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Immunohistochemical Analysis of Proteoglycan Biosynthesis during Early Development of the Chicken Cornea[1]

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Antibodies to core proteins of chicken corneal keratan sulfate proteoglycan and chondroitin sulfate proteoglycan were prepared and purified by use of an affinity column. Using these antibodies and monoclonal antibody 5-D-4 to keratan sulfate (commercial), the localization of proteoglycans in developing corneas (Days 5 to 17 of embryonic age and 2 days after hatching) was determined immunohistochemically. Keratan sulfate proteoglycan antigen was not detected in cornea on Day 5, but it was detected uniformly over the whole stroma on Day 6, *ca.* 12 h after invasion of the primary stroma by mesenchymal cells. The absence of the antigen in cornea of Day 5 was confirmed by Western blotting of the corneal extract. Immunohistochemistry with 5-D-4 antibody revealed that the keratan sulfate chain was undersulfated in corneas of Days 6 to 7, because the staining was much weaker than that in cornea of Day 8. In addition, keratan sulfate proteoglycan antigen was detected uniformly over the whole stroma on Days 7 to 17 and 2 days after hatching, but not in the epithelial layer on Day 13 and after: because the epithelial layer was clearly not observed on photomicrographs until Day 13, it is not known whether keratan sulfate proteoglycan was synthesized by the epithelium during Days 6 to 12. In contrast, chondroitin sulfate proteoglycan antigen was detected in cornea on Day 5 and also, like keratan sulfate proteoglycan, uniformly over the whole stroma on Day 6 through 2 days after hatching. Furthermore, the chondroitin sulfate proteoglycan was not detected in the epithelial layer on Day 13 and after. These results show that keratan sulfate proteoglycan is synthesized by the stromal cells which invade the primary stroma between Day 5.5 and 6, while chondroitin sulfate proteoglycan is synthesized by epithelial and/or endothelial cells before the invasion, and also by the stromal cells after the invasion.

Key words: chondroitin sulfate, corneal development, immunohistochemistry, keratan sulfate.

The adult cornea is characterized by transparency, which appears to depend upon the maintenance of collagen fibrils with a small and uniform cross-sectional diameter and of interfibrillar spaces with a small and uniform size (*1-3*). In the adult chicken corneal stroma, two classes of small proteoglycans (PGs) localize around these collagen fibrils: one with chondroitin sulfate/dermatan sulfate (CS/DS) side chains, which is identical to decorin (*4*), and the other with keratan sulfate (KS) side chains, which has been termed lumican (*5*). In bovine cornea, three types of KSPGs have been reported, lumican, keratocan, and mimecan (*6-8*). Corneal scar experiments (*9, 10*) suggested that two small proteoglycans, KSPG and CS/DS PG, may play an important role in determining collagen spacing and transparency of the corneal stroma. How KSPG and CS/DS PG participate in the transparency, however, is unknown.

In developing embryonic chick cornea, colonization of the stroma begins on Day 5.5 of development with the invasion of the primary stroma by neural crest-derived mesenchymal cells; and on about Day 14, the stroma begins to dehydrate and become more transparent, with full transparency being achieved just before hatching on Day 20-21 (*11, 12*). In relation to this corneal development, many studies on developmental changes in proteoglycans (PGs) have been reported. Studies on biosynthesis of PG (or glycosaminoglycan) by developing embryonic chick corneas using radio-labeled precursors (*13-15*) showed that KS appears suddenly in Day 7 corneas, continues to increase until Day 14, and then decreases slightly. It was also found that sulfation of KS continues to increase until hatching. In addition, developmental changes in PGs have been analyzed in developing chick cornea by means of immunohisto-

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Abbreviations: CS/DS, chondroitin sulfate/dermatan sulfate; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; KS, keratan sulfate; MES, 2-morpholinoethanesulfonate; PBS, phosphate buffered saline; PG, proteoglycan; PVDF, poly(vinylidene difluoride).

chemistry (*16, 17*) and immunoprecipitation (*18*). Funderburgh *et al.* (*16*) reported from immunofluorescent staining of paraffin-embedded tissue sections that KSPG antigen localized inside stromal cells on Day 6, but did not appear outside the cell yet that time, and that extracellular KSPG appeared first in the posterior stroma on Day 9. This result does not conflict with those described above. Doane *et al.* (*17*), however, reported that a small amount of KS could be observed immunohistochemically in the corneal endothelium and that the core protein of lumican (KSPG) was localized in cells of the corneal endothelium on Day 5 (stage 26): lumican core protein biosynthesis precedes stromal invasion. In contrast, immunoprecipitation of extracts from radio-labeled embryonic corneas by Cornuet *et al.* (*18*) indicated that lumican precursor protein synthesis increased dramatically between Days 7 and 9, but lumican with sulfated glycosaminoglycan chains (*i.e.*, KSPG) was not detected in corneal extracts until Day 15.

In this study, to explain the above discrepancy and clarify whether KSPG biosynthesis begins before or after the invasion of neural crest-derived cells (stromal cells), we prepared antibodies against the core proteins of chick corneal KSPG and CSPG and performed the immunofluorescent staining of cryostat tissue sections of embryonic chick eyes using these antibodies. We found that KSPG was first detected in corneal tissue on Day 6, not on Day 5, and that KS chains in corneas of Days 6 and 7 had a low degree of sulfation (undersulfated KS).

MATERIALS AND METHODS

Materials—Fertilized eggs of White Leghorn chick were obtained from Hattori Youkei-en, Nagoya. Chondroitinase ABC (*Proteus vulgaris*), keratanase (*Pseudomonas* sp.), and endo-β-galactosidase (*Escherichia Freundii*) were obtained from Seikagaku, Tokyo. DEAE-Sephacel, CNBr-activated Sepharose 4B, and HiTrap Q were purchased from Amersham Pharmacia Biotech, Tokyo. Mouse anti-keratan sulfate monoclonal antibody (5-D-4), horseradish peroxidase-conjugated goat anti-rabbit IgG, and FITC-conjugated goat anti-rabbit IgG were purchased from Seikagaku; and normal goat serum was from Vector Lab., Burlingame, USA. E-Z-SEP (antibody purification kit) was purchased from Amersham Pharmacia Biotech, Tokyo; and Horseradish Peroxidase (HRP) Conjugate Substrate Kit (for color development of Western blot), from Nippon Bio-Rad Lab., Tokyo.

