Changes in the Distribution of the 34-kdalton Tyrosine Kinase Substrate during Differentiation and Maturation of Chicken Tissues

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ABSTRACT We examined the distribution of the 34-kilodalton (34-kD) tyrosine kinase substrate in tissues of adult and embryonic chicken using both a mouse monoclonal antibody and a rabbit polyclonal antibody raised against the affinity purified 34 kD protein. We analyzed the localization by immunoblotting of tissue extracts, by immunofluorescence staining of frozen tissue sections, and by staining sections of paraffin-embedded organs by the peroxidase anti-peroxidase method.

The 34-kD protein was present in a variety of cells, including epithelial cells of the skin, gastrointestinal, and respiratory tracts, as well as in fibroblasts and chondrocytes of connective tissue and mature cartilage, and endothelial cells of blood vessels. The 34-kD protein was also found in subpopulations of cells in thymus, spleen, bone marrow, and bursa. The protein was not detected in cardiac, skeletal, or smooth muscle cells, nor in epithelial cells of liver, kidney, pancreas, and several other glands. Although most neuronal cells did not contain the 34-kD protein, some localized brain regions did contain detectable amounts of this protein. The 34-kD protein was not detected in actively dividing cells of a number of tissues. Changes in the distribution of the 34-kD protein were observed during the differentiation or maturation of cells in several tissues including epithelial cells of the skin and gastrointestinal tract, fibroblasts of connective tissue, and chondroblasts.

The transforming protein of Rous sarcoma virus, designated $pp60^{src}$, has been shown to be a protein kinase that specifically phosphorylates tyrosine residues on substrate proteins (reviewed in references 1–3). Recently, the transforming proteins of a number of other retroviruses have also been found to possess tyrosine kinase activity (4–7; reviewed in references 2–3). To gain an understanding of the biochemical mechanisms involved in cell transformation, it is important to identify and characterize the substrates of the tyrosine kinases. One of the better characterized substrates is a 34-kilodalton (34-kD)[1] protein first identified by Radke and Martin (8) and purified by Erikson and Erikson (9). This 34-kD protein is specifically phosphorylated at a tyrosine residue in cells transformed by several different retroviruses, but is phosphorylated only at serine residues in normal cells (9–15). The 34-kD protein is also phosphorylated at a tyrosine residue when the epidermoid carcinoma cell line A-431 is treated with epidermal growth factor (16, 17) and when particular fibroblast cell lines are stimulated with platelet-derived growth factor (18). In some cases, however, stimulation of fibroblasts with epidermal or platelet-derived growth factor (18–20) is not accompanied by increased 34-kD protein phosphorylation. In addition, phosphorylation of this protein does not appear to be required for transformation of some cells by tyrosine kinase encoding retroviruses; several cell lines transformed by Abelson murine leukemia virus do not contain detectable amounts of the 34-kD protein (21).

Recently, the 34-kD tyrosine kinase substrate has been localized at the inner surface of the plasma membrane by immunofluorescence staining (22–24) and subcellular fractionation studies (22, 24–27) and was observed by immunofluorescence staining to have a distribution in chick or human embryo fibroblasts similar to that of the cytoskeletal associated protein α-spectrin (22, 28). However, the role of the 34-kD protein in cellular transformation by retroviruses as well

[1] *Abbreviations used in this paper:* kD, kilodalton; PBS, phosphate-buffered saline; PAP, peroxidase anti-peroxidase.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 FEBRUARY 1984 473–486

as its normal cellular function remain to be elucidated. Because of its alteration by tyrosine kinases and its potential significance in cellular transformation and normal cell growth, it is of particular interest to trace the tissue distribution of this protein.

Using a monoclonal antibody that specifically recognizes the 34-kD protein (15) and a polyclonal rabbit antibody raised against the affinity-purified protein (22), in this study we investigated the tissue distribution of the 34-kD tyrosine kinase substrate in a variety of chicken organs. The results indicate that the 34-kD protein is not present in all cells of the chicken; rather, this protein is specifically localized in particular differentiated cells within a range of different embryonic and adult tissues.

