Amphibian Lens Histones and Their Relation to the Cell Cycle

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ABSTRACT Histones have been electrophoretically separated from acid extracts of the frog lens for the first time. The five conventional histone fractions, representing four electrophoretic bands (f1; f2b, f3; f2a2; and f2a1), are present in both the epithelial and fiber cells. In addition, a fifth fraction was isolated from both sources and the evidence suggests that it may be a tissue-specific histone, possibly related to the lysine-rich f2c fraction found previously only in nucleated erythrocytes. The epithelial cells contain a substantially greater amount of histone than the fiber cells. Moreover, the fibers, unlike the epithelium, manifest no net histone synthesis or turnover following lenticular explantation. Microspectrophotometric, radioautographic, and gel electrophoretic studies indicate that the histones are synthesized in frog lenses concurrently with DNA. Inhibition of DNA synthesis does not completely abolish that of histones but reduces it by about one-half. In the early stages of culture (prior to their synthesis and that of DNA) the histones appear to undergo alterations which are prevented by treatment with cycloheximide.

INTRODUCTION

Upon explanation of the bullfrog lens to a suitable culture medium, the epithelial cells on the anterior surface of the organ are observed to undergo bursts of DNA synthesis (48-52 hr postisolation) and cell division (87 hr postisolation) (27, 33). As demonstrated in earlier publications, both of the aforementioned events are preceded by increased RNA and protein synthesis (3, 32). The elevation in RNA synthesis begins within the first day of culture and attains maximum levels at 36 and 72 hr after explantation. Similarly, peak protein synthetic activity is reached at 36 and 87 hr following initiation of culture. When the increases in RNA and protein syntheses are blocked with actinomycin D and puromycin, respectively, DNA synthesis and subsequent mitosis are prevented (3, 31). Likewise, inhibition of DNA synthesis with fluorodeoxyuridine (FUDR) reduces, substantially, the increase in subsequent protein and RNA syntheses (37).

A possible interpretation of these observations is that events which occur...
following explantation of the lens reflect a sequential activation of previously repressed gene loci. A hypothesis of this sort has already been put forward with regard to the stimulation of mitosis in phytohemagglutinin-stimulated lymphocytes (10, 22, 28, 40), and evidence for it has been reviewed recently by Mitchison (24).

Since histones have been shown to be natural inhibitors of DNA-dependent–RNA synthesis (2, 4, 18, 20, 23), it is of interest to examine their behavior during the activation and reentry of lens epithelial cells into the generative cycle. The results to be presented below indicate that acid extracts of lenticular tissue contain histones. Furthermore, these macromolecules undergo significant and reproducible changes at different stages of the cell cycle.

MATERIALS AND METHODS

In vitro Culture of Frog Lenses Bullfrog lenses were cultured in vitro as previously described (30) except that a new culture medium ("R20") was used. R20 consists of the following ingredients (per 100 ml of solution): 71.7 ml H2O; 5.3 ml 10 times medium 199; 20.0 ml hemolyzed rabbit serum; 3.0 ml NaHCO3 (50 g/liter); 400 units/cc penicillin; 300 μg/cc streptomycin.

Fast Green Staining Procedure Nuclear histones were stained with fast green following the basic procedure set forth by Alfert and Geschwind (1). Lenses were removed from culture at the desired time. The capsules with attached epithelia were dissected from the rest of the lens and fixed in 25% neutral buffered formalin for 3 hr. After staining, specimens were spread on slides, cell side up, air-dried, and mounted in Permount.

Feulgen Staining Procedure. Lens epithelial whole mounts were made (30) following fixation for 1 hr in 3:1 absolute ethanol:glacial acetic acid and 1 hr in 70% ethanol. They were stained according to a modification of Feulgen and Rossenbeck's procedure (26). A 10 min optimal HCl hydrolysis time was experimentally determined for the system.

