Previously, we generated monoclonal antibodies against chicken corneal cells (Zak, N. B., and Linsenmayer, T. F. (1983) Dec. Biol. 99, 373). We have now observed that one group of these antibodies reacts with a developmentally regulated component of corneal epithelial cell nuclei. This component is the heavy chain of ferritin, as determined by analyses of immunosolated cDNA clones and immunoblotting of the protein. Immunoblotting also suggests that the nuclear ferritin may be in a supramolecular form that is similar to the iron-noblotting also suggests that the nuclear ferritin may be in a supramolecular form that is similar to the iron-noblotting also suggests that the nuclear ferritin may be in a supramolecular form that is similar to the iron-noblotting also suggests that the nuclear ferritin may be in a supramolecular form that is similar to the iron.

In vitro cultures and transfection studies show that the nuclear localization depends predominantly on cell type but can be altered by the in vitro environment. The appearance of nuclear ferritin is at least partially under translational regulation, as is known to be true for the cytoplasmic form of the molecule. The tissue and developmental distributions of the mRNA for the molecule are much more extensive than the protein itself, and the removal of iron from cultures of corneal epithelial cells with the iron chelator deferoxamine prevents the appearance of nuclear ferritin. At present the functional role(s) of nuclear ferritin remain unknown, but previous studies on cytoplasmic ferritin raise the possibility that it prevents damage due to free radical generation ("oxidative stress") by sequestering iron. Although it remains to be tested whether nuclear ferritin prevents oxidative damage, we find this an attractive possibility. Since the corneal epithelium is transparent and is constantly exposed to free radical-generating UV light, it is possible that the cells of this tissue have evolved a specialized mechanism to prevent oxidative damage to their nuclear components.

Nuclei contain a myriad of different proteins as well as nucleic acids. Nuclear proteins range from general ones, such as the histones that are universally present in cells and determine the structure of chromatin, to the DNA-binding regulatory proteins, some of which are cell-specific and developmentally regulated. In the present investigation, we provide evidence that within the corneal epithelium of embryonic chicks, ferritin is also a developmentally regulated nuclear protein. In other cell types that have been investigated, the molecule is cytoplasmic and is thought to function as a protective antioxidant by sequestering iron (1, 2) (see "Discussion").

In earlier work we sought to identify developmentally regulated components of the embryonic avian cornea by generating hybridomas against corneal cell suspensions (3). By immunofluorescence, one group of antibodies (including antibody 6D11) reacted with a corneal epithelial antigen but had little or no detectable reaction with other tissues in the embryo. During development, this antigen first became detectable by immunofluorescence in 12-day corneas, the time when the epithelium begins to stratify (4). We also found it to arise de novo in cultured corneas, and it has been used as a marker for corneal epithelial differentiation in studies of corneal development (5–7). In these previous studies, the immunofluorescence analyses for the epithelial antigen were done on cryostat sections of unfixed corneas. Under these conditions, the antigen showed diffuse reactivity throughout the cells. In the present study we have observed that when immunofluorescence analyses are performed on corneal tissue sections or cell cultures fixed with formaldehyde, the antigen is localized predominantly, if not exclusively, within the nuclei of the epithelial cells. Thus, the antigen recognized by this monoclonal antibody has the characteristics of a developmentally regulated nuclear protein.

We have characterized the nuclear antigen as the heavy chain of ferritin (ferritin-H) and have shown that it is assembled into the same supramolecular complex that is found in the cytoplasm of many other types of cells (1). Thus, it is potentially capable of sequestering iron. The nuclear molecule, like the cytoplasmic one, appears to be regulated largely by translational/post-transcriptional mechanisms. This regulation is responsive to iron and thus likely involves the well-characterized translational iron response element (8). We have also observed that the nuclear localization of endogenous ferritin, as well as that produced by transfected constructs, depends largely on cell type but can be modified by the cellular environment, at least in vitro.

At present the functional role(s) of the nuclear ferritin remain unknown, but previous studies on cytoplasmic ferritin raise the possibility that it prevents damage to cellular components from "oxidative stress" by sequestering iron and thus lowering the concentration of active oxygen species (AOS) (for reviews see Refs. 9 and 10). Although it remains to be tested whether the nuclear ferritin prevents oxidative damage, we find this an attractive possibility since the corneal epithelium is transparent and is constantly exposed to AOS-generating UV light (see "Discussion").

MATERIALS AND METHODS

Immunofluorescence Histochemistry—White leghorn chicken eggs were obtained from Spafas (Norwich, CT) and incubated at 38 °C in a humidified incubator. Embryos were removed and rinsed in Hank's...
balanced saline solution and staged according to Hamburger and Hamilton (11). Corneas were taken from different ages of embryos, fixed in ice-cold 4% paraformaldehyde for 15 min, washed, and embedded in Tissue Tek OCT. The blocks were serially sectioned at 8 μm, and the sections were mounted on 12-spot slides (Shandon Scientific, Sewickley, PA) and stored at −20 °C until use. The expression of bacterial fusion protein from the positive cDNA clones was induced by 1 mM IPTG. The control bacterial proteins were from cells not induced by IPTG. The cells were pelleted by centrifugation in aliquots of 1 ml of bacterial growth. The fusion proteins and bacterial proteins were extracted and quantitated as described above.