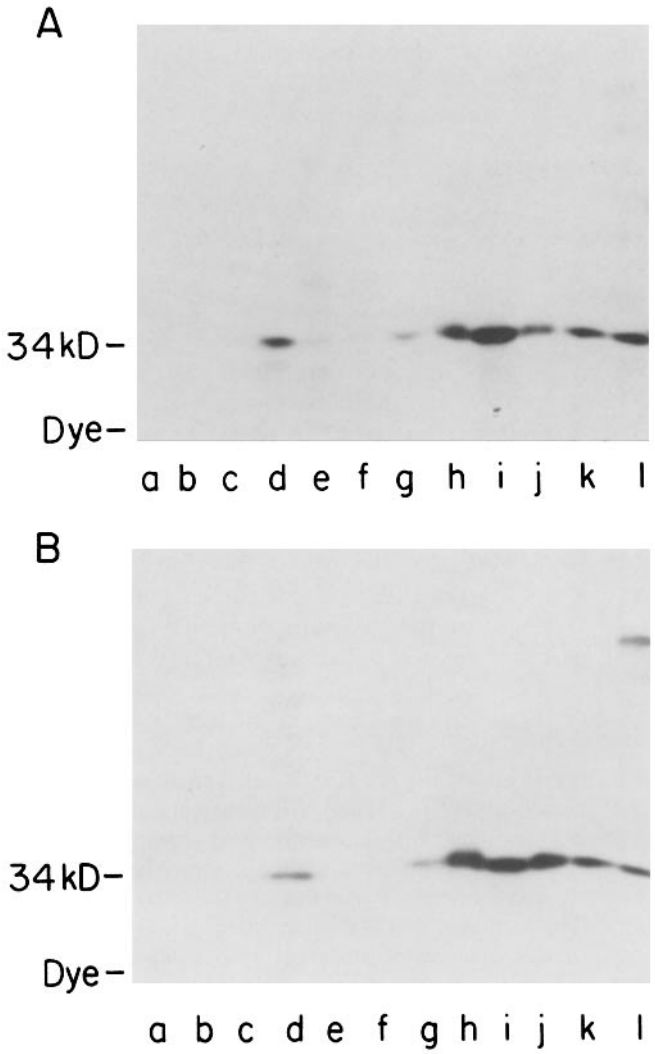


FIGURE 1 Immunoblot analysis of 34-kD protein content in various organs of adult chicken. Crude detergent extracts of tissues were prepared, fractionated by SDS gel electrophoresis, and blotted with monoclonal anti-34-kD protein antibody (*A*) or rabbit anti-34-kD protein antibody (*B*), followed by ^{125}I-protein A as described in Materials and Methods. The 34-kD protein was visualized by autoradiography. Gel lanes contained extracts of heart (*a*), brain (*b*), bone marrow (*c*), spleen (*d*), liver (e), pancreas (*f*), kidney (*g*), skin (*h*), intestine (*i*), adipose tissue (*j*), chicken embryo cells (*k*), and gizzard (*l*).

MATERIALS AND METHODS

Antibodies: The preparation of the monoclonal antibody to the 34-kD protein and the rabbit antibody to affinity-purified 34-kD protein have been described previously (15, 22). The production of monoclonal antibodies to vinculin has been described elsewhere (29).

Immunoblotting: Chicken organs were weighed and then homogenized, (1 g tissue per 5 ml boiling SDS sample buffer) in a Waring blender. Extracts were separated by electrophoresis on 8.5% SDS polyacrylamide gels following the procedure of Laemmli (30). Proteins were transferred to nitrocellulose and blotted, either with the monoclonal antibody to the 34-kD protein followed by rabbit anti-mouse IgG and then ^{125}I-protein A, or with the rabbit anti-34-kD protein antibody followed by ^{125}I-protein A as previously described (31). Binding of ^{125}I-protein A was visualized by autoradiography with Kodak SB-5 x-ray film at −70°C.

Immunofluorescence Staining: Organs from adult or 13-d-old embryonic chickens were frozen at −70°C in isopentane, embedded in O.C.T. compound (Tissue-Tek) and 4 μm sections cut in a Cryotome (Damon Corp., Needham Heights, MA) at −12°C to −20°C. The sections were air-dried overnight, fixed in 3.7% formaldehyde/phosphate-buffered saline (PBS) for 15 min and incubated for 15 min in 0.1 M glycine/PBS. After the sections were treated for 60 min with 5% goat serum in 0.1 M $NaKHPO_4$, pH 7.2, they were incubated for 3 h with the anti-34-kD protein monoclonal antibody (1:100 dilution of ascites fluid). Sections were washed five times with 0.1 M $NaKHPO_4$, pH 7.2, containing 1% goat serum and then incubated for 60 min with a 1:100 dilution of fluorescein-conjugated rabbit antibodies to mouse IgG (Miles Laboratories, Elkhart, IN). The sections were then washed, mounted in 90% glycerol/PBS, and visualized for immunofluorescence as previously described (22). Immunofluorescence staining of cultured cells was carried out as described (22).

Peroxidase Staining of Paraffin Sections: Organs from adult or 13-d-old embryonic chickens were dissected and immediately placed in Bouin's fixative for 24 h at 4°C. The organs were embedded in paraffin and thin sections cut following standard histological procedures. Sections were deparaffinized and stained with rabbit antibody to affinity-purified 34-kD protein or with preimmune serum from the same rabbit. The 34-kD protein staining was analyzed using the peroxidase anti-peroxidase (PAP) technique (32). In these experiments, all the sections were also stained with the anti-34-kD protein monoclonal antibody and similar but weaker staining patterns were obtained. The rabbit or mouse PAP complex (1 mg/ml) was obtained from Jackson Immuno Research (Avondale, PA) and used at a 1:500 dilution. The 34-kD protein staining was visualized by reaction with diaminobenzidine tetrahydrochloride (0.5 mg/ml). Subsequent to peroxidase staining, slides were counterstained with Gill's triple strength Hematoxylin (Polysciences, Inc., Warrington, PA). Stained sections were photographed under bright-field illumination using Panatomic-X Kodak film and a Wratten #55 green filter.