Microspectrophotometry The optical density of a standard circular area within fast green (630 μm) or Feulgen (570 μm) stained nuclei was measured using a Zeiss MPM microscope photometer with a standard universal microscope. 25 nuclei per specimen were analyzed and their optical densities were corrected for nuclear size (long and short nuclear axes times optical density of the standard area).

Treatment with Antimetabolites Two drugs were used in this study. Fluorodeoxyuridine (FUDR) (10^-6 M) was added to cultures 12 hr postisolation to study the relationship between DNA and histone synthesis. Cycloheximide (10^-1 μg/ml) was used.

1 Grand Island Biological Co., Grand Island, N. Y.
2 Pell-Freez Biologicals, Inc., Rogers, Ark.
3 Carl Zeiss, Inc., 444 Fifth Avenue, New York, N. Y.
4 Hoffmann-LaRoche, Inc., Nutley, N. J.
5 Upjohn Company, Kalamazoo, Mich.
introduced into cultures immediately following lens isolation to investigate the
dependence of fast green changes on protein synthesis. The former drug inhibits DNA
synthesis (17) and the latter has been used extensively as an inhibitor of protein
synthesis (8, 9, 34, 41, 42). The effective concentration used in this study had pre-
viously been determined6 (36, 38).

Radioautography Lenses were incubated in balanced salt solution containing
5 µCi/ml of DL-lysine-4,5-3H (specific activity 45 Ci/mM) 1 hr prior to fixation.
Following 18 hr in 1:3 glacial acetic acid–ethanol, epithelia were removed (30) and
washed for an additional 18 hr with frequent changes of 70% ethanol. Radioauto-
graphs of lens epithelial whole mounts were then prepared as previously described
(30). Preparations were exposed for 2 wk.

Isolation of Lens Epithelial and Fiber Cell Histone The epithelia from 36 lenses
were serially dissected from the fiber cells after a 1 hr incubation in balanced salt
solution containing either 5 µCi/ml of dl-lysine-4,5-3H (sp. act. 45 c/mM) or 3H-L-
amino acids. The epithelia and fiber cells were processed separately but simultane-
ously. After a 2 min homogenization in 20 ml of ice-cold 0.14 M NaCl: 0.01 M trisodium
citrate pH 7.2 (7, 19, 25), the solutions were centrifuged at 1000 g for 40 min at 0°C
and the supernatants were discarded. This step was repeated a second time in the case
of the fiber cells. The resultant pellets were washed once with 0.1 M Tris buffer pH 7.6
(7, 19, 25) and 95% ethanol (19). Each time they were centrifuged as before.

The final pellets were acid extracted three times (7 hr each) with 3–4 ml of 0.25 N
HCl (7). Following each extraction, the solution was clarified at 14,000 g for 1 hr,
the supernatants saved, and the precipitate reextracted. The supernatants were then
combined from each source, dialyzed 2 days against frequent changes of distilled
water, and lyophilized. The extraction procedure was shown to be reproducible, for
in 7 different extractions (36 lenses each) the relative proportion of each histone frac-
tion remained the same.

Polyacrylamide Gel Electrophoresis Histones were electrophoretically separated
using John’s procedure (21). Gels were subsequently stained for 18 hr in 1.0%
naphthol blue black (amido black) in 7% acetic acid. Destaining was accomplished
by diffusion against large volumes of 7% acetic acid.

Quantitative Determination of Protein Separated by Disc Electrophoresis Two equal
aliquots of acid-extractable protein from either epithelial or fiber cells were separated
electrophoretically. The protein bands from one of the identical tubes were excised
from the gel to determine the optical density of bound dye (21). Standard curves of
purified histone fraction verify that absorbance is directly related to protein content
(12, 21).