Both ferritin (1 μg) from the different tissues or from bacteria was solubilized in reducing sample buffer containing 1% SDS, 10% glycerol, 50 mM Tris-HCl, 1% β-mercaptoethanol, and 0.005% bromphenol blue, pH 6.8, separated on a 4–20% acrylamide Ready Gel (Bio-Rad) in Tris-glycine-SDS running buffer and electrototransferred to ECL nitrocellulose membrane (Amersham Corp.). For the nondenaturing and nonreducing gels, the proteins were solubilized in the sample buffer containing 2% SDS, without boiling or β-mercaptoethanol. Immunodetection was similar to that described for the immunoblotting of the cDNA library, except that the primary antibody 6D11 was diluted 1:1,000 in PBS (1.24 μg/ml).

**Purification of Adult Chicken Liver Ferritin by Gel Filtration Chromatography—**Adult chicken liver was minced, homogenized in 4 vol. of distilled H2O, and centrifuged at 50,000 g for 3 min. The supernatant was precipitated with ammonium sulfate (50% saturation), and the precipitate was resuspended and dialyzed into 50 mM Tris, pH 8.0. Protein samples were chromatographed on a Superose 6 gel filtration column (Pharmacia Biotech Inc.) in 50 mM Tris, pH 8.0. 2-ml samples were collected and compared with those of a horse spleen ferritin standard (Pharmacia). Further evaluation included examination by denaturing and nondenaturing SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining and immunoblotting with antibody 6D11.

**Cell Cultures, Plasmid Construction, and Transfection—**Cell cultures and transient transfections were performed using primary cells grown on 35-mm dishes. The corneal and skin epithelial cells were separated from stroma after treatment in 0.5% Dispase in PBS for 1 h at 4 °C. The epithelial cells were rinsed with PBS, further digested in 0.25% trypsin at 37 °C for 5 min, and cultured in Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (1:1). 20% fetal calf serum (heat-inactivated), 1% chicken serum, 5 μg/ml insulin, 10 ng/ml human recombinant epidermal growth factor, and penicillin and streptomycin. The corneal stromal fibroblasts and skin fibroblasts were prepared by further digestion of the separated tissues in collagenase (200 units/ml) for 2 h at 37 °C and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Liver cells were mixed cultures of hepatocytes and fibroblasts grown in William-E medium (Life Technologies, Inc.) with 10% fetal calf serum, penicillin, and streptomycin. Briefly, livers were cut into small pieces and rinsed with Hank’s balanced salt solution four to five times to remove the blood. 200–300 units/ml of collagenase (Worthington) was used to digest the tissues at 37 °C for 2 h, with one change of fresh enzymes after 1 h. The primary cell cultures were grown for 18–24 h before transfection.

Northern Blot Hybridization—Cornea, skin, liver, and heart were dissected from day 15 embryos and frozen immediately in liquid nitrogen. Corneal epithelia from different developmental ages were scraped from corneal epithelia, skin, liver, and heart were dissected from day 15 embryos and frozen immediately in liquid nitrogen. Corneal epithelium from different developmental ages was scraped from the different tissues or from bacteria was solubilized in reducing sample buffer containing 1% SDS, 10% glycerol, 50 mM Tris-HCl, 1% β-mercaptoethanol, and 0.005% bromphenol blue, pH 6.8, separated on a 4–20% acrylamide Ready Gel (Bio-Rad) in Tris-glycine-SDS running buffer and electrototransferred to ECL nitrocellulose membrane (Amersham Corp.). For the nondenaturing and nonreducing gels, the proteins were solubilized in the sample buffer containing 2% SDS, without boiling or β-mercaptoethanol. Immunodetection was similar to that described for the immunoblotting of the cDNA library, except that the primary antibody 6D11 was diluted 1:1,000 in PBS (1.24 μg/ml).

**Subcellular Fractionation—**Corneal, skin, and corneal stromal tissues from corneal epithelium, corneal stroma, and liver of day 14 embryos were grown on 100-mm dishes in epithelial culture medium (described above). The cultures were used after 48–72 h, at which time they had approached confluency. The cells were washed with ice-cold PBS and harvested with a cell scraper. The cell pellets were resuspended in a 5-fold volume of ice-cold hypotonic lysis buffer (20 mM
Hepes-KOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM EDTA, 1 mM PMSF). After 10 min on ice, the cells were homogenized by 8–15 strokes with a tight-fitting Dounce homogenizer. Lysis efficiency was monitored by trypan blue staining. After at least 80% lysis was achieved, the homogenates were centrifuged at 4,000 rpm for 10 min in an Eppendorf microcentrifuge. The supernatants (cytoplasmic fraction) were removed. The pellets (nuclear fraction) were resuspended in an equal volume of lysis buffer to which was added 0.1% SDS, 0.1% Triton X-100, and 1 mM PMSF, followed by homogenization. Both cytoplasmic and nuclear fractions were centrifuged at 12,000 rpm for 5 min to remove the insoluble materials, and the supernatants were dialyzed overnight against 0.1% SDS, 5 mM Tris-HCl, pH 7.5, and 1 mM PMSF. Protein was quantitated with a BCA kit (Pierce).