Isolation of Keratan Sulfate Proteoglycan (KSPG) and Chondroitin Sulfate Proteoglycan (CSPG)—Corneas were dissected from 578 male chickens 2 days after hatching. Corneal stromas were taken from the dissected corneas by scraping off the epithelial and endothelial layers. Proteoglycans were extracted from the corneal stromas as described previously (*19*). The extract was chromatographed on a DEAE-Sephacel column (2.5 × 6 cm) equilibrated with 7 M urea in 50 mM Tris-HCl, pH 7.5. Proteins were detected by absorbance at 280 nm. PGs were eluted from the column with a linear gradient of 0.2-0.6 M NaCl in the same 7 M urea buffer. The fractions of proteolgycans were combined and concentrated to about 8 ml by ultrafiltration at 4°C. The concentrated PG fraction was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 50 mM sodium acetate and 50 mM NaCl, and the dialyzed PG fraction was divided into two parts. One part was digested with 10 units (μmol/h) of keratanase in 50 mM Tris-HCl, pH 7.5, containing 0.36 mM pepstatin A, 5 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, and 10 mM EDTA at 37°C for 5 h. The other part was digested with 5 units (μmol/min) of chondroitinase ABC in the same 50 mM Tris-HCl (pH 7.5) containing protease inhibitors at 37°C for 4 h. Both digests were dialyzed against 7 M urea in 50 mM Tris-HCl, pH 7.5, and each digest was applied to a Superose 12 column (10 × 30 cm) equilibrated with 7 M urea in 50 mM Tris-HCl (pH 7.5)/1.0% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate. Proteins were detected by absorbance at 280 nm. The earliest-eluting peak on chromatography of chondroitinase ABC digest was recovered as KSPG, and the earliest-eluting peak on chromatography of keratanase digest was recovered as CSPG. The fractions containing KSPG or CSPG were combined and concentrated to about 8 ml by ultrafiltration at 4°C, dialyzed against Milli Q water and lyophilized. Each lyophilized PG was assayed for protein content by the method of Bradford (*20*).

A portion of the KSPG fraction was digested with keratanase/protease inhibitors, and a portion of the CSPG fraction was digested with chondroitinase ABC/protease inhibitors. These digests were subjected to SDS-polyacrylamide gel electrophoresis, and the molecular mass of the core protein (52 kDa for KSPG, 44 kDa for CSPG) was found respectively to coincide with that of KSPG and CSPG core protein reported previously (*18, 21*). These KSPG and CSPG fractions were used to raise antibodies against them in rabbits.

Preparation of KSPG Core Protein and CSPG Core Protein—The PGs (KSPG and CSPG) were extracted and prepared from corneal stromas of 405 male chickens 2 days after hatching as described above. Blochberger *et al.* (*22*) have reported that adult chickens contain two isoforms of decorin: one containing CS/DS side chains, and the other, a hybrid, containing both CS/DS and KS side chains. Therefore, in this study, KSPG and CSPG core proteins were isolated as follows. First, the PG fraction containing KSPG and CSPG was digested with 10 units of keratanase/protease inhibitors described above at 37°C for 6 h. This digestion was expected to eliminate KS side chains from both KSPG and hybrid type of CSPG, yielding KSPG core protein without KS and CSPG core protein with CS only. The digest was then dialyzed against 7 M urea in 20 mM 2-morpholinoethanesulfonate (MES) buffer, pH 5.1, and applied on a DEAE-Sephacel column (1.1 × 12.5 cm) equilibrated with 7 M urea in 20 mM MES buffer, pH 5.1 (*22*). The pass-through fractions were pooled as KSPG core protein, and the fractions eluted with a linear gradient of 0.3-0.8 M NaCl were pooled as CSPG. The KSPG core protein fraction was dialyzed against Milli Q water at 4°C and lyophilized. The CSPG fraction was dialyzed against 50 mM Tris-HCl, pH 7.5 containing 50 mM sodium acetate and 50 mM NaCl, and digested with 5.0 units of chondroitinase ABC/protease inhibitors at 37°C for 5 h. The digest was applied to a DEAE-Sephacel column (1.1 × 12.5 cm) equilibrated with 7 M urea containing 50 mM Tris-HCl, pH 7.5. The pass-through fractions were pooled as CSPG core protein, dialyzed against Milli Q water at 4°C, and lyophilized.

Both KSPG and CSPG core proteins were subjected to

SDS-polyacrylamide gel electrophoresis, and their molecular masses coincided with those reported previously (*18, 21*). These KSPG and CSPG core proteins were coupled to CNBr-Sepharose 4B to make an affinity-chromatography column for the antibodies described below.

Preparation of Polyclonal Antibodies against KSPG and CSPG Core Proteins—Antibodies were raised in rabbits as follows. The isolated KSPG (500 μg) or CSPG (500 μg) was dissolved in 1.2 ml of saline and mixed with 1.35 ml of Freund's complete adjuvant and 0.5 ml of saline suspension of inactive *Bordetella pertussis* (2×10^9 cells). This KSPG or CSPG emulsion was injected subcutaneously into a female rabbit (Japan White). A small amount of blood was taken as control serum before primary injection. After 14 days, a new emulsion consisting of 250 μg of KSPG or CSPG, 550 μl of incomplete Freund's adjuvant, and 0.5 ml of saline suspension of inactive *Mucobacterium butyricum* (5 mg), was given as the second injection. After another 14 days, a third injection was given in the same way as the second injection. Finally, after a further 14 days, the last injection was given in the same way as the primary injection but without bacterial suspension. Ten days after the last injection, whole blood was taken from two rabbits and serum was prepared therefrom. The serum was stored at −80°C until used.

Rabbit IgGs against PG core proteins were fractionated from the serum by the E-Z-SEP method (*23*). The IgG fractions obtained were separated from the reagents by HiTrap Q chromatography, dialyzed against 10 mM phosphate buffered saline (10 mM PBS; 0.45 g $NaH_2PO_4\cdot2H_2O$, 2.53 g $Na_2HPO_4\cdot12H_2O$, and 8.0 g NaCl in 1 liter, pH 7.0) at 4°C, and concentrated by ultrafiltration at 4°C. Each concentrated IgG fraction was then applied to a Sepharose 4B column (0.7×10 cm) to which KSPG core protein (1.7 mg) or CSPG core protein (1.1 mg) was bound by CNBr activation, and the pass-through fraction was circulated back through the column overnight. The column was then washed with 10 mM PBS, and the antibody (IgG) was eluted with 3 M KSCN/10 mM PBS. The antibody fraction was immediately dialyzed against 10 mM PBS. The yield of anti-KSPG core protein was 658 μg (2.0 ml), and that of anti-CSPG core protein was 449 μg (1.1 ml).