Liquid Scintillation of Acid-Soluble Lens Proteins and Whole Lens Epithelia Histone
bands from one of the two identical gels were removed and incorporation of radio-
active precursors was determined by liquid scintillation (39). These results were

6 Rothstein, H., and M. Bagchi. 1969. Synthesis of macromolecules in epithelial cells of the
cultured amphibian lens. III. Involvement of protein synthesis in mitotic activation. Arch. Int. Physiol.
Biochem. 77:717.
combined with those obtained from the protein determination and were expressed as cpm/0.1 OD unit.

Whole lenses were incubated in balanced salt solution containing 5 μCi/ml ³H-DL-lysine (sp. act. 45 c/mm) 1 hr prior to fixation. The epithelia were removed, their area was ascertained, and they were processed for scintillation analysis as previously described (30). Results were expressed as counts per minute per square centimeter of lens area. A Packard Tri-Carb liquid scintillation spectrometer (Model 3380) was used for all measurements.

RESULTS

Fast Green Staining

After 12 hr of culture, nuclear staining in the central epithelial cells increases slightly over 0 time and remains at this elevated level until 36 hr postisolation (Fig. 1). By 64 hr (near the end of the synthetic phase) the amount of bound dye is about twice as great as that observed immediately following explanta-
tion. The continuous presence of cycloheximide blocks both the 36 and 64 hr increases. By contrast FUDR exerts no obvious effect on the early change; it does, however, completely abolish the later one.

Feulgen Staining

Quantitative variations in DNA were monitored at various stages of the cell cycle to determine any correspondence between them and the fast green staining increases. The results are presented in Fig. 2. By 36 hr there is a slight increase in DNA content. At 52 and 64 hr the quantity of DNA increases substantially. In the presence of FUDR, the amount of nuclear DNA does not increase, but rather remains at the 0 hr level.

Scintillation Analysis of Lysine-\(^3\)H Incorporation into Whole Lens Epithelia

The incorporation of lysine-\(^3\)H was followed because of its abundance in histones. The controls show that there is no increase at 12 hr, but there is strong incorporation at 24 hr (Fig. 3). Following a decrease at 36 hr, there is
a second peak of incorporation during the S period at 52 hr. FUDR does not substantially affect the 24 hr peak, but it does lower the 36 hr level of incorporation. The 52 hr lysine-3H peak which is observed in controls is completely absent in the presence of FUDR.

Radioautographic Analysis of Lysine-3H Incorporation into Whole Lens Epithelia

This study was performed to determine the cellular localization of lysine-3H incorporation. Over the 0–36 hr period there is no increase in intranuclear lysine-3H content (Fig. 4). However, during the S phase (52 and 64 hr) the number of nuclear grains is twice as great as that seen in samples prepared at early stages of culture. Though nuclear uptake in 52 hr specimens (which had been exposed to FUDR) remains the same as in the drug-free controls, the cytoplasmic incorporation was found to be materially reduced.
**Lens Histone Isolation and Characterization**

The presence of histones in the acid extract of amphibian lenticular tissue has been demonstrated for the first time using polyacrylamide electrophoretic separation (Fig. 5). 36 epithelia yield approximately 15–30 μg of histone plus several micrograms of slower migrating contaminants. Fiber cells from the same number of lenses contain considerably less histone in comparison to contaminating proteins. Fig. 6 compares the electrophoretic separation of calf thymus histone and lens epithelial acid-soluble protein. As can be seen, four lens epithelial protein bands have the same electrophoretic mobilities as the calf thymus histone. In addition, there is an extraprominent lens band which migrates slightly faster than the fl fraction. This band was labeled “complex” for it was initially thought to be a combination of other fractions. Histones isolated in the same manner from rat liver, a more complex tissue, do not contain the complex band (Fig. 7). However, there are considerably more nonhistone contaminating proteins than are found in the acid extract of lens epithelia.
**FIGURE 5, left.** Diagram of the protein-banding pattern of electrophoretically separated lens epithelial (E) and fiber cell (F) histone (labeled with DL-\(^3\)H-lysine, 5 \(\mu\)Ci/ml; 45 Ci/mM), and isolated from lenses cultured 52 hr in the presence of FUDR from the 12th hr of culture. Histone fractions are labeled. Note that all five fiber cell histone fractions are visible. This drawing was made from the polyacrylamide gels shown on the right.