Total lysates of the different cell types were prepared as for the protein extracts described above.

15 mg of total proteins from nuclear and cytoplasmic fractions from different cells were used for immunoblotting under the nondenaturing condition in the presence of reducing reagents (as described above).

RESULTS

Nuclear Localization of the Corneal Epithelial Antigen

In tissue sections of paraformaldehyde-fixed corneas, the corneal epithelial antigen detected with monoclonal antibody 6D11 (3) is localized predominantly, if not exclusively, within the nuclei of the epithelial cells (Fig. 1A). For comparison, the same section stained with Hoechst dye shows the nuclei of both the epithelial (e) and the stromal (s) cells (Fig. 1B). The inset in Fig. 1A is a higher magnification taken by confocal laser scanning microscopy. It confirms that the immunofluorescence signal is confined to the nucleus where it is distributed throughout, except for nucleoli which are unreactive (Fig. 1A, inset, arrows). We also examined the tissue specificity and distribution of the antigen in fixed sections of embryonic liver (Fig. 1C), heart (Fig. 1E), and skin (not shown). The corneal epithelium was the only tissue that showed strong reactivity and the only one in which the reactivity was nuclear (compare with the Hoechst stained sections in Fig. 1, D and F). Liver showed some cytoplasmic reactivity within the hepatocytes and the erythrocytes; in heart the only reactivity was in the erythrocytes. In skin (not shown) very weak cytoplasmic reactivity was present, as was also true on close inspection of the fibroblasts of the corneal stroma.

Identification of the Antigen as the H-subunit of Ferritin

Isolation and Sequencing of cDNA Clones—To identify and further characterize the nuclear antigen, a corneal epithelial cDNA library was constructed in the expression vector lambda ZAP-II, and immunoscreening was performed using antibody 6D11. Fourteen of the 5 × 10⁶ plaques screened were positive. Eight of these were cloned, and three of the clones were sequenced. Each showed 100% identity in the open reading frame (540 nucleotides encoding the 180 amino acids) and the 3'-untranslated region (175 nucleotides plus the poly(A) tail) with the cDNA sequence of the heavy chain of ferritin (ferritin-H) from chicken reticulocytes (19). The 5'-untranslated region, however, was variably truncated with the longest clone being 30 nucleotides shorter than the reported sequence (121 nucleotides versus 151 nucleotides). This clone did, however, contain the highly conserved and functionally important iron response element (IRE) (19), so the shortening most likely represents a truncation in the cDNA resulting from incomplete reverse
plex result from different ratios of the H- and L-subunits (21), with a molecular mass of approximately 240 kDa (1). Tissue-specific forms of the human protein comprise the conventional iron-storage form, which is a supramolecular structure composed of 24 subunits (the anti-HeLa cell ferritin in Fig. 2A). The homology of the same subunit of ferritin among species is greater than 85% (1). Similar to chicken ferritin being composed of only one type of subunit, closely related to the H-subunit of other species (boiling in 1 or 2% SDS plus 1% 2-mercaptoethanol) are preabsorbed with IPTG-induced bacterial fusion proteins from the avian ferritin-H-positive cDNA clone.

**Immunofluorescence with Polyclonal Anti-ferritin Antibodies**—Independent confirmation of the nuclear antigen as the ferritin H-subunit was achieved by several additional criteria, such as immunofluorescence with polyclonal antibodies against ferritins from HeLa cells, rat heart, and human liver (provided by Dr. James Drysdale). The polyclonal antibodies against HeLa cell and rat heart ferritin were directed predominantly against the H-subunit (ferritin-H); those against the human liver ferritin were directed predominantly against the light, L-subunit (ferritin-L). The homology of the same subunit of ferritin (H or L) among species is greater than 85% (1). Similar to the nuclear immunoreactivity detected by monoclonal antibody 6D11 (Fig. 2A), in day 14 corneal epithelial cells the polyclonal antisera directed predominantly against the H-subunit (ferritin-H); those against the human liver ferritin were directed predominantly against the light, L-subunit (ferritin-L). The homology of the same subunit of ferritin (H or L) among species is greater than 85% (1).

**Immunoblocking of the Nuclear Staining with the Bacterial Fusion Protein of the cDNA Clones**—Immunoblocking experiments provided further confirmation that the corneal epithelial antigen recognized by monoclonal antibody 6D11 is indeed ferritin-H. The nuclear immunofluorescence produced by monoclonal antibody 6D11, as expected, could be blocked by preincubation with the IPTG-induced fusion protein produced by the cDNA clones (Fig. 2C). More importantly (see “Discussion”), the nuclear reactivity produced by the polyclonal antibodies against the human ferritin-H-subunit could also be blocked by preincubation with this fusion protein (Fig. 2D). In control incubations in which the antibodies were preincubated with non-IPTG-induced bacterial proteins, the nuclear immunoreactivity was not blocked (not shown).

**Immunoblotting**—In human, the conventional iron-storage form of ferritin is a supramolecular structure composed of 24 H- and L-subunits, with a molecular mass of approximately 450 kDa (1). Tissue-specific forms of the human protein complex result from different ratios of the H- and L-subunits (21), with the liver form being predominantly ferritin-L (1). The chicken liver ferritin aggregate, however, while having a molecular mass approximately the same as that of the human, has been reported to contain only the H-subunit (22 kDa) (20).