Cell Culture: All established cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco Laboratories, Grand Island, NY). The preparation of primary cell cultures of fibroblasts (33), glial cells from forebrain (34), myotubes and myoblasts (35), and retinal cells (36) was previously described. Human platelets were obtained from the New York Blood Center and human sperm from The Population Council through the courtesy of Dr. Marc Goldstein. HeLa cells were provided by Dr. Joseph Nevins of The Rockefeller University.

RESULTS

Tissue Distribution of the 34-kD Tyrosine Substrate Analyzed by Immunoblotting

The overall content of the 34-kD protein in a range of different chicken tissues was analyzed by immunoblotting (Fig. 1). Detergent extracts of organs from embryonic and adult chickens were fractionated on SDS polyacrylamide gels. The proteins were transferred to nitrocellulose and then reacted with either a monoclonal antibody to the 34-kD protein or a rabbit anti-34-kD protein antibody. As shown in Fig. 1, the 34-kD protein is present in significant quantities in chicken skin, intestine, adipose tissue, gizzard, and spleen, and to a lesser extent in kidney, pancreas, bone marrow, skeletal muscle, and liver. Very low levels of the 34-kD protein were found in chicken heart and brain and no 34-kD protein

was detected in retina, lens, pigmented epithelium, erythrocytes, nor in human platelets or sperm (Table I, Fig. 1). These results indicated that the 34-kD protein was broadly but not universally distributed throughout the organism. The immunoblotting procedure further established that, in all the tissues examined, the anti-34-kD protein antibodies recognized a unique antigen with an apparent molecular mass of 34,000.

TABLE I

34-kD Protein Content of Additional Tissues and Cultured Cells

Tissues	Origin	34-kD protein	Method*
Erythrocytes	5-d-old chicken embryo	−	I
	Adult chicken	−	I
Retina	5–14-d-old chicken embryo	−	I, F, P
Adrenal gland	13-d-old chicken embryo	−	I
Pigmented epithelium	5–14-d-old chicken embryo	−	I
Lens	Adult chicken	−	F
Sperm	Human	−	I, F
Platelets	Human	−	I
Cells in culture			
Retinal cells	7-d-old chicken embryo	−	I, F
RSV-transformed retinal cells‡	7-d-old chicken embryo	+	I, F
RSV-transformed cerebellar cells	Rat	+	I
Neuroblastoma	Mouse	+	I
Forebrain glial cells	14-d-old chicken embryo	+	I, F
C6 glioma	Rat	+	I
Myoblasts and myotubes	11-d-old chicken embryo	+	F
HeLa cells	Human	+	I
Fibroblasts	11-d-old chicken embryo	+	I, F
Splenocytes	Mouse	+	I

* 34 kD content was determined by immunoblotting (*I*), immunofluorescence staining (*F*), or by the PAP staining technique (*P*) as described in Materials and Methods.

‡ *RSV*, Rous sarcoma virus.

Immunofluorescence and Immunohistochemical Localization of the 34-kD Tyrosine Kinase Substrate in Skin

Immunofluorescence microscopy and immunohistochemistry were used to determine the cellular distribution of the 34-kD protein in different organs. All of the tissues illustrated in Figs. 2–9 except bursa and urinary tract were examined using both techniques. The two methods gave virtually identical results which were also consistent with those obtained by immunoblot analysis. Additional tissues and cells were examined by one or more of these methods and the results are summarized in Table I.

The 34-kD tyrosine kinase substrate has previously been identified in cultured fibroblasts and epithelial cells; in our initial studies we therefore chose to examine skin from embryonic and adult chickens. The results of an experiment in which thin frozen sections of avian tongue were stained with the anti-34-kD protein monoclonal antibody are shown in Fig. 2*A*. The 34-kD protein appears to be concentrated in the epidermal regions of the tongue. The squamous epithelial cells of the stratum germinativum are brightly stained while the keratinized layer is unstained. Similar staining of squamous epithelium was seen when thin sections of the chicken footpad (Fig. 2*B*) were incubated with the anti-34-kD protein antibody. When paraffin sections were stained by the PAP method it was possible to localize the 34-kD protein to particular layers of the epidermis. In the chicken tongue (Fig. 3, *C* and *D*) the basal epithelial cells of the stratum germinativum were lightly stained with the anti-34-kD protein antibody while the second layer, the stratum spinosum, which is composed of the more differentiated prickly cells and is undergoing the process of keratinization, was intensely stained. The flattened cells of the stratum granulosum, which are more highly keratinized and are beginning to exhibit nuclear degeneration, are unstained with the anti-34-kD pro-