**FIGURE 5, right.** Photograph of two sets of identical polyacrylamide gels corresponding to the diagram on the left. One of the two identical gels was processed to determine the lysine-\(^3\)H incorporation into the protein bands, and the other gel was analyzed for the protein-bound dye content of each band.
FIGURE 6, left. Diagram of the two polyacrylamide gels shown in the accompanying photo. The gel on the right (CT) contains only calf thymus histone. The gel on the left (CT + E) contains a combination of calf thymus and lens epithelial histone run simultaneously. Histone fractions are labeled. Note the additional band present (complex) which is contributed by the lens histone. All other fractions from the two sources have the same electrophoretic mobilities.

FIGURE 6, right. Photograph of the two polyacrylamide gels drawn on the left. The gel on the right is the electrophoretic separation of calf thymus histone (Sigma type II). The one on the left is a combination of calf thymus and lens epithelial histone run simultaneously. Note the extra complex band.
FIGURE 7, left. Diagram of the electrophoretic separation of rat liver histone (isolated in the same way as lens histone). Histone fractions are labeled. This drawing corresponds to the polyacrylamide gel whose photograph appears on the right.

FIGURE 7, right. Photograph of a polyacrylamide gel following the electrophoretic separation of rat liver histone. The histone bands correspond to those labeled in the accompanying diagram. Lens histone contains an extra band not found in rat liver histone.

Incorporation of Labeled Precursors into Histone Fractions during the Cell Cycle

The incorporation of $^3$H-DL-lysine into each epithelial histone fraction at different times during the cell cycle yielded raw counts too low (0–50 cpm above
background) to give meaningful data. In an attempt to increase the incorporation into histones, similar extractions were performed following incubation of lenses in \(^{3}H\) reconstituted protein hydrolysate (Fig. 8). Each fraction at 52 hr incorporates approximately eight times more activity than the same fraction at 0 time. In the presence of FUDR, histone synthesis at 52 hr is depressed to a level three times as great as at 0 time.

It appears that the same histone bands found in the lens epithelium are faintly present in the fiber cell acid extracts. However, histones obtained from this source never contained any radioactivity above background levels.

**DISCUSSION**

The results reported indicate that the histones of frog lenses undergo several changes when the epithelial cells are stimulated to divide. The increment in fast green staining that occurs prior to 36 hr probably does not represent augmented synthesis of histones. If histones were being made one would expect to find an elevated incorporation of lysine-\(^{3}H\) into the nucleus. This was not observed. Instead, the peak lysine incorporation into whole lens epithelia at 24 hr was preceded by a change in fast green staining, shown to be mainly cytoplasmic, and occurred in the absence of DNA synthesis.

Furthermore, the increased staining most probably does not represent the
accumulation of nonhistone basic protein. If this were the case, one would also expect increased nuclear incorporation of lysine. As stated above, this did not occur. Second, basic ribonucleoprotein does not stain with this procedure (1), and other basic proteins, common to the cytoplasm and nucleus, were effectively negated by using the cytoplasm for blank microspectrophotometric readings.

We are inclined to conclude, on a tentative basis, that the increment in fast green staining detectable between 12 and 36 hr postisolation reflects some type of configurational change in the deoxyribonucleoprotein complex rather than histone accumulation or nonspecific staining. This could be visualized as a rearrangement or change in the binding of acidic and basic chromosomal components resulting in the greater availability of basic groups for fast green staining following the removal of DNA. The fact that cycloheximide impedes the change in stainability shows, however, that the phenomenon responsible for it depends, in some way, upon protein synthesis.