Antibody titer was determined by ELISA on 96-well plates as follows. Each well was coated with KSPG core protein (0.2 μg) or CSPG core protein (0.2 μg) in 200 μl of Vollers buffer (2.96 g $NaHCO_3$ and 1.92 g Na_2CO_3 in 1 liter, pH 9.6). The plate was rinsed with PBS-Tween 20 (2.6 g $NaH_2PO_4\cdot2H_2O$, 29 g $Na_2HPO_4\cdot12H_2O$, 80 g NaCl, 2.0 g KCl, and 5 ml Tween 20 in 1 liter) and incubated with the affinity-purified anti-KSPG core protein or anti-CSPG core protein diluted variously in 200 μl of PBS-Tween 20 per well for 0.5-1 h at room temperature. The wells were then washed with PBS-Tween 20 and incubated with 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in 200 μl of PBS per well without Tween 20 for 1 h at room temperature. The wells were washed with PBS-Tween 20, and peroxidase in each well was quantitated with *o*-phenylenediamine/H_2O_2 (*24*). The absorbance at 490 nm of each well was measured by use of a plate reader (Bio-Rad). The titer of anti-KSPG core protein was 4,050 and that of anti-CSPG core protein was 12,150 (Fig. 1). Each antibody cross-reacted weakly with the other core protein, probably because both corneal KSPG (lumican) and CSPG (decorin) are small interstitial PGs containing leucine-rich repeats, and the primary structures of the core proteins are similar to each other (*5*). Keratan sulfate (bovine cornea; 1.0 μg/well), however, did not inhibit the reaction by anti-KSPG core protein, and chondroitin 4-sulfate (whale cartilage; 1.0 μg/well) did not inhibit the reaction by anti-CSPG core protein (not shown).

Immunohistochemistry—Fertilized White Leghorn chicken eggs were incubated at 37°C from 0 day to 17 days. At Days 5 through 17, chicken embryos were dissected as follows. Heads were dissected at Day 5; the eye balls at Days 6 to 13; corneas at Days 14 to 17 and 2 days after hatching. These dissected tissues were frozen immediately in OTC (Tissue Tik) with liquid nitrogen and stored at −80°C until use. The frozen tissues were serially sectioned at 10 to 14 μm in thickness by cryostat and mounted on slide glasses. The slides were rinsed with 28 mM ice-cold phosphate buffer, pH 7.2, and dried in the hood. Before staining, all sections were blocked for 4 min with 40 μl of 28 mM phosphate buffer, pH 7.2, containing 5% normal goat serum and 1% BSA, and rinsed once with 28 mM phosphate buffer, pH 7.2. Then 40 μl of anti-CSPG core protein (1:20 dilution) or 40 μl of anti-KSPG core protein (1:20 dilution) in 28 mM phosphate buffer, pH 7.2, was overlayed on each section, and the sections were incubated at 4°C for 20 h. They were then rinsed with the same buffer five times and dried in the hood. Next, 40 μl of FITC-con-

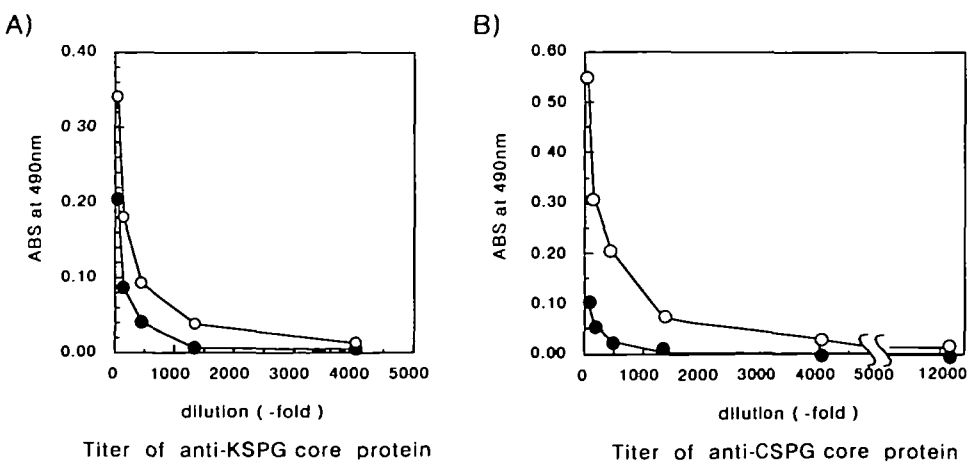

Fig. 1. **Titers of antibodies against KSPG core protein (A) and CSPG core protein (B).** Open circles represent KSPG core protein; closed circles, CSPG core protein.

jugated goat anti-rabbit IgG (1:40 dilution) in the same buffer was overlayed on each section, and the sections were incubated at room temperature in the dark for 30 min, rinsed again with the same buffer three times, and dried in the hood. Fluorescence on the sections was examined by use of an epifluorescence and Normarski differential contrast microscope (Axio-Phot; Zeiss) with a laser scanning confocal imaging system (Bio-Rad).

Western Blotting—At Days 5, 6, and 7 of embryonic age, and 2 days after hatching, corneas (35 to 70 eyes per experiment) were dissected and minced. PGs were extracted from the minced corneas with 4 M guanidine hydrochloride as described above. Extracts were dialyzed against 7 M urea/50 mM Tris-HCl, pH 7.5/protease inhibitors as described previously (*19*) and concentrated by ultrafiltration at 4°C. The concentrated extracts were dialyzed again against 50 mM Tris-HCl, pH 7.5, then digested with 0.01 unit (μmol/min) of endo-β-galactosidase and 0.05 unit (μmol/min) of chondroitinase ABC in 50 mM Tris-HCl, pH 7.5, containing the protease inhibitors described above at 37°C for 5 h. The digest (60 μl) of each embryonic age was mixed with 60 μl of the sampling buffer (4% SDS, 0.4%