**Northern Blot Analysis**—To determine whether the nuclear ferritin of the corneal epithelium is organized in the conventional supramolecular form, we performed immunoblotting with monoclonal antibody 6D11 on gel-separated denatured (Fig. 3A) and non-denatured (Fig. 3B) protein extracts from embryonic chicken tissues. Extracts of corneal epithelium were compared with those from corneal stroma, liver, heart, and skin, as well as to the chromatographically purified protein (20). Under denaturing conditions (boiling in 1 or 2% SDS plus 1% β-mercaptoethanol)
(Fig. 3A), the extracts of corneal epithelium (CE) showed a strong 22-kDa band, a molecular mass identical to that of the monomer of the purified chicken ferritin. Extracts of liver and heart also showed a band with the same molecular mass and those from corneal stroma showed a very faint band. Although the skin extracts showed no detectable reactivity at this molecular mass, there probably is a small amount as determined by nondenaturing gels (described below). The 120-kDa band seen in some lanes most likely represents nonspecific binding of the labeled secondary antibody, since we sometimes observed this in the absence of the primary antibody. We are not certain about the identity of the material near the top of the gel (arrowhead) in the samples from liver, heart, and skin. It may, however, represent incompletely dissociated ferritin complex, since it was present even in purified liver ferritin when run under nonreducing conditions (data not shown). No such material was ever seen in samples from CE.

In immunoblots of extracts run under nondenaturating conditions (Fig. 3B), extracts of all tissues behaved as described previously for purified chicken liver ferritin (20). Samples run in 2% SDS, without boiling and without reduction, gave a 240-kDa band (data not shown); in identical samples run in the presence of reducing agents, the band was shifted to 260 kDa (Fig. 3B). This mobility shift in the presence of reducing agents suggests that thiol groups are involved in the maintenance of the compact ferritin structure from all of these sources. Thus, the nuclear ferritin of the corneal epithelium appears to share the same supramolecular structure as the cytoplasmic molecule of the other tissues.

The immunoblotting data is consistent with, and supports, the immunohistochemistry, the one apparent exception being liver. Whereas tissue sections of liver show only modest reactivity for ferritin (in the cytoplasm), the immunoblots of tissue extracts show strong bands. In liver the relatively low immunofluorescence signal could reflect the fact that in hepatocytes the ferritin is distributed throughout the cytoplasm, whereas in the corneal epithelial cells it is concentrated within the nucleus. Alternatively, the epitope in hepatocyte cytoplasm in situ may be partially masked.

Steady-state Levels of Ferritin-H mRNA in Different Tissues—Previous studies have shown that ferritin expression is largely regulated at the translational level (22). We tested this for the tissues we examined by comparing immunoblots of ferritin protein to corresponding Northern blots of ferritin-H mRNA (Fig. 3C). Relative mRNA levels were determined by normalization to mRNA for G3PDH (Fig. 3C). The mRNA from CE, corneal stroma, liver, heart, and skin, when hybridized with a ferritin-H cDNA probe, each gave a single 1-kilobase band. When these were quantified by densitometry and normalized, the intensities of the ferritin-H signals among the different tissues showed less than a 2-fold difference. Thus, the tissue differences noted in ferritin-H protein are likely to result from translational and/or posttranscriptional regulation (experimentally demonstrated later).

Developmental Regulation of Ferritin-H in Corneal Epithelium

Translational/posttranscriptional regulation is also likely to be involved in the developmental regulation of ferritin-H within the corneal epithelium. Our previous immunofluorescence studies showed that developmentally the epithelial antigen first appeared on day 12 (3). This, we observed, is true for the fixed tissues of cornea as well, with the nuclear ferritin-H first becoming detectable at 11.5 to 12 days (not shown). By day 14 (see Fig. 1A), the nuclear immunoreactivity is detectable throughout the corneal epithelium, including the outermost, flattened cells of the periderm layer; this persists throughout the remainder of embryonic development and into the adult (data not shown).

Fig. 4. A, immunoblot of protein extracts (40 μg) of corneal epithelia from day 9, day 12, day 15, and day 19 chick embryos, reacted with monoclonal antibody 6D11 as in Fig. 3A. B, Northern blot analysis of total RNA (20 μg) from corneal epithelia from day 9, day 12, day 15, and day 19 embryonic chickens, hybridized with 32P-labeled cDNA probes for ferritin-H and G3PDH as in Fig. 3C.

Consistent with the immunofluorescence results, immunoblots of protein extracts from epithelia at different developmental ages showed no 22-kDa ferritin-H band until 12 days (Fig. 4A). This increased until just before hatching (day 19). In contrast, the mRNA for ferritin-H (Fig. 4B) was already present at day 6, the earliest time examined (not shown). When normalized to mRNA for G3PDH, the steady state level of mRNA for ferritin-H from different stages of epithelia (days 9, 12, 15, and 19) showed less than a 2-fold variation. This indicates that the developmental regulation of the nuclear ferritin is also controlled largely at the translational/posttranscriptional level.