Other investigators have found similar nucleoprotein changes following activation of quiescent cells to reenter the cell cycle or vice versa. In particular, work with liver cells of newborn rats (5) and phytohemagglutinin-stimulated lymphocytes (10, 22, 28, 40) suggests that there is an alteration in the DNP complex, which allows for the transcription of new segments of DNA.

The increased fast green staining observed at 52 and 64 hr undoubtedly represents histone synthesis. This conclusion is supported by the finding that peak lysine-3H incorporation, as determined on whole mounts, by scintillation and nuclear radioautographic analyses, corresponds to the increment in nuclear fast green staining. This point was further confirmed by evaluation of isotope uptake into histones that had been electrophoretically separated from the total acid-extractable lens epithelial protein. In accord with expectation, the major increase in fast green staining occurs at the same time as does the increase in DNA (determined by the Feulgen reaction). Both staining procedures indicate a slight increase at 36 hr with maximal levels reached at 52 and 64 hr.

When the initiation of DNA synthesis is completely blocked by continuous culture with FUDR, histone synthesis at 52 hr still is appreciably higher than with 0 hr unactivated cells. However, this level of synthesis is less than one-half that of the 52 hr control. This decrease is also manifested by the reduction in cytoplasmic incorporation of lysine into FUDR-treated lenses. This suggests that histones are made in the cytoplasm, for nonhistone protein synthesis has previously been shown not to be affected by FUDR at 52 hr (37). The suggested site of histone synthesis in the lens agrees with the findings in HeLa cell cultures (15, 29).

The level of net histone synthesis (as opposed to turnover) in the presence of FUDR was not precisely characterized. The decreased fast green staining ob-
served at 64 hr in the presence of FUDR probably represents a true decrease in synthesis. If the incorporation represented histone turnover, one would not expect an increase in staining. It is, however, possible that net histone synthesis is taking place in the presence of FUDR but that the material fails to stain because of the lack of proper interaction with DNA (whose production has been blocked).

Our results agree with those in the literature which suggest that histone synthesis does continue at a reduced level in the absence of concurrent DNA synthesis (15, 16, 29, 35, 43). This is in disagreement with Bloch and co-workers, and Flamm and Birnstiel who found histone synthesis continuing at the normal rate in the presence of DNA synthetic blocking agents (6, 14).

The presence of histones in acid extracts of amphibian lenticular tissue has been electrophoretically confirmed for the first time. The epithelium contains five electrophoretic bands which are also faintly visible in acid extracts of fiber cells. Four of these (f1; f2b, f3; f2a; f2a1) have the same electrophoretic mobility as those from purified calf thymus histone. The extra complex band may be a tissue-specific histone fraction but unfortunately not enough histone could be isolated for amino acid analysis. This would be interesting because it has an electrophoretic mobility similar to that of the f2c lysine-rich histone fraction previously isolated only from nucleated erythrocytes (12, 13).

The question whether the complex band was a contamination artifact was investigated indirectly by comparing the electrophoretic separation of acid extracts made in exactly the same manner from a more complex tissue, rat liver. It was found that this tissue did not contain the complex band even though the bands were fuzzy and surrounded by a substantial number of minor contaminating bands. The lens epithelium, in contrast, is a very homogeneous monolayer of cells with very large nuclei and little cytoplasm. The histone bands from this source were very sharp, discrete bands with few or no minor bands in their general vicinity to interfere with radioactive analyses.

In our investigations with whole mounts (by scintillation counting and radioautographic technique) Carnoy's fluid was used as a fixative. Because of its acidic nature the possibility exists that this fixative may extract large quantities of histones. However, Dick and Johns have noted that Carnoy's fluid removes only 7-8% of the total histone from calf thymus tissue (11).

This work was supported by U. S. Public Health Service Grant NB 05425-05 from the National Institute for Neurological Diseases and Blindness.

Received for publication 19 September 1969.

REFERENCES