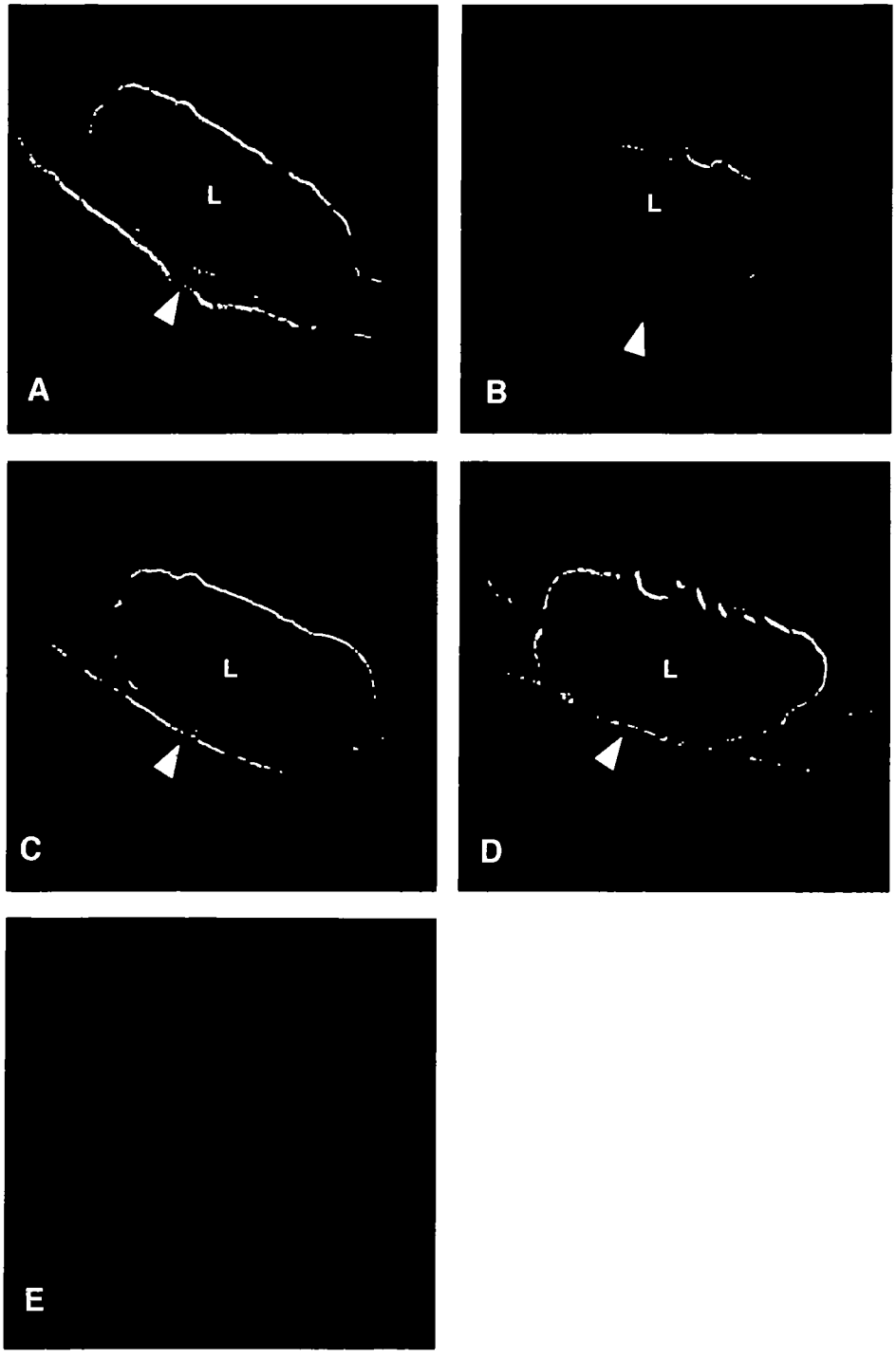

Fig. 2. **Immunofluorescent staining of chick embryonic eye of Day 5.** Tissues were stained with: (A) anti-CSPG core protein, (B) the same antibody blocked with CSPG core protein, (C) anti-KSPG core protein, (D) the same antibody blocked with KSPG core protein, and (E) monoclonal antibody 5-D-4. Arrowheads indicate corneal epithelial side; L, lens. Magnification is ×200 for all micrographs.

glycerol, 0.4 mM dithiothreitol, 1×10^{-4}% bromophenol blue, and 50 mM Tris-HCl, pH 7.5) and incubated at 70°C for 30 min. The mixture was electrophoresed in 7.5% acrylamide gel (Ready gel SJ; Bio-Rad) containing 0.1% SDS at a constant current of 10 mA for 35 min, then at 20 mA for 45 min. Proteins in the gel were then transferred to PVDF membrane using a semi-dry type blotting unit (Amersham Pharmacia) at 50 mA for 100 min. The blotted membrane was pretreated with 3% skim milk in 10 mM PBS at 37°C for 2 h, then exposed to anti-KSPG (diluted 1:250) or anti-CSPG core protein (diluted 1:100) in 10 mM PBS/5% skim milk/0.3% Tween 20 at 4°C for 16 h. The membrane was then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5,000 in the same buffer at room temperature for 2 h. The color was developed with 4-chloro-1-naphthol (HRP Conjugate Substrate Kit) (*25*).

RESULTS

Developmental Appearance of PGs—Sections of chick embryonic eyes of Days 5 and 6 were immunostained, and