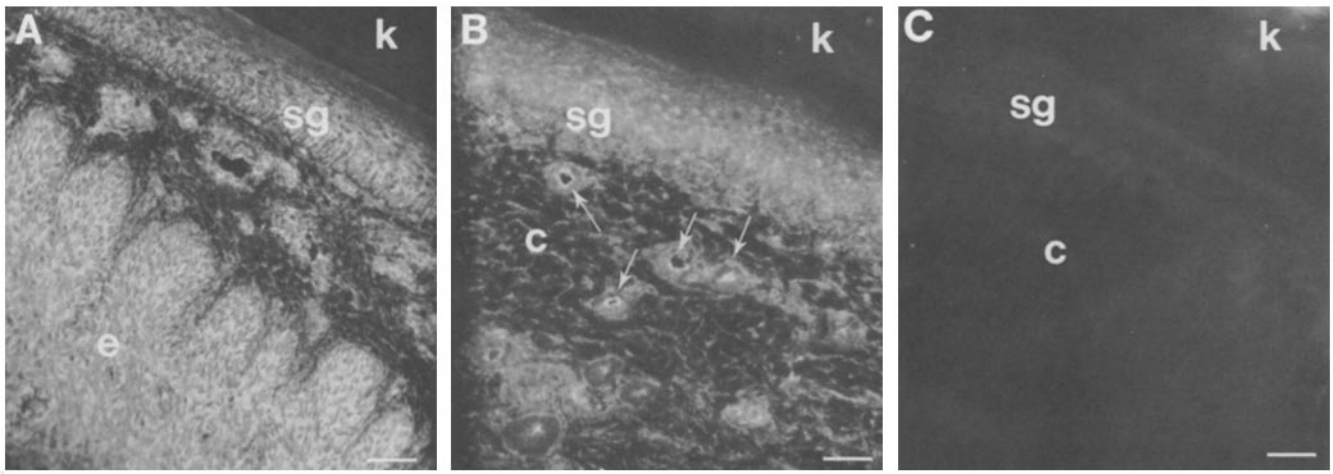


FIGURE 2 Localization of the 34-kD protein in adult chicken skin by immunofluorescence staining of thin frozen sections. 4-μm transverse frozen sections of chicken tongue (*A*), or footpad (*B*), were stained with the monoclonal anti-34-kD protein antibody as described in Materials and Methods. Footpad stained with a control monoclonal antibody is shown in C. Bright staining was observed in epithelial cells of the stratum germinativum (*sg*) and thick dorsal squamous epithelium of the tongue (*e*). The staining appeared to be concentrated along the edges of the epithelial cells. Arrows indicate endothelial cells of blood vessels that were also brightly stained. The connective tissue of the dermis is indicated (*c*), the keratinized layer (*k*). Bars, 50 μm. × 134.