Expression of Ferritin-H in Vitro—In primary cell culture, and in cells transiently transfected with a full-length ferritin-H construct, most of the cell types expressed ferritin-H similar to that observed in vivo. Some cell types, however, differed in their content and/or distribution of ferritin-H, suggesting that the cellular environment can also have an effect.

Expression of Ferritin-H in Primary Cell Culture—Consistent with the in vivo observations, ferritin-H was observed in the nuclei of embryonic corneal epithelial cells in primary cultures (Fig. 5A). In these cells, as in vivo, the ferritin-H was distributed throughout the nucleus but was absent in the nucleoli (arrow). Almost all of the cultured cells showed nuclear ferritin even though the fluorescence intensity varied from cell to cell (A). The cells showed little if any detectable immunoreactivity in the cytoplasm, and occasional cells were essentially devoid of nuclear immunoreactivity. One difference from the in vivo state was the precocious appearance of nuclear ferritin-H in cultures of corneal epithelia from 5-day embryos (data not shown), the earliest stage examined (experimentally investigated below). Even when the time in culture (2 days) is considered, the nuclear ferritin-H appears earlier than it does in vivo.
Effects of Iron on Nuclear Ferritin in Cultured Corneal Epithelial Cells—The expression of cytoplasmic ferritin is known to be translationally regulated by iron bound to an iron response protein (IRP). Our observations suggesting that translational regulation was also likely to be involved in the synthesis of the nuclear molecule raised the possibility that iron might be involved. If true, changes in environmental iron might also alter the expression of ferritin in cells. Since the early embryonic corneal epithelial cells expressed nuclear ferritin in vitro, but not in vivo, it is possible that the precocious appearance of nuclear ferritin is dependent on the iron concentration in the culture environment.

To test this, the concentration of iron in the culture media was either reduced by the addition of the chelator deferoxamine or increased by the addition of ferrous sulfate. As described earlier, a major difference between the temporal expression of the molecule in vivo and in vitro was its precocious appearance in the nuclei of 7-day corneal epithelial cells when put in culture. Consistent with an involvement of iron, the addition of deferoxamine to such cultures prevented the appearance of the ferritin (Fig. 7A). This was not due to any toxic effect of the chelator, since the addition of equimolar concentrations of ferrous sulfate along with the chelator overcame the inhibition (Fig. 7B). The in vitro appearance of the nuclear ferritin in 7-day corneal epithelia was rapid, occurring at least by 16 h in culture, the earliest time point examined. Once present, however, the nuclear ferritin seems to be long lived (not shown). When deferoxamine was added to the medium of 7-day corneal epithelia that already had accumulated nuclear ferritin (after 16 h in culture), the addition of deferoxamine had no noticeable effect. Likewise, when nuclear ferritin-containing 14-day corneal epithelia were cultured in the presence of deferoxamine, the nuclear ferritin was still present there after 4 days in culture (the longest time examined).

Expression of Ferritin-H in Transiently Transfected Cells—From the in vivo and in vitro results, it seemed likely that the expression of the ferritin-H depended predominantly on cell type, but this could be modified by alterations in the cellular environment.

To investigate this further, and to provide a basis for future experiments on the nuclear localization and function of ferritin-H, we performed transient transfections of the cell types examined above. The transfected construct contained the full-length coding sequence for the ferritin-H, plus, at the 3’-end, a sequence coding for an eight amino acid sequence of human c-MYC. The use of an anti-myc monoclonal antibody (9E10) directed against this eight amino acid sequence (19) allowed us to identify immunohistochemically the myc-tagged ferritin-H produced by the transfected construct and to distinguish it from the endogenous molecule.

In most of the cell types, the product of the transfected construct behaved like that of the endogenous ferritin-H. In transfected corneal epithelial cells from day 14 (Fig. 8A) and day 7 (not shown) embryos, the anti-myc immunofluorescence was almost entirely nuclear. Again, it was distributed throughout the nucleus, except for the nucleoli (arrow). A small pro-
portion of cells with strong nuclear immunoreactivity also had some weak cytoplasmic immunoreactivity. In an occasional cell, the immunoreactivity was seen only in cytoplasm. Conversely, in transfected fibroblasts from corneal stroma (Fig. 8B) and skin (not shown), and in epidermal keratinocytes (Fig. 8C), the immunoreactivity for the product of the transfected construct was predominantly, if not exclusively, cytoplasmic. Occasional fibroblasts did show both cytoplasmic and nuclear immunoreactivity. Even after an additional 24 h in culture, corneal stromal fibroblasts did not show an increased level of nuclear localization of the transfected gene product. In cultures of liver (Fig. 8D), some transfected hepatocytes showed the transfected product to be exclusively nuclear (small arrows), whereas other hepatocytes showed it to be predominantly, if not exclusively, cytoplasmic (large arrow). None of the cells showed appreciable amounts of the transfected product in both locations. In all cases, nontransfected, parental control cells showed no reactivity with the anti-myc antibody (not shown).

DISCUSSION

The results of the present study show that during development of the avian corneal epithelium, ferritin is a developmentally regulated nuclear protein. Previously, there have been reports of ferritin having a nuclear localization, but these were in pathological conditions, such as within hepatocytes of mice subjected to iron overloading (24, 25) and reticular cells of bone marrow and spleen of blood transfused mice (26). In corneal epithelial cells and in that subpopulation of cultured hepatocytes showing nuclear ferritin-H reactivity, the ferritin was distributed throughout the nucleoplasm, except the nucleoli from which it was excluded. This distribution is reminiscent of certain other nuclear proteins (27), including the SV-40 large T-antigen (28), v-Jun (29) and the heterogeneous nuclear ribonucleoprotein A1 (30).

