monitored microscopically by examining the slide prior to fixation. Optimally, NOR regions on chromosomes should be stained black while the chromosome arms should be stained yellow.

Results and Discussion

Examples of NOR-stained metaphases using our silver staining procedure are shown in Figs. 1–3. As illustrated, the silver stained NORs are cleanly differentiated, and only in a few instances is even a minor silver precipitate noticeable elsewhere on the slide. Immediately obvious advantages to the method are that (i) the overall morphology of the chromosomes is essentially unchanged from preparations stained only with Giemsa, and (ii) only rarely is silver found elsewhere on chromosomes than at NOR sites.

Our modifications to Howell and Black's (1980) technique include the sodium thiosulfate fixation step and alterations in the silver development conditions. The

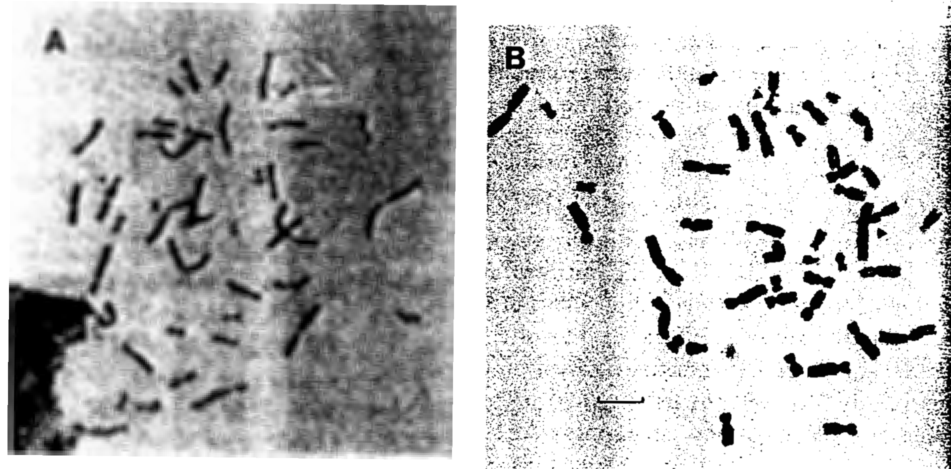

Fig. 1. Silver stained human metaphases showing (A) 7 NORs including one D-G association (arrow) and (B) 6 NORs including two D-G associations (arrows). Bar = 5 microns.

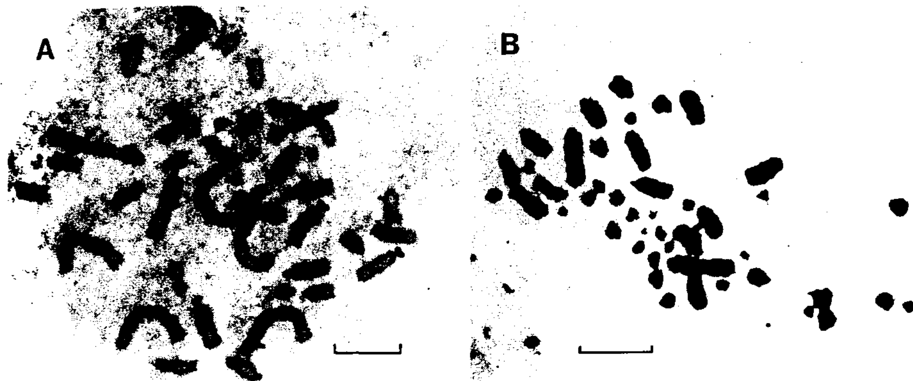

FIG. 2. Silver stained metaphases of (A) *Myotis daubentonii* (vespertilionid bat) showing conspicuous NOR regions on the short arms of four subtelocentric chromosomes, and (B) *Platemys platycephala* (chelid turtle) showing an association between the two NOR bearing microchromosomes. Bar = 5 microns.