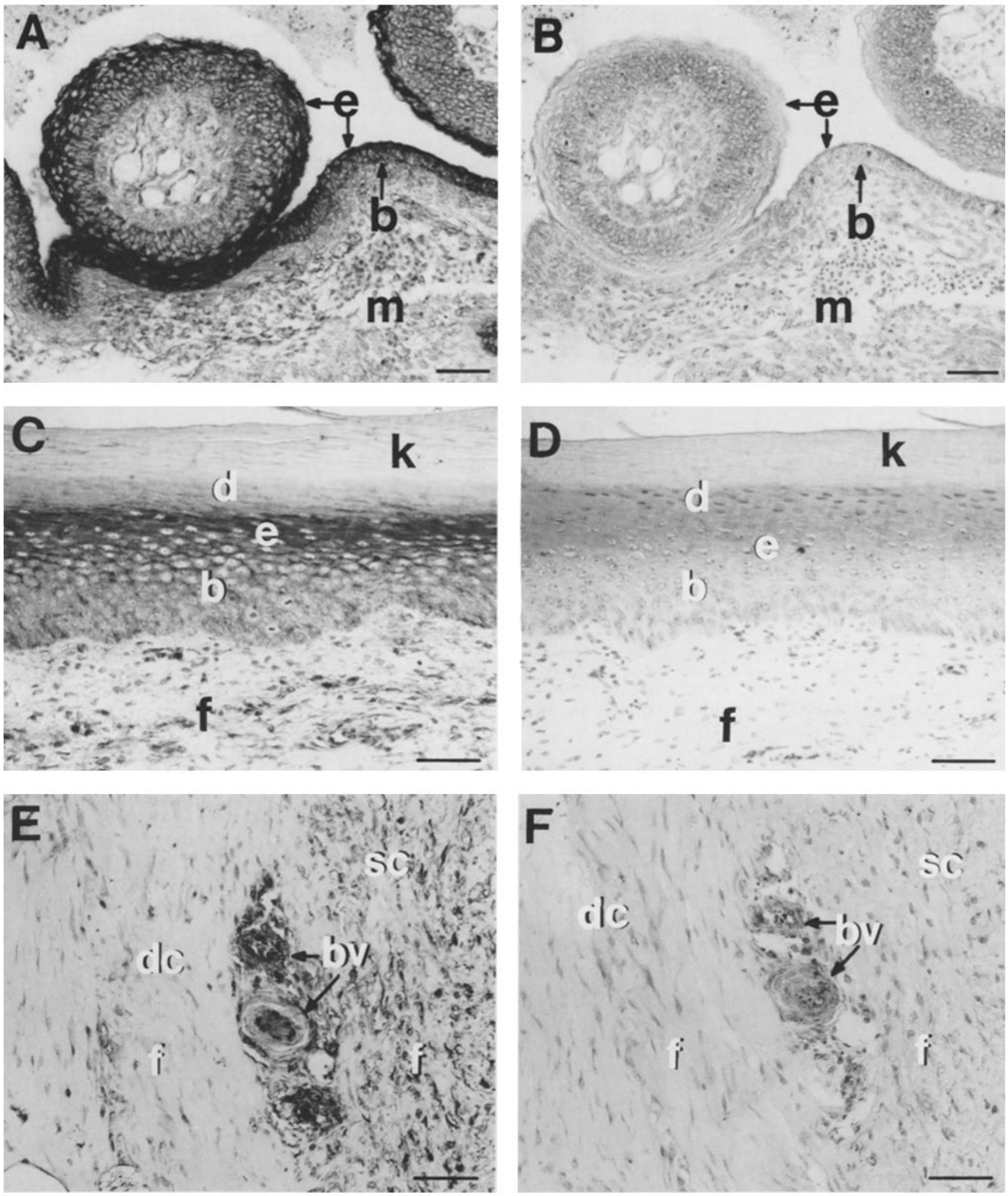


FIGURE 3 The presence of the 34-kD protein in embryonic and adult chicken skin visualized by the peroxidase antiperoxidase staining method. Staining with rabbit anti-34-kD protein antibody (*A*, *C*, and *E*); with preimmune antibody (*B*, *D*, and *F*). Intense staining was seen in the superficial epithelial cell layer (*e*) of embryonic skin and feather rudiments (*A* and *B*). Lower levels of staining were detected in the basal epithelial cell layers (*b*). Mesenchymal cells (*m*) were not appreciably stained. Staining of a longitudinal section of adult tongue is shown in *C* and *D*. The basal epithelial layer (*b*) is lightly stained while the more mature epithelial cells (*e*) are intensely stained. Cells in the early stages of keratinization and nuclear degeneration (*d*) and the fully keratinized layer (*k*) are unstained. Significant staining of fibroblasts (*f*) of connective tissue was observed. Sections through an area of adult chicken comb that reveal variations in the intensity of staining of fibroblasts (*f*) are shown in *E* and *F*. Fibrocytes present in areas of dense collagen (*dc*) were frequently unstained, whereas fibroblasts in areas of sparse collagen (*sc*) or in the vicinity of blood vessels (*bv*) were intensely stained. In sections stained by the PAP method, positive staining was indicated by the presence of a brown diaminobenzidine reaction product. All sections were counterstained with Gill's hematoxylin. The difference between the brown reaction product and the blue counterstain, while not always dramatic in reproductions was readily apparent under bright field microscopic illumination. Bars, 50 μm. (*A* and *B*) × 180. (*C–F*) × 225.

tein antibody as is the outermost keratinized layer. When similar sections were incubated with control antibodies, only low levels of staining were detectable by either the PAP (Fig. 3, *B*, *D*, and *F*) or immunofluorescence (Fig. 2 *C*) techniques.

In addition to epithelial cells, the anti-34-kD protein antibody specifically stained fibroblasts present in the dermal connective tissue as well as endothelial cells of blood vessels (Figs. 2 *B* and 3, *E* and *F*). It is significant, however, that not all fibroblasts present in the dermis expressed detectable amounts of the 34-kD protein. Fibroblasts and possibly pericytes in the vicinity of blood vessels and in areas of connective tissue where collagen was sparse appeared to contain significant amounts of the 34-kD protein (Fig. 3, *E* and *F*) while fibrocytes in areas of dense collagen did not express appreciable amounts of this protein (Fig. 3, *E* and *F*). This variation in the level of the 34-kD protein in the fibroblasts present in different regions of connective tissue may reflect differences in the age and/or differentiation state of these cells.

The localization of the 34-kD protein was similar in embryonic and adult skin. When a section of embryonic skin (Fig. 3, *A* and *B*) was stained by the PAP method, the 34-kD protein was again concentrated in the epithelial cells of the epidermis. The basal layer of epithelial cells was lightly stained with the anti-34-kD protein antibody while the outer layer of squamous epithelium was intensely stained. A similar staining pattern was seen in the developing feather rudiments (Fig. 3, *A* and *B*). In contrast to the fibroblasts of the adult skin, the embryonic mesenchymal cells, which eventually give rise to connective tissue fibroblasts, did not contain detectable amounts of the 34-kD protein.

These initial findings indicated that in skin, the 34-kD protein was present in several different cell types, most notably epithelial cells, fibroblasts, and endothelial cells. To extend and generalize these observations, we carried out further experiments in which the tissues of a range of chicken organs were tested for their 34-kD protein content.

