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Chicken Erythrocytes as an Internal Reference for Analysis of DNA Content by Flow Cytometry in Grass Carp

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Abstract.—We estimated the nuclear DNA content of unfixed erythrocytes of the grass carp *Ctenopharyngodon idella* by fluorescence flow cytometry using propidium iodide as a fluorochrome. Frozen erythrocytes from the domestic chicken *Gallus gallus* were thawed and used as an internal reference for simultaneous analysis with grass carp samples. The nuclear DNA content of erythrocytes from diploid grass carp (mean ± SD) was 2.00 ± 0.01 pg/cell, whereas triploids possessed 2.97 ± 0.03 pg/cell. Variations in the nuclear DNA content of different chickens were standardized in relation to the DNA content of fresh human leukocytes (7.00 pg/cell). The value for DNA content of the chicken erythrocytes (around 2.5 pg/cell) occupied a position intermediate between the two grass carp values, and thus provided a clear reference for discrimination between diploids and triploids.

Grass carp *Ctenopharyngodon idella* are used worldwide for control of aquatic vegetation. Diploid grass carp are highly fecund, and thus pose a potential risk in systems into which they are introduced. The production of triploid grass carp by thermal or pressure treatment of eggs has been accepted as a means of generating sterile fish suitable for stocking (Allen and Wattendorf 1987). Screening grass carp for verification of ploidy and removal of diploid individuals is necessary, however, because no technique is successful in producing only triploids. Screening is most commonly accomplished by measurement of nuclear volume with a Coulter counter (Wattendorf 1986), but it can also be performed with microscopic nuclear volume measurements, karyotyping, microspectrophotometric methods, or flow cytometry.

Nuclear DNA content in several species of fish has been estimated by use of flow cytometry. This technique allows detection of interspecific differences in cellular DNA (Johnson et al. 1987) and ploidy levels (Thorgaard et al. 1982; Allen 1983). The nucleated erythrocytes of fish can be analyzed in a few minutes by flow cytometry with less than 1 μL of whole blood. An internal reference, such as nucleated erythrocytes from another species, is analyzed simultaneously with a sample to enable calculation of DNA content. Calculations of DNA mass can be performed directly, in relation to an internal reference standard, or indirectly, in relation to a third-party reference standard that has a known relationship to the internal reference. Mosaicisms, chromosomal polymorphisms, and DNA aneuploidies can be detected as small variations in nuclear DNA mass.

Because the quantification of nuclear DNA by

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flow cytometry is relative, accurate determinations require a suitable internal reference standard (Coulson et al. 1977; Jakobsen 1983; Lee et al. 1984). To reduce possible zero shift error, the internal reference should have a DNA content close to that of the sample being analyzed (Vindeløv et al. 1983), but it should not be so close that the DNA values overlap, because the overlap itself causes measurement errors. Internal references reduce variation in measurements by correcting for factors such as laser beam fluctuations, minor variations in staining conditions, or changes in sample flow characteristics that cause spurious DNA content estimations (Hoehn et al. 1977; Tannenbaum et al. 1978).

Analysis by flow cytometry of fresh erythrocytes from the domestic chicken *Gallus gallus* and formalin-fixed grass carp erythrocytes revealed that diploid and triploid fish each possessed a DNA content lower than that of the chicken reference standard; values for triploid grass carp, however, overlapped considerably with values for the chicken (Allen and Stanley 1983). In the present study, the objective was to measure the nuclear DNA content of grass carp in order to evaluate a modified technique for identification of diploids and triploids. We report that chicken erythrocytes, stored at −80°C, provide a useful internal reference for high-resolution analysis of DNA content and determination of ploidy in unfixed grass carp erythrocytes.

Methods

Healthy 3- and 4-year-old diploid ($N = 16$) and triploid ($N = 39$) grass carp 4.5–10.0 kg in mass and 65–95 cm in fork length were studied. We collected blood samples in May of 1988 and 1989 by caudal puncture, using blood-banking solution as an anticoagulant (from Becton-Dickinson Vacutainer 4606). Samples were kept at 4°C in 3-mL Vacutainers and transported from Florida to Memphis, where DNA analyses were conducted within 4 d of collection. We have found that samples treated similarly yield consistent results for at least a week provided they are kept cold.

We modified the method of Krishan (1975) according to Elias et al. (1988) and Kent et al. (1988). In brief, we suspended grass carp and chicken blood cells in 0.5 mL of a solution containing 25 μg propidium iodide, 0.1% sodium citrate, 25 μL buffered RNase (1 mg/mL), and 0.1% Triton X-100. All samples were mixed, filtered through 20-μm nylon mesh, and analyzed within 15 min. We used propidium iodide as a fluorochrome because its staining properties are not influenced by DNA base composition; other stains, such as 4′, 6-diamidino-2-phenylindole (DAPI), bind only to specific nucleotides within DNA (Taylor and Milthorpe 1980; Lee et al. 1984).