Identification and Characterization of Nuclear Ferritin—All of our evidence suggests that the nuclear component recognized by the corneal epithelial monoclonal antibody 6D11 is the ferritin-H subunit. The three immunoisolated cDNA clones sequenced show complete identity with the published sequence for chicken ferritin-H subunit (19). Since the longest clone is missing only 30 nucleotides from the 5′-untranslated region of the published sequence, it is likely derived from a full-length mRNA. However, Northern analyses and immunoblotting produced single bands with the expected size for the ferritin-H mRNA and the expected molecular weight for the protein, respectively. Lastly, immunofluorescence histochemistry with the polyclonal antibodies against these human ferritins that are predominantly H-subunit produced nuclear immunoreactivity identical to that of monoclonal antibody 6D11. This immunofluorescence could be eliminated by preincubation of the antibodies with the IPTG-induced fusion protein from the cDNA clones. It is likely that the anti-human ferritin polyclonal antibodies are directed against multiple epitopes within the ferritin-H subunit. The complete inhibition of nuclear immunofluorescence by the fusion protein shows that all of the epitopes recognized by these polyclonal antibodies are also present within the fusion proteins. This adds additional strong confirmation to its identity as the H-subunit of chicken ferritin.

The nuclear ferritin of the corneal epithelium may lack an L-subunit as appears to be true for other chicken tissues (20). Polyclonal antibodies against human liver ferritin, which are directed largely against ferritin-L, failed to react with the corneal epithelial cell nuclei, whereas strong reactivity was observed with two different anti-human ferritin polyclonals predominantly against the H-chain. The anti-human L-chain antibodies, however, may have a lower titer against the putative chicken protein, so this negative result should be considered as tentative. It is likely that the nuclear ferritin of the corneal epithelium is assembled into the same supramolecular complex as the cytoplasmic ferritin of the other tissues. Immunoblotting of nondenatured ferritin from corneal epithelium gave a band with a molecular weight identical to that of purified chicken liver ferritin and to ferritins from other chicken tissues. Also, in the presence of reducing agents this band shifted slightly, as described previously for chicken liver ferritin (20). Thus, the nuclear ferritin appears to be in a form capable of sequestering iron, but whether this occurs remains to be determined.

Regulation of Expression—Our studies suggest that during corneal development, translational or other post-transcriptional regulation is likely to be involved in the expression of nuclear ferritin. By Northern analysis, the steady-state level of mRNA for ferritin-H in the corneal epithelium was similar at all stages examined, including times well before the appearance of the protein within the nucleus. Also, the mRNA of ferritin-H is present in other tissues in which the protein was undetectable, or at low levels. Previous studies by others (52) have also shown that the expression of cytoplasmic ferritin is largely regulated at the translational level in response to the concentration of iron. This is generally effected through the binding of iron regulatory proteins (IRPs) (31, 32) to iron response elements (IREs) in the 5′-untranslated region of the mRNA (8). Studies have also shown transcriptional regulation of ferritin-H by tumor necrosis factor (33), thyrotropin (34), and cAMP (35).

The cDNA clones for the corneal epithelial ferritin do contain the 5′-IRE, and in vitro experiments with deferoxamine clearly show that the precocious appearance of nuclear ferritin in cultured 7-day corneal epithelial cells is dependent on iron in the medium. This suggests that the expression of nuclear ferritin is most probably regulated translationally by the IRP-IRE interaction as is known for the conventional cytoplasmic ferritin (8). It is unclear, however, if in vivo the appearance of the nuclear ferritin in the nuclei of the corneal epithelial cells at 11 days of development is due solely to iron. Biochemical measurements reported in the early literature suggest that the concentration of iron in embryonic fluids (including the amniotic fluid that bathes the anterior eye) remains relatively constant throughout development (36). In addition, any systemic increase in iron should be reflected in a concomitant increase in ferritin in the other tissues containing the mRNA, unless the uptake of iron itself is tissue-specific and developmentally regulated. Recent studies on liver cells suggest that iron transport into nuclei is an ATP-dependent process, most probably involving a nuclear membrane Fe-ATPase (37). Whether corneal epithelial cells have evolved a special mechanism for the developmentally regulated uptake of iron remains to be determined. Alternatively, recent studies have identified other factors that may be involved in the translational up-regulation of ferritin.

FIG. 7. Fluorescence micrographs of primary cultures of cells from 7-day corneal epithelium reacted with monoclonal antibody 6D11. A was cultured in the presence of 100 μm deferoxamine; B was in the presence of 100 μm each of deferoxamine and ferrous sulfate. Bar in B = 25 μm.
These positive regulators include ascorbic acid (38), hypoxia (39), interleukin-1β (40), and most recently thyroxin (41). Whether any of these factors are involved in the expression of nuclear ferritin during corneal epithelial development is unknown, but it may be of interest that thyroxin has been shown to have a profound effect on the temporal development of the cornea (42, 43).