Localization of the 34-kD Protein in Cells of the Gastrointestinal and Respiratory Tracts

The distribution of the 34-kD protein was next surveyed in several organs that contain well developed epithelial cell layers. Fig. 4, *A* and *C*, show that substantial amounts of the 34-kD protein were present in the epithelial cells lining adult trachea and embryonic intestine, respectively. In the embryonic intestine (Fig. 4 *C*) the 34-kD protein was concentrated at the edges of the cuboidal epithelial cells lining the lumen, consistent with a plasma membrane localization for the 34-kD protein in these cells. The 34-kD protein was absent from the underlying mesenchyme that ultimately gives rise to smooth muscle and connective tissue. The anti-34-kD protein antibody staining of the adult intestine (Fig. 4 *B*) was somewhat different. The 34-kD protein was not detected in the epithelial cells of the crypts of Lieberkuhn except perhaps along the apical surface. Along the intestinal villi (not shown) the 34-kD protein content of the epithelial cells appeared to increase as cells migrated from the stem to the tip of the villi. The connective tissue, including the intestinal mucosa and the cores of the intestinal villi (Fig. 4 *B*), was brightly stained with the anti-34-kD protein antibody. Changes in the distribution of the 34-kD protein during development were also observed when embryonic and adult proventriculus (glandular stomach) were compared. In embryonic proventriculus (Fig. 5, *A* and *B*) the 34-kD protein was restricted to the epithelial cells lining the lumen; the epithelial cells of the developing tubular glands were not stained nor were the mesenchymal cells. In the adult proventriculus (Fig. 5, *C* and *D*), the anti-34-kD protein antibody primarily stained fibroblasts of connective tissue and the endothelial cells while the epithelial cells lining the lumen were unstained.

The distribution of the 34-kD protein in the intestine was different from that of another potential tyrosine kinase substrate, the cytoskeletal-associated protein, vinculin. The anti-34-kD protein antibody stained the connective tissue fibroblasts, epithelial cells of the villi, and endothelial cells of blood vessels, but did not stain smooth muscle cells (Fig. 4 *B*). In contrast, a monoclonal antibody to vinculin stained smooth muscle cells intensely, but stained intestinal epithelial and connective tissue to a limited extent (Fig. 4 *D*).

The location of the 34-kD protein in tissues of the respiratory tract was found to be similar to that seen in the digestive organs. In the adult trachea (Fig. 4 *A*) and lung (Fig. 5, *E–H*), significant anti-34-kD protein antibody staining of epithelial cells was detected. Several different cell-types present in adult trachea (Fig. 4 *A*) contained the 34-kD protein. The tracheal wall consists of a pseudostratified epithelial layer made up of ciliated, basal, and mucous secreting cells. All three types of epithelial cells exhibited intense edge staining with the 34-kD antibody by either the fluorescent or PAP methods. Quite notably, however, the ciliated epithelial cells were not stained along the cilia. The chondrocytes present in adult trachea were also found to contain significant amounts of the 34-kD protein. In addition, cells of the lamina propria, a highly vacularized connective tissue layer separating the epithelial and cartilage layers of the trachea, were found to contain substantial amounts of the 34-kD protein.

The distribution of the 34-kD tyrosine kinase substrate in adult lung is shown in Fig. 5, *E–H*. Fig. 5, *E* and *F*, shows the results of incubating a section of a tertiary bronchus with the anti-34-kD protein antibody or preimmune serum, respectively. The epithelial cells lining the airway are specifically stained. The epithelial cells of the parabronchial wall and the atria also contain the 34-kD protein as seen in Fig. 5 *G*. In addition, throughout the lung, a low level of specific staining of endothelial cells of blood capillaries and fibroblasts of connective tissue was also detected.

Survey of Tissues That Contained Low or Nondetectable Amounts of the 34-kD Protein

The apparent 34-kD protein content of epithelial cells varied considerably among different chicken tissues. Several organs involved in either secretion or absorption were observed to have little or no 34-kD protein in their epithelial cells. The 34-kD protein was not detected in the cells lining the proximal, distal, or collecting tubules of the adult kidney, but was observed to be present in the endothelial cells of the glomerulus (Fig. 6 *C*). The 34-kD protein was also found in significant amounts at the basal aspect of the transitional epithelial cells lining the ureter (Fig. 6, *A* and *B*). The acinar cells of the exocrine pancreas and the islet cells of the endocrine pancreas (Fig. 6, *E* and *F*) did not contain significant amounts of the 34-kD protein. Moreover, we were unable to detect the 34-kD protein in hepatocytes (Fig. 6, *G* and *H*). The 34-kD protein detected in kidney, pancreas, and liver by immunoblotting (Fig. 1) appears to be restricted to the endo-