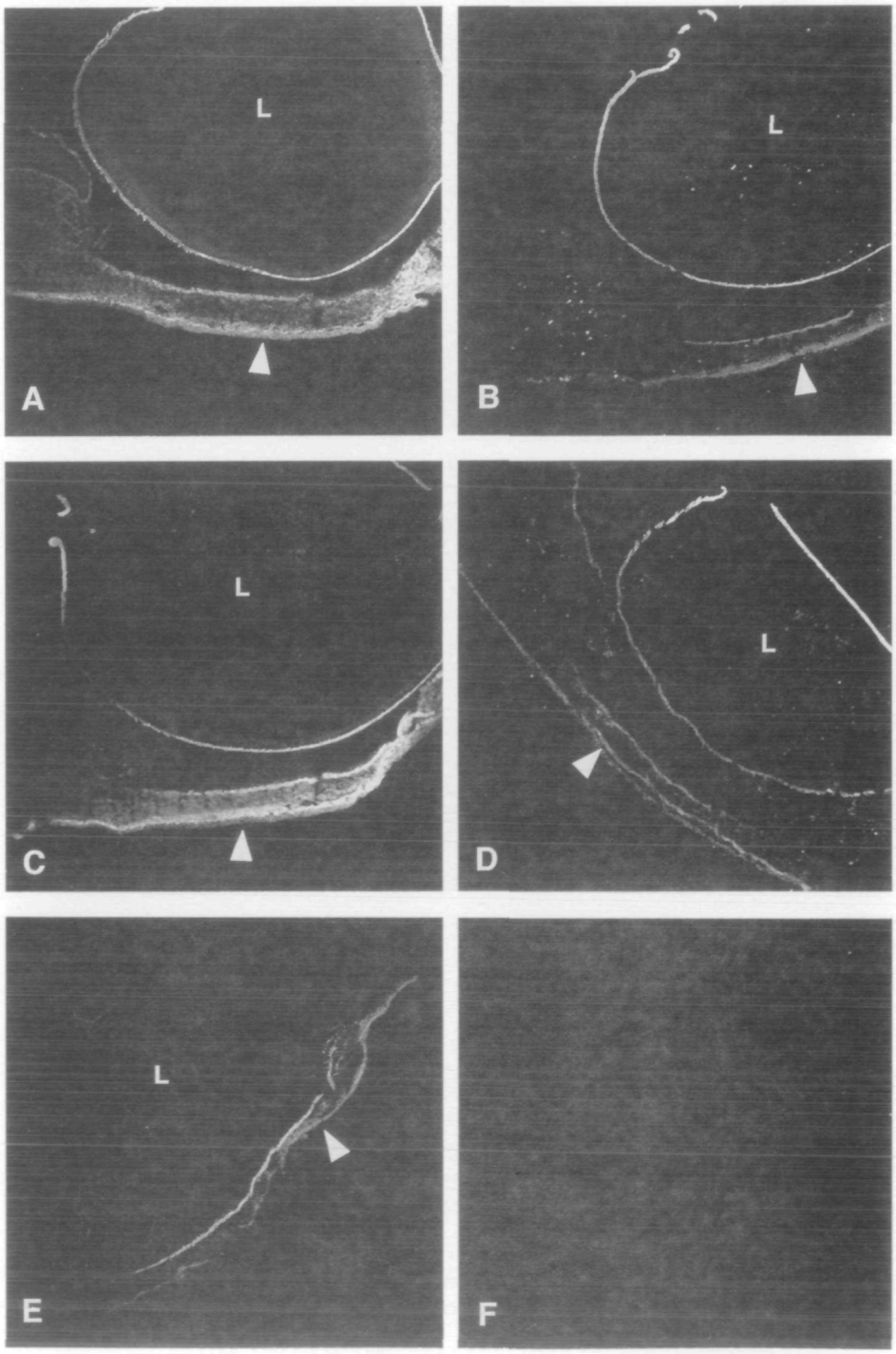

Fig. 3. **Immunofluorescent staining of chick embryonic eye of Day 6.** Tissues were stained with: (A) anti-CSPG core protein, (B) the same antibody blocked with CSPG core protein, (C) anti-KSPG core protein, (D) the same antibody blocked with KSPG core protein, (E) monoclonal antibody 5-D-4, and (F) secondary antibody only (without 5-D-4). Symbols and magnification are as in Fig. 2.

the day of appearance of KSPG or CSPG in the cornea was examined. Figure 2 shows the immunostaining of Day 5 chick embryo. The corneas were stained with both anti-CSPG and anti-KSPG core proteins (Fig. 2, A and C). However, when the antibodies were blocked by CSPG or KSPG core protein before incubation with the sections, the staining with anti-CSPG core protein disappeared (Fig. 2B), while that with anti-KSPG core protein remained (Fig. 2D). This result means that CSPG core protein (probably with CS side chains) is actually present in the cornea, but KSPG core protein is not. Because neural cell-derived mesenchymal cells have not yet invaded the primary stroma on Day 5, CSPG of Day 5 cornea must have been synthesized by epithelial or endothelial cells. The immunofluorescence on panel C probably derives from non-specific binding of anti-KSPG core protein. As seen in Fig. 2, A and C, the surfaces of lenses were also stained with both anti-CSPG and KSPG core proteins. The identity of this staining substance is unknown. It was found during this study that such stainable unknown substances were present in early developmental eye tissues. The cornea of Day 5 chick embryo was not stained with monoclonal anti-KS (5-D-4)

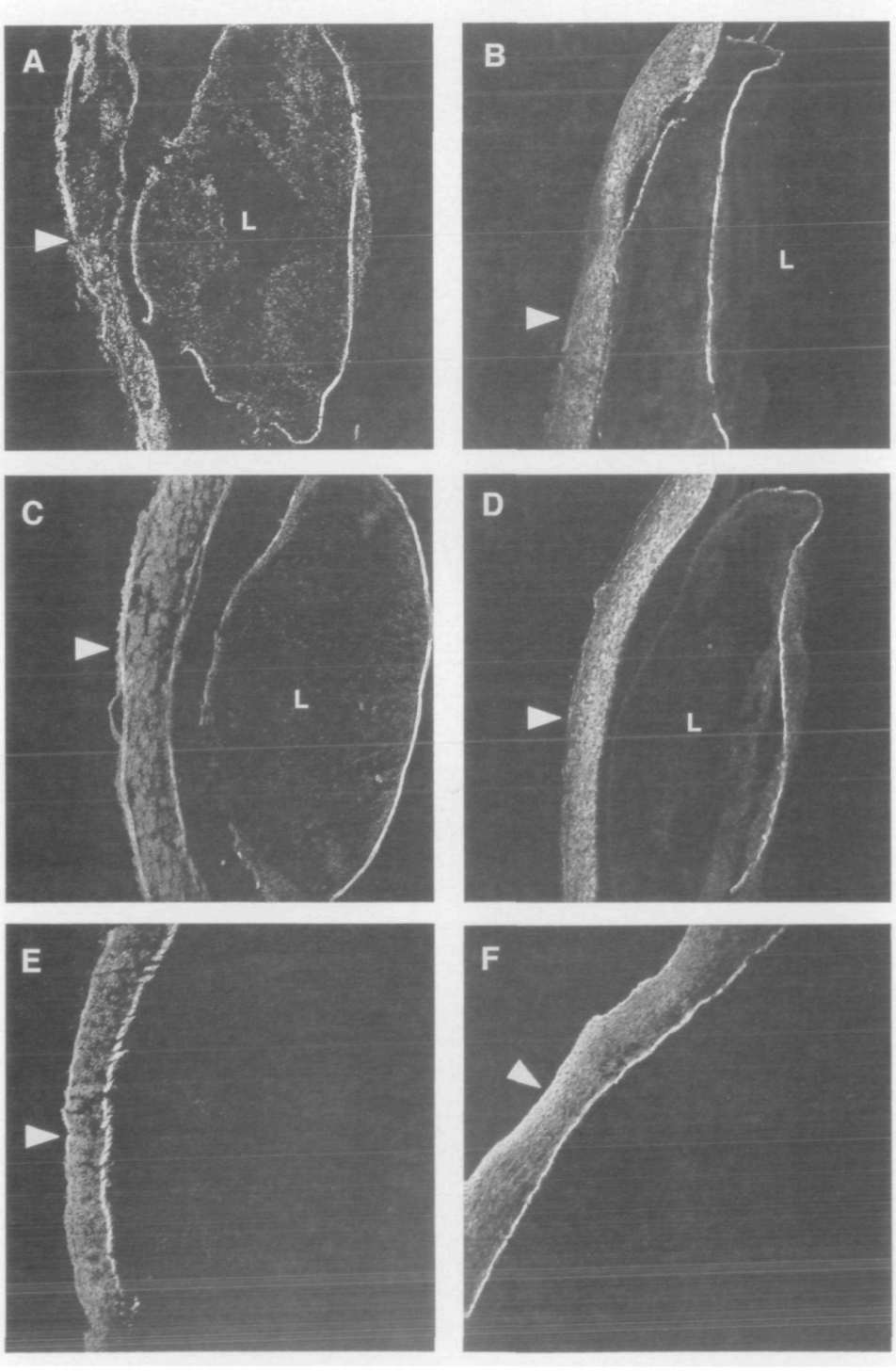

Fig. 4. **Immunofluorescent staining of chick embryonic eyes of Days 7 and 8.** Tissues were stained with: (A) and (B) anti-CSPG core protein, (C) and (D) anti-KSPG core protein, (E) and (F) monoclonal antibody 5-D-4. (A), (C), and (E) Day 7; (B), (D), and (F) Day 8. Symbols are as in Fig. 2, and magnification is ×100.

