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Direct observation of δ-crystallin accumulation by laser light-scattering spectroscopy in the chicken embryo lens

(differentiation/protein synthesis)

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ABSTRACT **By using the technique of laser light-scattering spectroscopy, direct observation has been made on the intracellular accumulation of a crystallin protein within the cells of chicken embryo lens during the process of development. Appearance of δ-crystallin has been detected as early as day 4, and its concentration reaches a plateau at day 19. The measurements constitute a noninvasive determination of accumulation of protein molecules that specifically characterize the process of cell differentiation.**

Rapid progress is being made in the understanding of molecular events of cellular differentiation, particularly at the level of gene expression. Lens cell differentiation has been a favorable system of investigation because it is associated with a marked change in morphology (cell elongation) and differential synthesis of the soluble proteins (crystallins). The crystallins, or structural proteins within the lens, comprise as much as 90% of the soluble protein of the lens. There are four families of crystallins (α-, β-, γ-, and δ-crystallin), with δ-crystallin being confined to birds and reptiles (1, 2). Reviews on lens differentiation and lens crystallins can be found elsewhere (3–6).

In the chicken lens, δ-crystallin is the first crystallin to appear (7) and can be detected already at the lens placode stage of development (8, 9). This protein accumulates in the embryonic lens until it comprises 70–80% of the soluble protein in the developing lens fiber cells (10–12). δ-Crystallin synthesis gradually ceases during the first few months after hatching (13–15). Consequently, the concentration of δ-crystallin is high in the center (nucleus) of the adult lens, which contains cells deposited early in development, and is low or absent at the periphery of the lens (cortex), which contains the cells deposited later in development (16) (Fig. 1). The current knowledge of δ-crystallin and its nucleic acids has been reviewed recently (17).

Experimentation on crystallin accumulation during lens development has been confined to biochemical and immunological methods that disrupt the tissue. The biochemical methods lose spatial resolution, and the *in situ* methods (i.e., immunofluorescence) are semiquantitative at best. Both methods may introduce artifacts such as protein denaturation or aggregation. In the present investigation, we show that crystallin accumulation may be followed *in vivo* by the noninvasive technique of laser light-scattering spectroscopy. Laser light-scattering spectroscopy allows observation of the Brownian motion of macromolecules inside the cytoplasm of lens cells. The rate of the molecular Brownian motion is directly proportional to the rate of intensity fluctuations of laser light scattered from randomly moving macromolecules (18–20). From the amplitude and relaxation time of the scattered light-intensity fluctuations, it is possible to determine the concentration and size distribution of the macromolecules within the lens cells. Using this technique, we have succeeded in determining the time course of intracellular accumulation of macromolecules, mostly δ-crystallin, in intact chicken lenses during their development.

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MATERIALS AND METHODS

Fertile eggs of white Leghorn chickens were obtained from Spafas (Norwich, CT) and were incubated in a humidity-controlled incubator at 37.5°C. The lens of the appropriate age was immersed in silicon oil in a cuvette for laser light-scattering spectroscopy. The beam from an argon laser was focused onto the lens, and the light scattered from a volume of approximately $(50\ \mu m)^3$ was imaged onto a photomultiplier tube. The temporal fluctuations of the scattered light intensity $I(t)$ were analyzed in the form of the time correlation function defined as

$$C(t) = \langle I(t')I(t'+t)\rangle_{t'},$$

where the angle brackets denote the time average over t'. For a solution of monodispersed macromolecules, the correlation function can be expressed in terms of diffusion coefficient D of the macromolecules

$$C(t) = A\exp(-2Dq^2t)+B,$$

where $q = (4\pi/\lambda)\sin(\theta/2)$ is the scattering wave number, λ is the wavelength of the laser light in water, θ is the scattering angle ($\theta = 90°$ in this experiment), and A and B are constants proportional to the square of concentration and molecular weight of the macromolecules. The diffusion coefficient is further related, via the Stokes–Einstein–Kawasaki–Ferrel formula, to the correlation length, a, of the macromolecules

$$D = kT/6\pi\eta a,$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of water. The correlation length a is the average distance beyond which the motion of neighboring macromolecules becomes independent. When interaction is negligible, the correlation length is simply the hydrodynamic radius of the macromolecules.

When there are macromolecules of different sizes and, therefore, different diffusion coefficients, the correlation function is given by the superposition of the correlation functions corresponding to each size. By precise analysis of the correlation function, it is, in principle, possible to determine the size distribution and the concentration of the macromolecules.