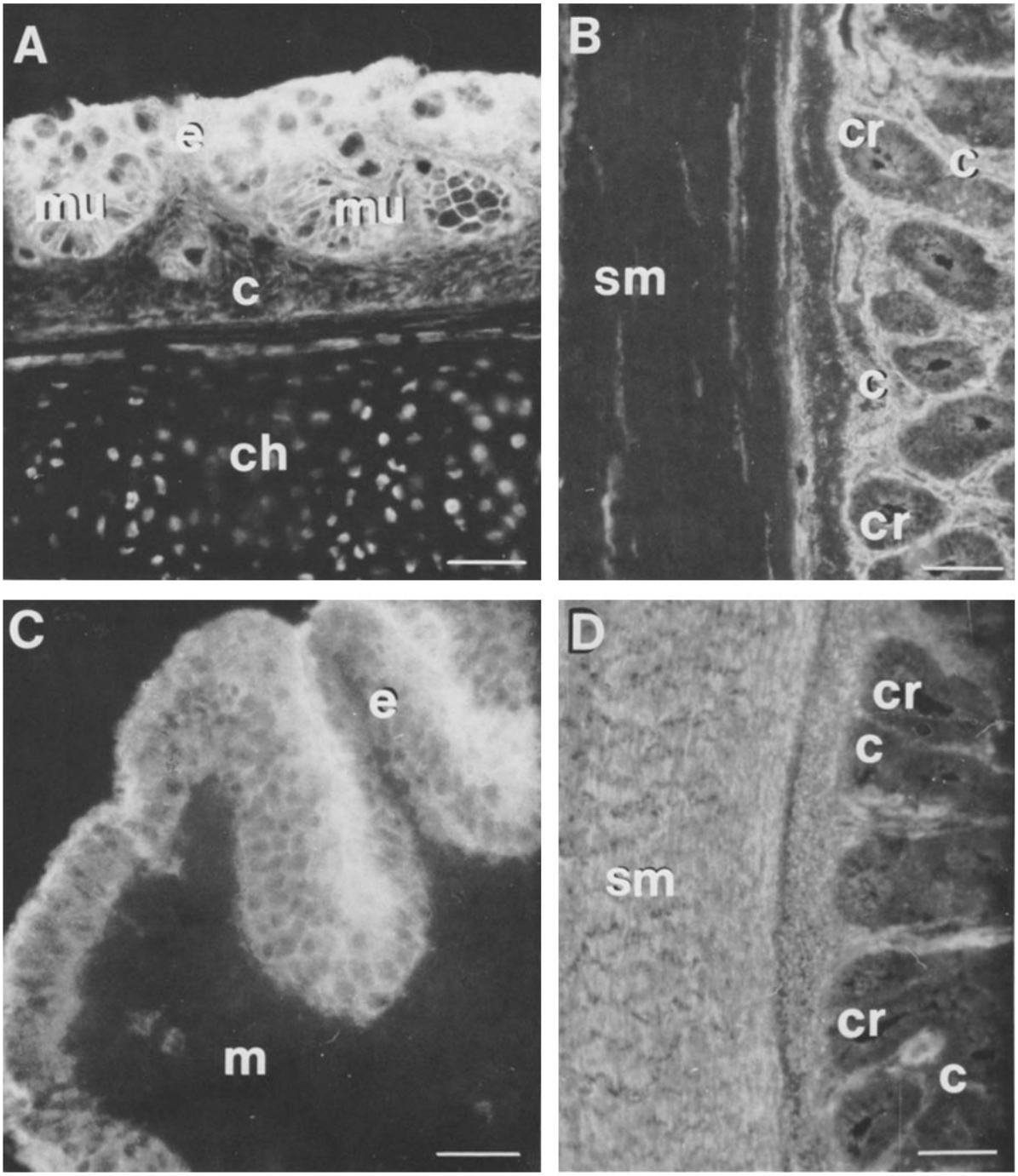


FIGURE 4 Immunofluorescence localization of pp60^{src} substrates in trachea and intestine. Sections incubated with monoclonal anti-34-kD protein antibody, (*A*, *B*, and *C*); with monoclonal antivinculin antibody (*D*). In the adult trachea (*A*), significant staining of epithelial cells (e) mucous secreting epithelial cells (*mu*), connective tissue (*c*), and chondrocytes (*ch*) was observed. The localization of the 34-kD protein and vinculin in adult intestine is compared in *B* and *D*. *sm*, smooth muscle; *cr*, crypts of Lieberkuhn; *c*, vascularized connective tissue. A section of embryonic intestine is shown (C). *e*, cuboidal epithelial cells lining intestinal lumen; *m*, mesenchymal precursors to smooth muscle and connective tissue. Bars, 50 μm. × 246.

thelial cells that line the blood vessels and to the fibroblasts present in the connective tissue of these organs.

The 34-kD protein was also absent from most neural cells examined. For example, in cerebellum (Fig. 7, *E* and *F*), cells of the granular and Purkinje layers and fibers of the molecular layer contained little, if any, 34-kD protein. However, some localized regions in the brainstem (Fig. 7, *E* and *F*) were enriched in the 34-kD protein. This protein was also found in large ganglion neurons of the embryonic dorsal root ganglion (Fig. 7, *C* and *D*). As in other tissues, endothelial cells of blood vessels and fibroblasts in the meninges contained substantial amounts of this protein.