(Fig. 2E), showing that KS chains are absent at this stage. On the other hand, the immunostaining photographs of sections of Day 6 chick embryo eyes shown in Fig. 3 reveal that corneas were strongly stained with both anti-CSPG and anti-KSPG core proteins (Fig. 3, A and C), and that these stainings were substantially reduced by blocking with CSPG or KSPG core protein (Fig. 3, B and D). Clearly, both CSPG and KSPG core proteins are present in the cornea of Day 6. The stainings with anti-CSPG and anti-KSPG core protein were stronger on both the peripheral epithelial and endothelial sides than in the central region of the corneas. This may have been caused artificially: because the cornea is not rigid in the early embryonic stage, the central region may have become thinner than the peripheral region as a result of sectioning. If this is the case, it means that corneas of Day 6 were stained uniformly in all regions and that the stromal cells begin to synthesize PGs simultaneously in all regions. Furthermore, the cornea was stained with 5-D-4 antibody (Fig. 3E), although much more weakly than with anti-KSPG core protein, showing that the core protein has KS side chains and suggesting that these KS chains are undersulfated, because the epitope for 5-D-4 contains

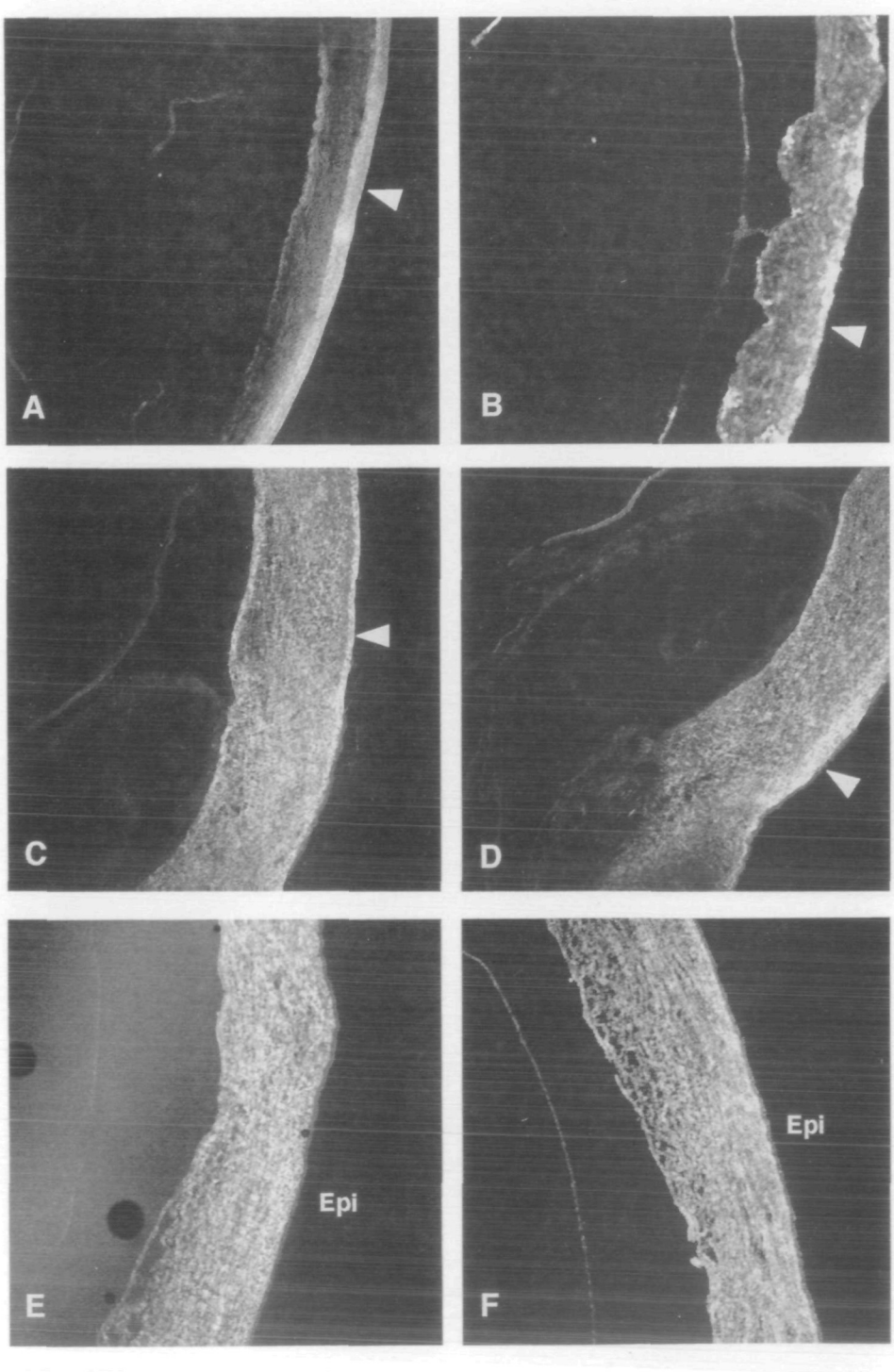

Fig. 5. **Immunofluorescent staining of chick embryonic eyes of Days 10-13.** Tissues were stained with: (A), (C), and (E) anti-KSPG core protein; (B), (D), and (F) with anti-CSPG core protein. (A) and (B) Day 10; (C) and (D) Day 12; (E) and (F) Day 13. Epi indicates epithelium. Other symbol and magnification are as in Fig. 4.

sulfate (*26*).

Extent of Staining of Early Developmental Corneas with Monoclonal Anti-KS (5-D-4)—Figure 4 shows immunostaining photographs of Days 7 and 8 embryonic eyes. Corneas of Days 7 and 8 were stained with both anti-CSPG and KSPG core proteins (Fig. 4, A to D), and these stainings disappeared by blocking with CSPG and KSPG core proteins (not shown), showing that CSPG and KSPG are synthesized uniformly in all regions of Days 7 and 8 corneas. Figure 4, E and F, shows photographs of Days 7 and 8 corneas stained with 5-D-4 antibody. The intensity of 5-D-4-staining clearly increased from Day 6 (Fig. 3E) through to Day 8. In the same period, the intensity of anti-KSPG core protein-staining of the corresponding corneas (Figs. 3C, 4C, and 4D) remained virtually unchanged. This indicates that the sulfation of KS chains probably increased from Day 6 to Day 8, although the possibility remains that the core protein without KS chain was converted to the protein with KS chain in this period (see below).