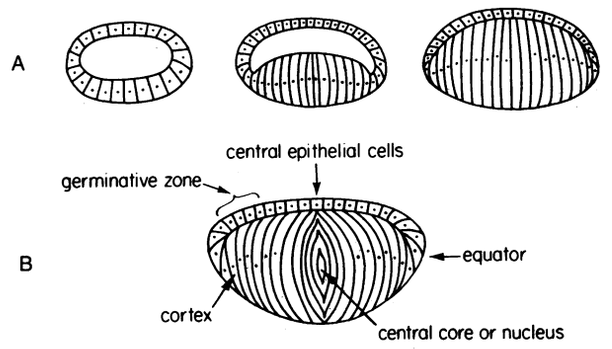

FIG. 1. Schematic illustration of lens development. (*A*) Early in development, the lens appears as a vesicle with anterior epithelial cells and posterior primary fiber cells. The cells at the posterior region elongate further into the hollow of the vesicle and differentiate into elongated fiber cells. The anterior cells of the lens vesicle remain epithelial and mitotically active. They contribute new cells at the equator, which differentiate into secondary fiber cells. (*B*) Later in development, mitosis is restricted to the germinative zone that encircles the central epithelial cells. This process of fiber cell formation continues throughout the life of the lens.

RESULTS

The measured correlation functions of laser light scattered from the center of the chicken lenses are shown as a function of age from 4 to 40 days (hatching is at 21 days) in Fig. 2*A*. The analysis of the correlation functions shows that there are mainly two components within the cells, one with a hydrodynamic radius of about 65 Å and one (probably organelles) with a hydrodynamic radius of about 6,700 Å. We assumed that the smaller particles are δ-crystallins due to their abundance in the lens cells.

In order to test this assumption, the radius of purified δ-crystallin was determined by electron microscopy. An electron micrograph of the protein negatively stained with 2% uranyl acetate is shown in Fig. 3. The area of 100 protein particles was determined by planimetry with a graphics calculator (Numonics, North Wales, PA, Model 224-116). The average radius of the proteins calculated from the areas (assuming a circle) was 41.6 ± 5 Å. δ-Crystallin stained with 2% sodium silicotungstate had an average radius of 41.9 ± 4 Å. Considering δ-crystallin a sphere with a density of 1.26 [calculated from its amino acid composition (21)], a radius of 40 Å gives a molecular weight of 203,000 for the native protein. This agrees remarkably well with a molecular weight near

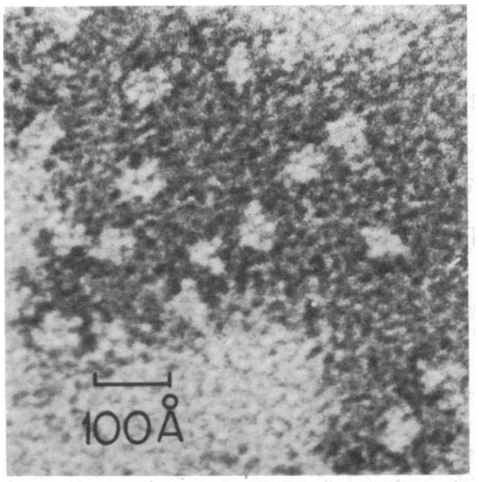

FIG. 3. Electron micrograph of δ-crystallin purified from 15-day old embryonic chicken lenses by isoelectric focusing in a sucrose density gradient (21). The δ-crystallin was greater than 90% pure as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis. The protein was dialyzed against deionized water and spread on thin carbon floated off mica that was supported on a thick carbon film with holes in it. [Bar = 100 Å (1mm = 10 Å).]

200,000 for δ-crystallin determined by gel filtration and by centrifugation at sedimentation equilibrium (21). We also have determined by laser light-scattering spectroscopy an average hydrodynamic radius of 45 Å on embryonic chicken δ-crystallin in a dilute solution (0.1 mg/ml). Taken together, these data are consistent with the identification of the smaller intracellular particles (average radius near 65 Å) as δ-crystallin with laser light-scattering spectroscopy on intact cells. It is reasonable that by using this technique, the apparent hydrodynamic radius of intracellular δ-crystallin is a little larger than the size obtained by any of the other methods mentioned here because of the interactions among the crystallins at the high concentrations inside the cell (22).

We further determined the relative concentration of the smaller particles, mostly δ-crystallin, to that of organelles within the lens as a function of age (Fig. 4*A*). The result clearly shows (*i*) the presence of δ-crystallin at day 4 of development and (*ii*) the increase in its concentration until hatching. After hatching, the concentration of δ-crystallin remains constant. The dashed line to the abscissa of the fig-