**Regulation of the Nuclear Localization**—Our results suggest that once the translation of ferritin-H is initiated, whether the chain remains within the cytoplasm or is transported into the nucleus is largely a cell-specific function but one that can be modified in some cell types by the environment. Corneal epithelial cells showed rapid and essentially quantitative nuclear transport both in vitro and in vivo. In contrast, most cell types examined had no nuclear ferritin in vivo. And, under “normal” in vitro conditions, if cells showed any nuclear ferritin at all, it was found in only a small percentage of them. The transfection experiments with the myc-tagged ferritin-H construct confirmed these results. In general the myc-tagged ferritin-H was found in the same subcellular location as the endogenous ferritin-H and in transfected cells that had little detectable endogenous ferritin, the majority of the product of the transfected construct remained cytoplasmic. When cells are cultured in high iron medium, however, we see variable increases in both cytoplasmic and nuclear ferritin in the cell types examined (hepatocytes and corneal stromal fibroblasts). This is reminiscent of the appearance of nuclear ferritin in hepatocytes of mice loaded with iron in vivo (24, 25).

It is unlikely that a conventional nuclear localization signal (NLS) is involved in the nuclear translocation of the ferritin-H. A characteristic feature of such a signal is a cluster of basic amino acids (27), and no such region is found in the sequence of ferritin-H. It is also difficult to envision how a consensus NLS could produce the high degree of tissue specificity observed for the nuclear localization. One possible alternative is that the ferritin-H-subunits, or their supramolecular aggregate, become associated with another corneal epithelial molecule that has an NLS. This NLS-containing molecule would then function as a tissue-specific nuclear-transport chaperone. The feasibility of this mechanism is shown by the nuclear translocation studies of Lanford et al. (28). For these studies, a system was devised in which a cytoplasmic protein could be made to translocate into the nucleus through conjugation with a synthetic peptide homologous to the NLS of SV40 T-antigen. The protein they chose was the 450-kDa form of ferritin; this suggests that such a mechanism could effect such a translocation. Recently, studies have identified a receptor that mediates nuclear transport, independently of an NLS (44). Thus there most likely exist multiple pathways for nuclear protein importation. Our preliminary transfection experiments with deletion constructs of the ferritin-H transcript suggest that short amino- and carboxy-terminal portions of the molecule can be deleted without preventing the nuclear localization, whereas large deletions result in inhibition. The precise sequence required for nuclear localization is yet to be defined, but in the proposed model its function would be to bind to the putative chaperone. Alternatively, the nuclear membrane or the nuclear pore complex of the corneal epithelial cells may contain a component necessary for the transport of ferritin.

**Physiological Roles**—At present any function proposed for nuclear ferritin is speculative. Numerous studies have shown that cytoplasmic ferritin, as the supramolecular complex, regulates intracellular iron through sequestration (for review, see Ref. 45). In this manner it protects against active oxygen species (2). The cells of the corneal epithelium are under oxidative stress from ultraviolet radiation and thus may require specific mechanisms to counteract this hazard. In liver, following pathological iron overloading, ferritin is found within hepatocyte nuclei (25). Likewise, some bacteria contain inducible proteins that have structural domains for binding both iron and DNA and are thought to confer resistance to peroxide damage during periods of oxidative stress (46). Transparent ocular tissues such as those of the cornea and lens are known to contain high concentrations of a number of components known to diminish damage from AOS, including superoxide dismutase (47, 48). Numerous studies have demonstrated that iron greatly exacerbates AOS damage to DNA (49–51). Since the corneal epithelium is the initial ocular tissue exposed to UV light, possibly its cells have evolved nuclear ferritin as a specialized mechanism to directly prevent such damage to their DNA. We are currently testing this hypothesis.
Acknowledgments—We thank Dr. James Drysdale for providing the polyclonal antibodies to the human ferritins, along with Dr. Marion Gordon for helpful discussions. We also thank Eileen Gibney and Rita Hahn for technical assistance and Dr. John Fitch for his critical reading and comments on the manuscript.