Staining of Later Developmental Corneas with Anti-CSPG and Anti-KSPG Core Proteins—Corneas of Days 10

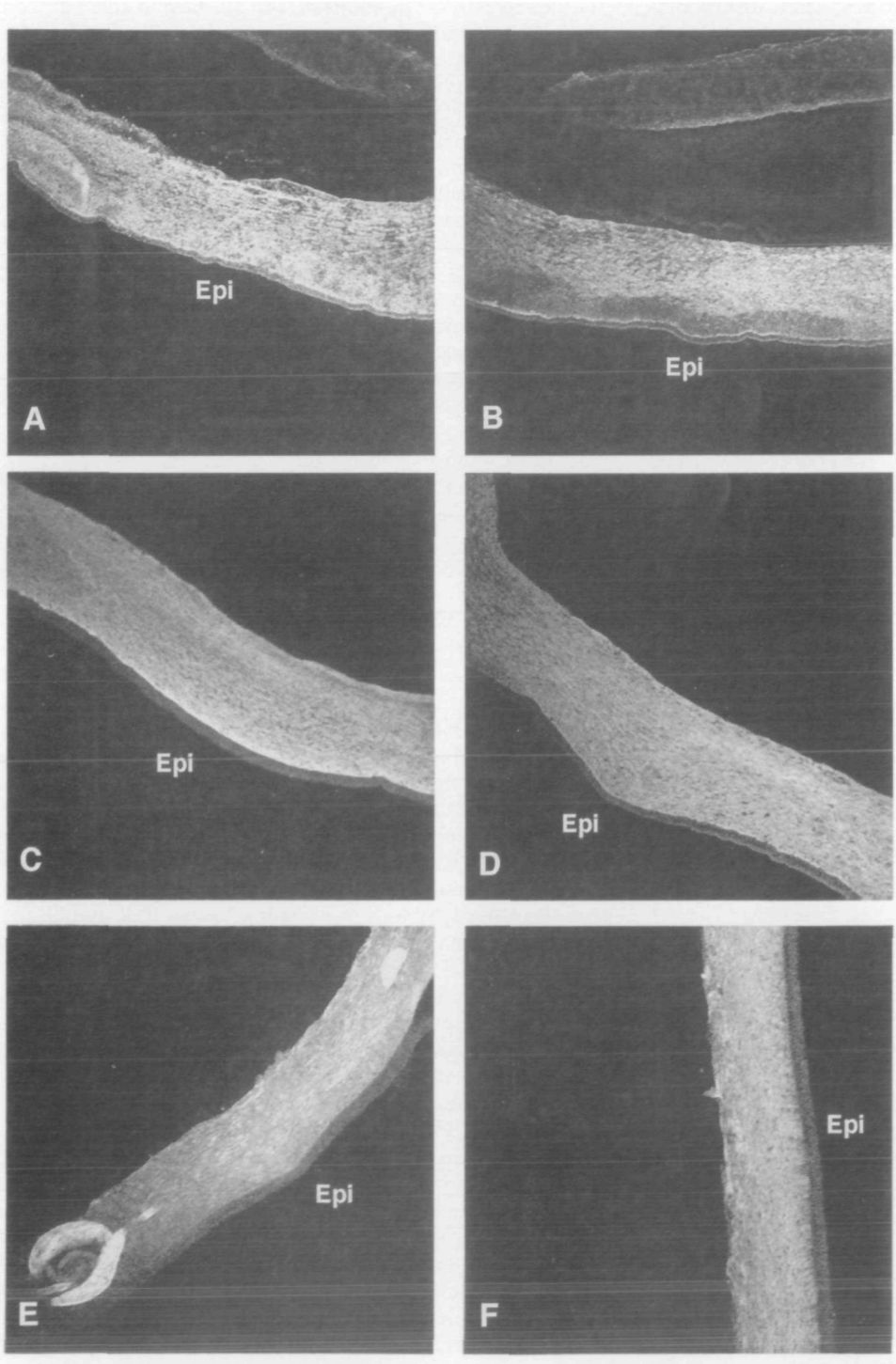

Fig. 6. **Immunofluorescent staining of chick embryonic eyes of Days 15-17 and 2 days after hatching.** Tissues were stained with: (A), (C), and (E) anti-KSPG core protein; (B), (D), and (F) anti-CSPG core protein. (A) and (B) Day 15, (C) and (D) Day 17, (E) and (F) 2 days after hatching. Symbol (Epi) and magnification are as in Fig. 5.

to 17 and of Day 2 after hatching were stained with anti-CSPG and anti-KSPG core proteins and the localizations of CSPG and KSPG core proteins in these corneas were examined. The results are shown in Figs. 5 and 6. All corneas were stained over the whole stroma with both anti-CSPG and anti-KSPG core proteins, showing that the stromal cells actively synthesize both CSPG and KSPG core protein at this time. It is known that the corneal epithelium becomes multilayered between Days 13 and 14 (*12*). Consistent with this fact, as seen from Figs. 5 and 6, the epithelial layer was clearly observed in corneas of Day 13 and after. These epithelial layers were not stained with either anti-CSPG or anti-KSPG core protein. It was reported previously (*14, 19, 27*) that the corneal epithelial cells of embryos and newly hatched synthesize CSPG. The above result shows, therefore, that the core protein of epithelial CSPG synthesized on Day 13 and after is different from that of stromal CSPG (probably, decorin). Whether the CSPG core protein is present in the corneal epithelial layer before Day 13 is not discernible from photographs. It seems probable that KSPG core protein is not synthesized by the epithelium at any period of development.

Western Blotting—Western blotting confirmed that the corneas of Day 5 do not synthesize KSPG. The extracts from corneas of Days 5, 6, 7, and 2 days after hatching were digested with both endo-β-galactosidase and chondroitinase ABC, electrophoresed on SDS-polyacrylamide gel, then transferred to PVDF membrane as described under "MATERIALS AND METHODS." The membrane was then immunostained with anti-KSPG or CSPG core protein. The result is shown in Fig. 7. As control, the extracts without endo-β-galactosidase and chondroitinase ABC digestions were treated in the same way. As seen in Fig. 7A (staining with anti-KSPG core protein), because a relatively low dilution of antibody was used, many bands were stained on each lane, and a very strongly staining band was observed on lane 8 (enzymatic digest of extract from corneas of 2 days after hatching). This strongly staining band is the corneal KSPG core protein. On lane 4 (digest of extract from corneas of Day 6) and lane 6 (digest of extract from corneas of Day 7), bands were observed at the same position as the strongly staining band on lane 8. On lane 2 (digest of extract from corneas of Day 5), however, no such band was observed. This shows that corneas of Day 5 do not synthesize KSPG. In addition, the absence of this band on lanes 1, 3, 5, and 7 (undigested extracts) indicates that the core protein without KS chain or polylactosamine is absent in these corneas. On the other hand, staining with anti-CSPG core protein (Fig. 7B) yielded two strongly staining bands on lane 8: the faster-moving band (arrow) is CSPG core protein, and the slower-moving one (arrowhead) is KSPG core protein, detected by the cross-reaction with anti-CSPG core protein. A weakly staining band was observed at the position of CSPG core protein on lane 2 (digest of Day 5), showing that CSPG core protein is present in cornea of Day 5, while KSPG core protein was again not detected. On lanes 4 and 6 (digests of Days 6 and 7), the staining pattern confirmed that both CSPG and KSPG core proteins are present in corneas of Days 6 and 7. The above experiment was also performed by digesting corneal extracts with keratanase and chondroitinase ABC, instead of endo-β-galactosidase and chondroitinase ABC. Electrophoresis and Western blotting of the resultant digests yielded similar results to Fig. 7 (not shown).