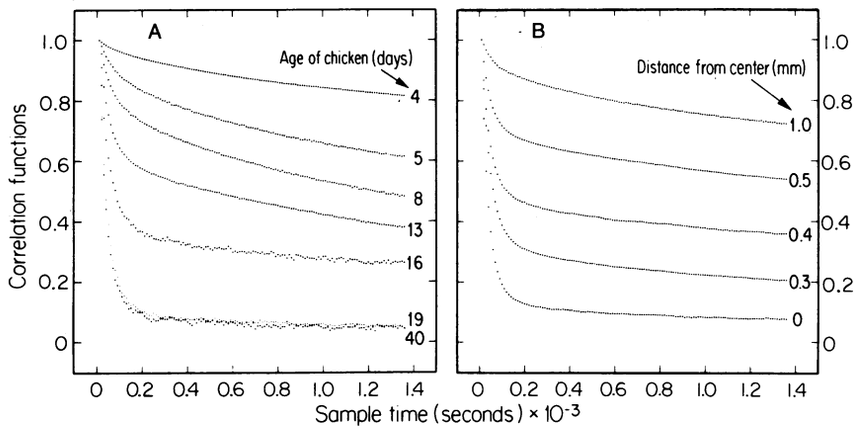

FIG. 2. (*A*) Correlation functions of laser light scattered from the fiber cell cytoplasm at the center of chicken lenses of ages from 4 to 40 days. As age increases, the amplitude of the fast-decay component, corresponding to light scattered from δ-crystallin, increases gradually. (*B*) Correlation functions for a 19-day old chicken embryo lens at various positions in the lens along a radial line from the center to the equatorial periphery. The correlation function for the center has the largest amplitude for the fast-decay component, indicating the largest concentration of δ-crystallin.

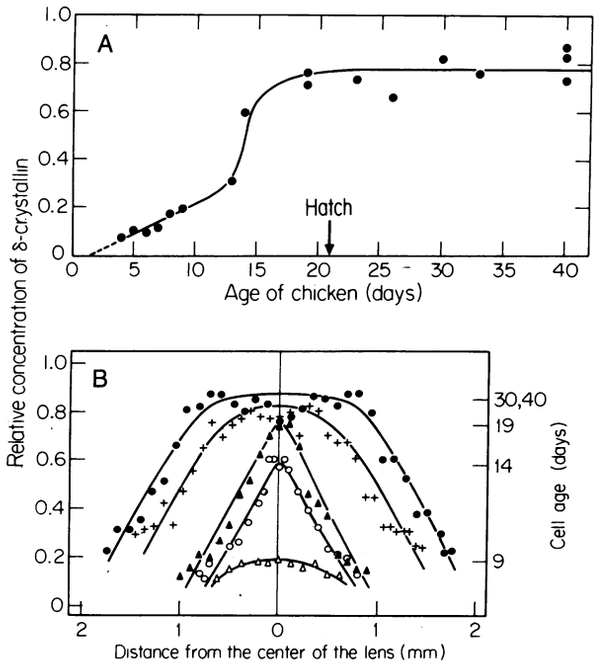

FIG. 4. (*A*) The relative concentration of δ-crystallin at the center of the lens is plotted as a function of lens age. The presence of δ-crystallin is clearly seen at day 4, and its concentration increases and plateaus after 19 days of development. The dashed line to the abscissa is extrapolated and suggests that δ-crystallin synthesis begins at about 1.4 days of development. (*B*) The position dependence of the relative concentration of δ-crystallin and organelles in chicken lenses of age 9 (△), 14 (○), 19 (▲), 30 (+), and 40 (●) days. Progressive increase of δ-crystallin concentration as the position moves toward the lens center reflects the correlation between the cell age and the radial position within the lens. Corresponding cell age obtained from Fig. 2*A* is indicated to the right of the figure.

ure is extrapolated and suggests that δ-crystallin synthesis begins at about 1.4 days of development. This is in good agreement with earlier biochemical analyses of δ-crystallin mRNA accumulation in the developing lens (8, 9).

We also have measured the position dependence of the correlation function inside the lens (Fig. 2*B*). In Fig. 4B we show the relative concentration of the δ-crystallin and the 6700 Å particles for lenses of chickens of age 9, 14, 19, 30, and 40 days. The concentration of the δ-crystallin is low at the periphery and progressively increases toward the center, where it reaches a maximum. This reflects the fact that δ-crystallin is synthesized continuously in the embryo, and thus the concentration of the protein inside the fiber cells increases with cell age as new fiber cells are deposited at the lens periphery. Thus, the morphogenesis of the lens can be examined by studying the position dependence of δ-crystallin content within the lens. The high spatial resolution of the relative amount of δ-crystallin achieved by laser light-scattering spectroscopy provides a new and sensitive measure of lens development.

DISCUSSION

We have developed a technique of microscopic laser light spectroscopy that can probe a cytoplasmic region as small as $(2\ \mu m)^3$ (23). This new technique will make it possible to examine intracellular events within an intact single cell during the process of lens differentiation of epithelial cells as well as fiber cells. We believe that laser light-scattering spectroscopy will lead to a quantitatively better understanding of lens development.

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