Erythrocytes from individual chickens were prepared for use as internal references by dilution of whole blood (collected with anticoagulant) in phosphate-buffered saline (GIBCO Laboratories 310-4190) containing a final concentration of 8% dimethyl sulfoxide. Suspended cells were aliquoted, frozen in liquid nitrogen, and stored at −80°C for at least 4 months before use. A male chicken of unknown strain provided cells used as an internal reference in 1988, and a male chicken of a Rhode Island Red × Barred Rock cross provided cells used in 1989. Repeated measurements of the DNA content of these two chickens (calculated in reference to human leukocytes) were 2.45 ± 0.00 (mean ± SD) pg/cell (1988) and 2.53 ± 0.00 pg/cell (1989). Cells from a female chicken of a Rhode Island Red × Barred Rock cross were prepared in 1989 for comparison with male values. Aliquots of similarly cryopreserved blood cells from channel catfish *Ictalurus punctatus* and mosquitofish *Gambusia affinis* were thawed daily. These cells were mixed with thawed cells from the chicken, and with fresh leukocytes from porcine and human males, to calibrate the flow cytometer.

Analyses were performed with a Coulter EPICS V flow cytometer (Coulter Electronics) with argon ion laser set at a wavelength of 488 nm at an output power of 300 mW in light-stabilized mode. The optical filters consisted of two 515-nm long pass barriers, a 560-nm dichroic splitting filter, and a 570-nm long pass filter placed before the photomultiplier tube. Individual nuclei of lysed erythrocytes (particles) were analyzed at a rate of 100–200 particles/s. The fluorescence of each particle was transmitted as an analog signal to a computer, and the signal was digitized to generate pulse-height histograms that represented at least 40,000 cells. For greatest resolution and accuracy, the fluorescence peaks of cell populations in the G_0 and G_1 stages of the cell cycle (stages before the synthesis of new DNA) were collected near channel 130 for diploid fish, channel 165 for the chicken, and channel 200 for triploid fish.

We used the Coulter PARA 1 program to calculate the fractional mode channel of each histogram fluorescence peak to four decimal places. Grass carp nuclear DNA content was expressed as fluorescence intensity relative to chicken fluo-

rescence or as picograms of DNA per cell in relation to an assigned value of 7.0 pg for fresh human leukocytes (from one of us [R.W.C.]). This value is based on 24 determinations in cells from various normal human tissues in 11 separate studies cited by Shapiro (1976), and on absorption cytophotometry of Feulgen-stained cells (Atkin et al. 1965; Allison et al. 1981; Rasch 1985). Values for DNA mass were calculated by the formula: nuclear DNA content (pg) = (A/B)(C/D) × 7.0; A, B, C, and D are all fractional mode channel values, B is the value of the chicken internal reference analyzed simultaneously with a particular grass carp (A), and C is value of the chicken internal reference analyzed simultaneously with fresh human leukocytes (D). Thus, the chicken values are cancelled during this calculation and the grass carp value is expressed in relation to the DNA content of fresh human cells. The Mann–Whitney *U*-test was used for statistical comparisons; $P \leq 0.05$ was selected as the criterion for significance.

Results and Discussion

The nuclear DNA content of chicken erythrocytes (about 2.5 pg/cell) was intermediate between the DNA contents of erythrocytes of diploid and triploid grass carp, and thus provided a useful reference for determination of ploidy in the fish. No significant difference was found between the DNA contents of the diploids studied in 1988 and in 1989, and there was no difference between the triploids studied for the same two years. Also, no significant difference in DNA content was found between male and female grass carp of either ploidy. The coefficient of variation for fluorescence peaks (calculated at 50% maximum height) was less than 4% for all grass carp studied. Mosaicism was not detected, although an apparent "spontaneous triploid" (Goudie 1988) was found in the diploid group of 1989. When the data from the two years were pooled, nuclear DNA content, calculated in relation to the human, was 2.00 ± 0.01 pg/cell for diploids and 2.97 ± 0.03 pg/cell for triploids (Figure 1). Triploids possessed 1.48 times the amount of DNA found in diploids (a value not significantly different from 1.50).

Nuclear DNA contents reported previously for grass carp are lower than those found in this study. Formalin-fixed erythrocytes of grass carp used in a ploidy study by Allen and Stanley (1983) yielded diploid and triploid DNA contents that were lower than that of fresh chicken erythrocytes. In that study, the triploid DNA content was only 1.34 times larger than the diploid value, which was

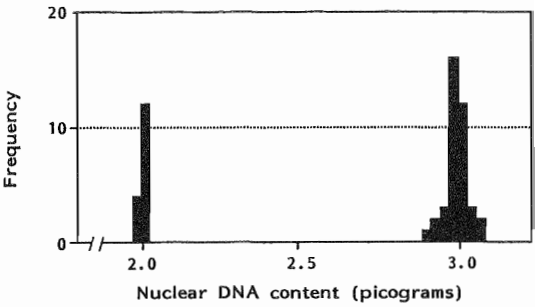

FIGURE 1.—Frequency histogram of DNA content of erythrocyte nuclei from diploid ($N = 16$) and triploid ($N = 39$) grass carp analyzed by flow cytometry.