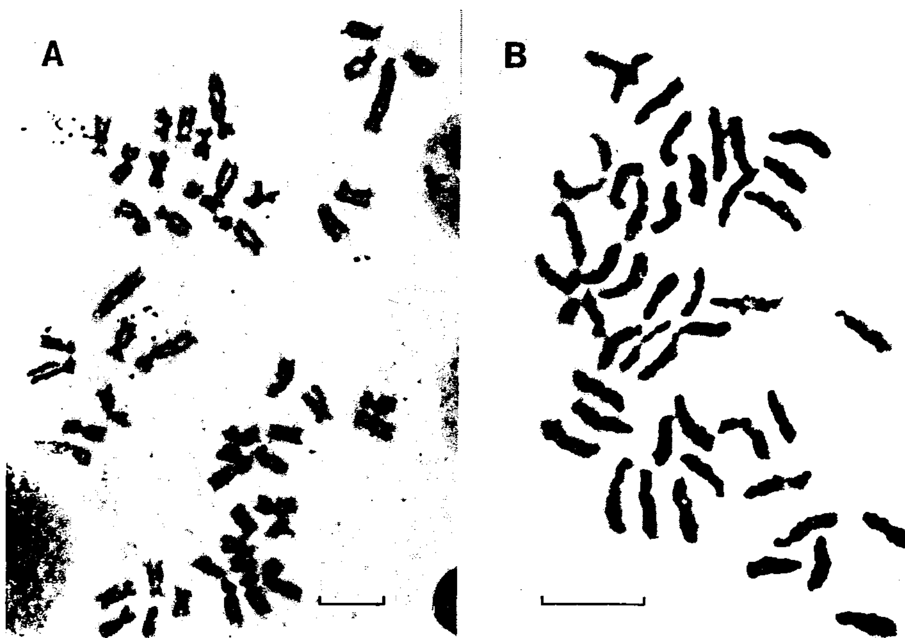

FIG. 3. Silver stained metaphases of (A) *Notemigonus crysoleucas* (cyprinid fish) showing an association between the two NOR bearing acrocentric chromosomes, and (B) *Pomoxis annularis* (centrarchid fish) showing NORs on the short arms of four subtelocentric chromosomes. Bar = 5 microns.

fixation step initially was added to ensure the removal of residual ionic silver (Carrol *et al.* 1980) and to prevent further silver reduction elsewhere on the slide. Its major benefit, however, is that since most of the unreduced silver is apparently removed, Permounted slides become more or less permanent. We have, in fact, examined silver stained material over a month old and noticed no adverse effects.

Our modifications to the silver development conditions were prompted by the

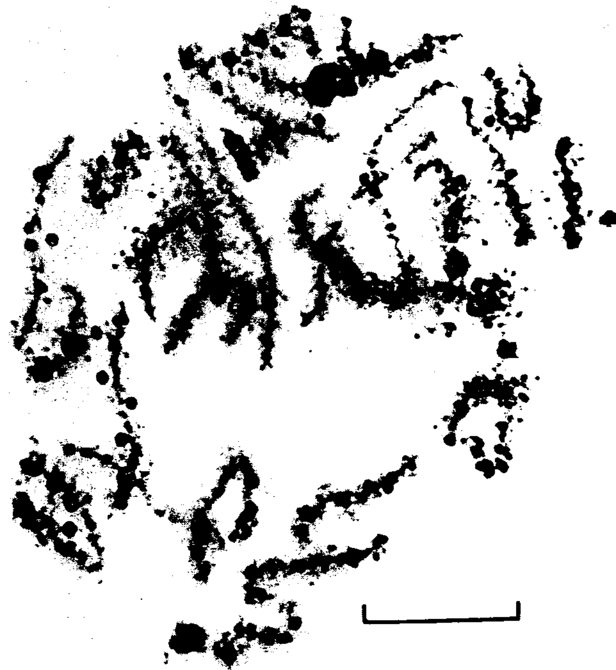

FIG. 4. Silver stained mid-late pachytene chromosomes of *Geomys attwateri* (geomyid rodent). M: terial was prepared following Tucker *et al.* (1981); silver staining was carried out for 7–8 min at 45 (see Methods). Bar = 10 microns.

apparent ill effects on the very small chromosomes of fish and turtle of Howell an Black's 70 C incubation. In both groups, most of the chromosomes less than tw microns in length were clearly distorted and very poorly stained even after on! two minute's treatment. The effect lessened with increased storage time of slide but nonetheless suggested that less harsh conditions might yield better result Accordingly, we silver stained several different preparations of both human ar fish chromosomes at different temperatures ranging from 40–60 C and for perio ranging from 2–14 minutes. As expected, a roughly linear inverse relationsh between temperature and time in terms of optimal NOR differentiation w observed. The best results in our hands generally were obtained with the conditio specified in Methods, but we expect that minor adjustments will be necessary f different species.

The central advantages of the NOR technique as described are that it is rap reliable, simple, and inexpensive. Additional benefits include both consistent a uniform staining at the NOR sites as well as the absence of silver depositi elsewhere. With the refinements added, there are fewer disruptive effects in gene on chromosome morphology and stainability, and the preparations are more less permanent. Finally, we should note that our method has been used successfu

to stain synaptonemal complexes of pachytene chromosomes (Fig. 4). As discussed by Pathak and Hsu (1979), procedures to demonstrate synaptonemal complexes with light microscopy should greatly facilitate studies of meiosis in a number of species.

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