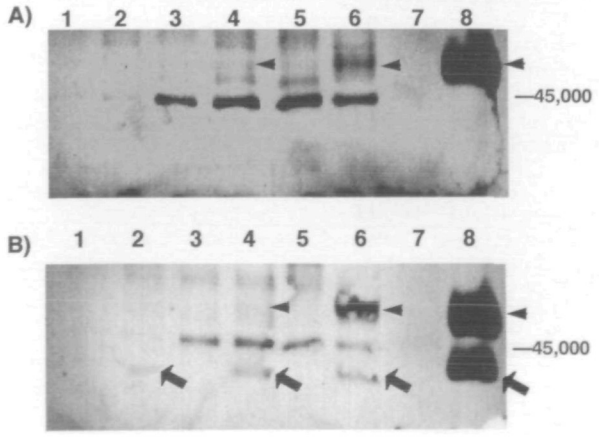

Fig. 7. **Western blotting of extracts from corneas of Days 5-7 and 2 days after hatching.** One portion of each extract was digested with endo-β-galactosidase and chondroitinase ABC containing protease inhibitors, the digest was electrophoresed, blotted to PVDF membrane, and stained with anti-KSPG core protein (A) or anti-CSPG core protein (B). The other portion was electrophoresed and immunoblotted without the enzymatic digestion. Lane 1 is the extract of Day 5 cornea without the digestion; lane 2, Day 5 cornea with the digestion; lane 3, Day 6 cornea without the digestion; lane 4, Day 6 cornea with the digestion; lane 5, Day 7 cornea without the digestion; lane 6, Day 7 cornea with the digestion; lane 7, cornea of 2 days after hatching without the digestion; lane 8, cornea of 2 days after hatching with the digestion. The arrowheads indicate the position of KSPG core protein; arrows, the position of CSPG core protein. Bar and value (kDa) on the right side show the positions and molecular mass of ovalbumin as standard, respectively.

DISCUSSION

In this study, KS chains and KSPG core protein were not detected in corneas of Day 5. This is inconsistent with the result reported by Doane *et al.* (*17*), who found that corneas of Day 5 (stage 26) were stained strongly with anti-lumican (KSPG) core protein. However, they did not examine the effect of blocking the anti-lumican core protein with lumican core protein. If their antibody is polyclonal, the strong staining of Day 5 corneas could be caused by the non-specific binding of the antibody, as observed in our experiment. The identity of the substance that was stained in Day 5 corneas remains to be clarified. Our results show that the mesenchymal cells (stromal cells) that invade the primary stroma at Day 5.5 to 6 synthesize KSPG core protein and KS chains after the invasion. On the other hand, we detected CSPG core protein in corneas of Day 5, and a similar result was reported by Doane *et al.* (*17*). Because mesenchymal cells have not invaded the primary stroma at this stage, CSPG core protein must be synthesized by epithelial and/or endothelial cells. Doane *et al.* reported that CSPG (decorin) core protein was present subjacent to the epithelium at Day 5. It seems likely that the CSPG core protein is synthesized by the epithelium on Day 5. But whether the epithelium continues to synthesize the same species of CSPG core protein after Day 5 is unknown. As seen in Figs. 5 and 6, epithelial layers were discriminated clearly in corneas of Day 13 through 2 days after hatching and not immunostained with anti-CSPG core protein. Funderburgh *et al.* (*16*) stated that the period of

Days 11-16 is a time during which the epithelium is undergoing marked changes. Therefore, it is likely that the epithelial cells synthesize the CSPG (decorin) core protein until about Day 13 and another species of CSPG core protein after that time. The corneal stromal cells continue to synthesize the CSPG (decorin) core protein from Day 6 until after hatching. The absence of KSPG core protein and the presence of CSPG core protein in corneas of Day 5 was confirmed by the Western blotting experiment (Fig. 7).

Cornuet *et al.* (*18*) reported that, while lumican with polylactosamine (nonsulfated KS) was detected in extracts of corneas as early as Day 7, lumican with sulfated KS (*i.e.*, proteoglycan) was not detected in corneal extracts until Day 15. In our experiment, however, sulfated KS was detected in corneas even at Day 6. Because it appears that only a small amount of KSPG core protein with sulfated KS is present in corneas in the early days of development, the core protein might have been lost during their experimental procedures (immunoprecipitation, electrophoresis, and Western blotting). Because polylactosamine (nonsulfated KS) chain cannot be detected by the method used in our experiment, it is possible that both core proteins with polylactosamine and with sulfated KS are both present in corneas of Days 6 to 15. Furthermore, it is likely that KS chains are undersulfated during Days 6 to 7. Sulfotransferase activities may be much less than glycosyltransferase and core protein-synthetic activities in corneas in the early days of development, and an increase in sulfotransferase activities during development may lead to the cornea becoming transparent (*15, 18*).

Funderburgh *et al.* (*16*) reported from immunohistochemistry with monoclonal antibody (I22) to KS that KSPG antigen localized inside stromal cells on Day 6, that extracellular KS was first seen in the posterior stroma on Day 9, and that KS accumulated in a posterior to anterior direction during subsequent development. In our experiment, however, corneas of Day 6 were stained uniformly with anti-KSPG core protein (polyclonal antibody), and corneas of Day 7 were stained uniformly with 5-D-4 (monoclonal antibody to KS). We cannot explain this discrepancy, but one possibility is that it derives from the different procedures used for immunostaining: in their experiment, tissue was fixed in 3.5% formaldehyde, embedded in paraffin, incubated with primary antibody at 37°C for 1-2 h, and fluorescence was detected by use of an epifluorescence microscope; in our experiment, tissue was fixed and frozen in OTC, incubated with primary antibody at 4°C for 20 h, and fluorescence was detected by use of a laser scanning confocal-imaging system. The mesenchymal cells invade the posterior portion of the stroma first, and then invade the posterior to anterior portion (*12*). If the cells begin to synthesize and accumulate PGs immediately after invasion, a concentration gradient of the PG antigens would be formed in a posterior to anterior direction. Our method may not discriminate clearly this gradient because of the long incubation time with the primary antibody (20 h), and because a small amount of the antigen in the anterior region may be detected by the laser scanning confocal system.

In conclusion, KSPG begins to be synthesized by stromal cells over the whole stroma immediately after the cells have invaded the primary stroma between Days 5.5 and 6. The KS chains synthesized between Days 6 and 7 are, however, undersulfated. On the other hand, CSPG (decorin) is synthesized by the epithelial and/or endothelial cells before the invasion by mesenchymal cells, and also by the stromal cells (differentiated from mesenchymal cells) over the whole stroma immediately after the invasion.

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