REFERENCES

1. Theil, E. C. (1987) Annu. Rev. Biochem. 56, 289–315
2. Cairo, G., Tachibana, K., Pogliahi, G., Anzen, E., Tomasi, A., and Bernelli-Zambra, A. (1995) J. Biol. Chem. 270, 700–703
3. Zak, N. R., and Linsenmayer, T. F. (1983) Dev. Biol. 105, 390–398
4. Hay, E. D. (1983) Exp. Cell Res. 159, 390–398
5. Zak, N. B., and Linsenmayer, T. F. (1985) Dev. Dyn. 195, 143–150
6. Zak, N. B., and Linsenmayer, T. F. (1985) Dev. Dyn. 195, 143–150
7. Cai, C. X., Fitch, J. M., Svoboda, K. K. H., Birk, D. E., and Linsenmayer, T. F. (1988) J. Biol. Chem. 263, 1564–1570
8. Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., and Hoffman, A. (1988) J. Biol. Chem. 263, 1564–1570
9. Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) J. Cell Biol. 100, 121–129
10. Gutteridge, J. M. (1994) Free Radic. Biol. Med. 15, 435–445
11. Hamburger, V., and Hamilton, H. L. (1951) J. Morphol. 88, 137–156
12. Fitch, J. M., Mentzer, A., Mayne, R., and Linsenmayer, T. F. (1988) Dev. Dyn. 191, 121–133
13. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) J. Cell Biol. 100, 121–129
14. Schmitt, T. M., and Linsenmayer, T. F. (1985) J. Cell Biol. 100, 598–605
15. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 471–510, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
17. Chen, Q., Johnson, D. M., Haudenschild, D. R., Tondravi, M. M., and Goetinck, P. P. (1995) Mol. Biol. Cell 6, 1743–1753
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 16,32–16,36, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Stevens, P. W., Dodgson, J. B., and Engel, J. D. (1987) Mol. Cell. Biol. 7, 1751–1758
20. Passaniti, A., and Roth, T. F. (1989) Biochem. J. 258, 413–419
21. Drysdale, J. W., Adeleman, T. G., Arosio, P., and Yokota, M. (1976) Birth Defects 12, 105–122
22. Curtis, D., Lehmann, R., and Zamore, P. D. (1995) Cell 81, 171–178
23. Krek, W., Maridor, G., and Nigg, E. A. (1992) J. Cell Biol. 116, 43–55
24. Richter, G. W. (1961) J. Biophys. Biochem. Cytol. 9, 263–270
25. Smith, A. G., Cartherw, P., Francis, J. E., Edwards, R. E., and Dinsdale, D. G. (1990) J. Biol. Chem. 265, 666–670
26. Richter, G. W. (1961) Proc. Natl. Acad. Sci. U. S. A. 53, 206–215
27. Garcia-Bustos, J., Heitman, J., and Hall, M. N. (1991) J. Biol. Chem. 266, 11743–11753
28. Fitch, J. M., Mentzer, A., Mayne, R., and Linsenmayer, T. F. (1988) Dev. Dyn. 191, 121–133
29. Kim, H.-Y., Klausner, R. D., and Rouault, T. A. (1995) J. Biol. Chem. 270, 201–205
30. Siomi, H., and Dreyfuss, G. (1995) J. Cell Biol. 129, 551–560
31. Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) J. Cell Biol. 100, 121–129
32. Leibold, E. A., and Munro, H. N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2171–2175
33. Kwak, E. L., Larochelle, C., Torti, S. V., and Torti, F. M. (1995) J. Biol. Chem. 270, 15283–15289
34. Chazenbalk, G. D., Wadsworth, H. L., and Rapoport, B. (1990) J. Biol. Chem. 265, 666–670
35. Liu, G., Chan, L. M., and Peng, P. (1991) J. Biol. Chem. 266, 18819–18826
36. Romanoff, A. L. (1967) Biochemistry of the Avian Embryo, p. 172, John Wiley & Sons, Inc., New York
37. Gurgueira, S. A., and Meneghini, R. (1996) J. Biol. Chem. 271, 13616–13620
38. Toth, I., Rogers, J. T., McPhee, J. A., Elliott, S. M., Abramson, S. L., and Bridges, K. R. (1995) J. Biol. Chem. 270, 2846–2852
39. Qi, Y., and Dawson, G. (1994) J. Neurochem. 63, 1485–1490
40. Rogers, J. T., Andristakis, J. L., Lacroux, L., Durniowicz, G. P., Kasschau, K. D., and Bridges, K. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4983–4986
41. Leedman, P. J., Stein, A. R., Chin, W. W., and Rogers, J. T. (1996) J. Biol. Chem. 271, 12017–12023
42. Coulombre, J. A., and Coulombre, J. L. (1958) J. Cell. Comp. Physiol. 51, 1–11
43. Poullard, V. W., Michael, W. M., Nakielny, S., Sion, M. C., Wang, F., and Dreyfuss, G. (1996) Cell 86, 985–984
44. Harrison, P. M. O., and Bullerjahn, G. S. (1995) J. Cell Biol. 129, 22478–22482
45. Hayden, B. J., Zhu, L., Sens, D., Tapert, M. J., and Crouch, R. K. (1999) Exp. Eye Res. 68, 511–516
46. Redmond, T. M., Duke, E. J., Coles, W. H., Simson, J. A., and Crouch, R. K. (1984) Exp. Eye Res. 38, 369–378
47. Golconda, M. S., Ueda, N., and Shah, S. V. (1993) J. Cell Biol. 122, 1228–1234
48. Latzke, K., Lernzto, F. A., Byrn, H. W., Antholine, W. E., and Petering, D. H. (1994) J. Biol. Chem. 269, 655–661
49. Kubota, T., Watanabe, N., Kanai, Y., and Stellar, D. B. (1996) J. Biol. Chem. 271, 655–661
50. White, K., and Munro, H. N. (1988) J. Biol. Chem. 263, 8938–8942
Ferritin Is a Developmentally Regulated Nuclear Protein of Avian Corneal Epithelial Cells
Cindy X. Cai, David E. Birk and Thomas F. Linsenmayer

J. Biol. Chem. 1997, 272:12831-12839.
doi: 10.1074/jbc.272.19.12831

Access the most updated version of this article at http://www.jbc.org/content/272/19/12831

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 23 of which can be accessed free at http://www.jbc.org/content/272/19/12831.full.html#ref-list-1