reported to be about 2 pg. Triploid cells thus apparently contained 2.68 pg DNA per cell. An assumed nuclear DNA content of 2.5 pg for the chicken internal standard employed in that study would, however, lead to calculated values of 1.70 pg for diploids and 2.28 pg per cell for triploids. These calculations are based on the relative fluorescence value of 68% reported for the diploids and the relative fluorescence value of 91% reported for the triploids. The differences in DNA content reported in these two studies may be due to intraspecific variation in genome size in the grass carp, differences in DNA content in the chickens used, or methodological disparities. One possible explanation is the use of formalin for fixation of the grass carp cells. Crissman et al. (1978) found that formalin fixation of cells reduced the fluorescence intensity of subsequent staining procedures; in addition, increases in the coefficient of variation for formalin-fixed samples have been reported (Crissman et al. 1978; Deitch et al. (1982). In a study with unfixed fish cells, Allen et al. (1986) obtained relative values for grass carp that closely approximated the expected relative values for haploid, diploid, and triploid cells, although chicken cells were not used as an internal reference.

Direct calculations of grass carp DNA content in relation to an internal standard may be complicated by variation in the DNA mass of the chicken. The chicken internal reference of 1988 had a nuclear DNA mass that was 3% greater than that of the chicken used in 1989. In 1988, relative fluorescence in relation to the chicken was 82% for diploid grass carp erythrocytes and 122% for the erythrocytes of the triploids. In 1989, diploids possessed a 79% relative fluorescence value, and triploids possessed a value of 117%. The values for diploids were significantly different for the two

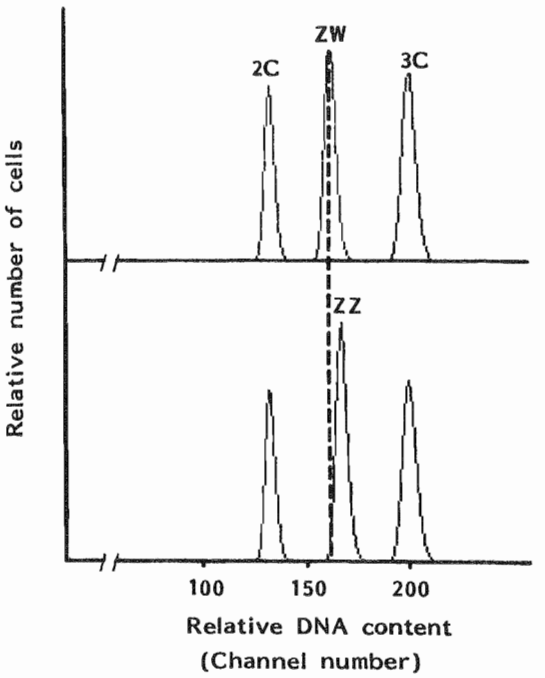

FIGURE 2.—Male–female differences in DNA fluorescence histograms of erythrocytes from diploid (2C) and triploid (3C) grass carp analyzed by flow cytometry in a mixture with erythrocytes of female (ZW) or male (ZZ) chickens.

years, as were those for triploids. Thus, direct calculation of DNA content in relation to different chickens can yield different values. However, standardization of the DNA values in relation to the same human each day that analyses were performed corrected for this difference. The reason for the difference in DNA content between the chickens is unknown. It could be due to changes in the instrument over the year, minor differences in the reagents used, or strain differences between the chickens.

Because of the small size of their W chromosome, hens possess less DNA than roosters. The male chicken used in 1989 had 2.7% more DNA than the female chicken we used in that year (Figure 2). This difference could represent the intraspecific variability of DNA content that exists in the domestic chicken. However, in an unpublished study, we compared the DNA content of fresh blood cells from 11 male and 7 female chickens of a Rhode Island Red × Barred Rock cross and found that these males possessed 2.7% more DNA than the females. Furthermore, Vindeløv et al. (1983) found 2.5% more DNA in the male of a pair of chickens, and Rasch (1976) found differences of 4–6% between male and female cranes of the genus *Grus* with the use of microspectrophotometry of Feulgen-stained nuclei. These observations suggest that there is indeed a measurable sex-related difference in the DNA content of avian cells. Accordingly, direct computation of DNA content in grass carp relative to a male or female chicken will yield different values.

In conclusion, chicken erythrocytes stored at −80°C provided a useful internal reference for high-resolution analysis of genome size in unfixed erythrocytes of grass carp. Because many other teleost species have nuclear DNA contents of about 2–3 pg (Hinegardner and Rosen 1972), chicken erythrocytes should be useful for high-resolution analysis in those species also.

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