Muscle cells of the various organs from 13-d-old embryonic or adult chickens were not stained with the anti-34-kD protein antibody. Immunofluorescence staining of thin sections of cardiac muscle, skeletal muscle of the breast, and intestinal smooth muscle incubated with the anti-34-kD monoclonal antibody are shown in Fig. 7, *A* and *B*, and Fig. 4*B* respec-

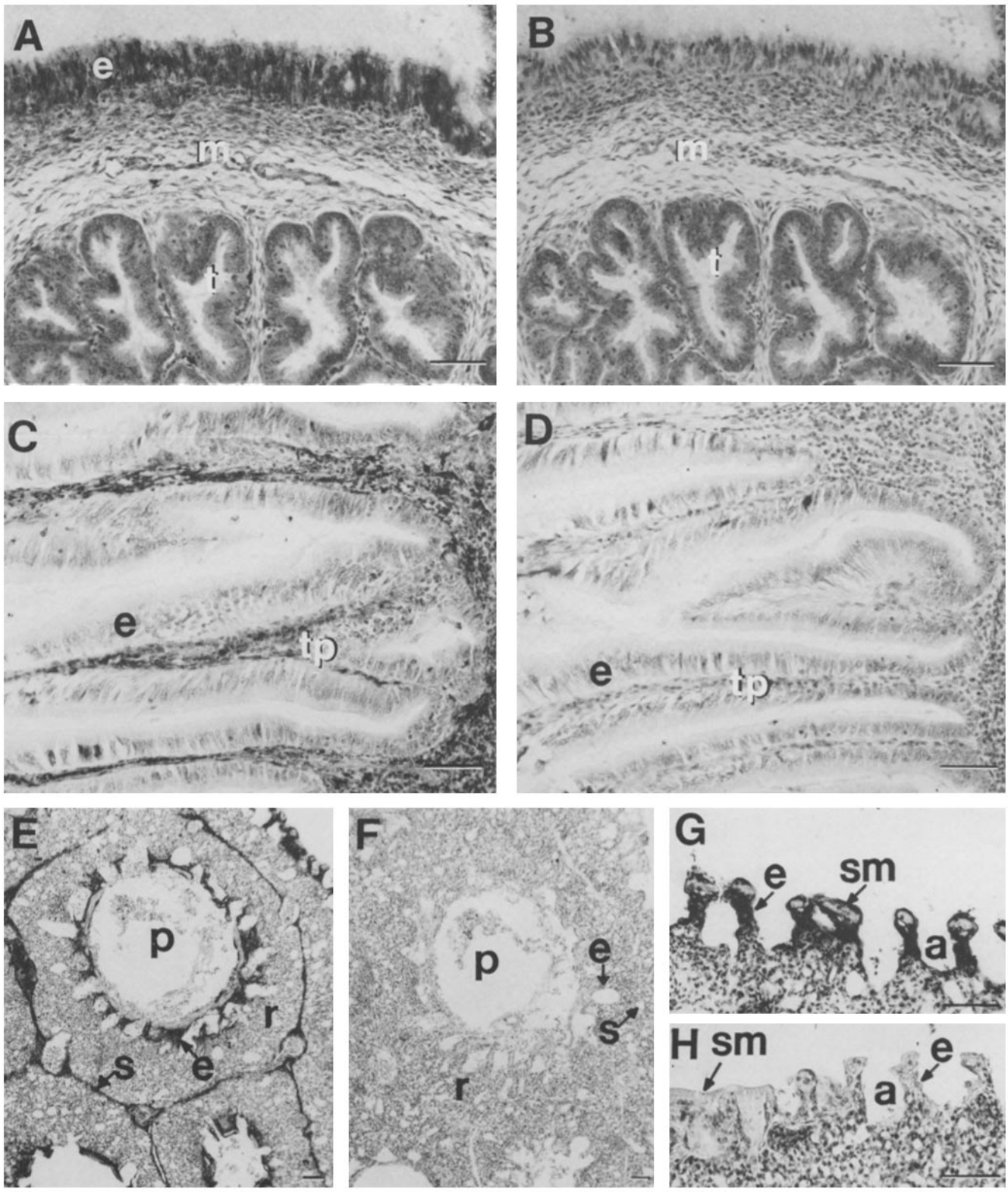


FIGURE 5 PAP localization of the 34-kD protein in gastrointestinal and respiratory tracts. Staining with rabbit anti-34-kD protein antibody (*A*, *C*, *E*, and *G*); preimmune antibody (*B*, *D*, *F*, and *H*). Embryonic proventriculus, (*A* and *B*). Lumenal epithelial cells (*e*) are intensely stained. *m*, mesenchymal cells; *t*, epithelial cells of tubular glands are not stained, although the counter staining of the sections with hematoxylin yields the dark color seen in the control panel. (C and *D*) Adult proventriculus. Lamina propria (*tp*) forming the cores of the villi is positively stained; the mucosal epithelial cells (*e*) lining the lumen are unstained. (*E* and *F*) Sections of adult lung showing one complete lobule and parts of others. Epithelial cells (*e*) lining the parabronchus (*p*) and vascularized connective tissue of the interlobular septum (*s*) are also stained. *r*, respiratory and blood capillaries. High magnification view of cells lining the parabronchus. Epithelial cells (*e*) lining the atria (*a*) are stained. Smooth muscular layer of the parabronchus (*sm*) is unstained. (*r*) Respiratory and blood capillaries. Bar, 50 μm. (*A–D*, *G*, and *H*) × 200. (*E* and *F*) × 66.

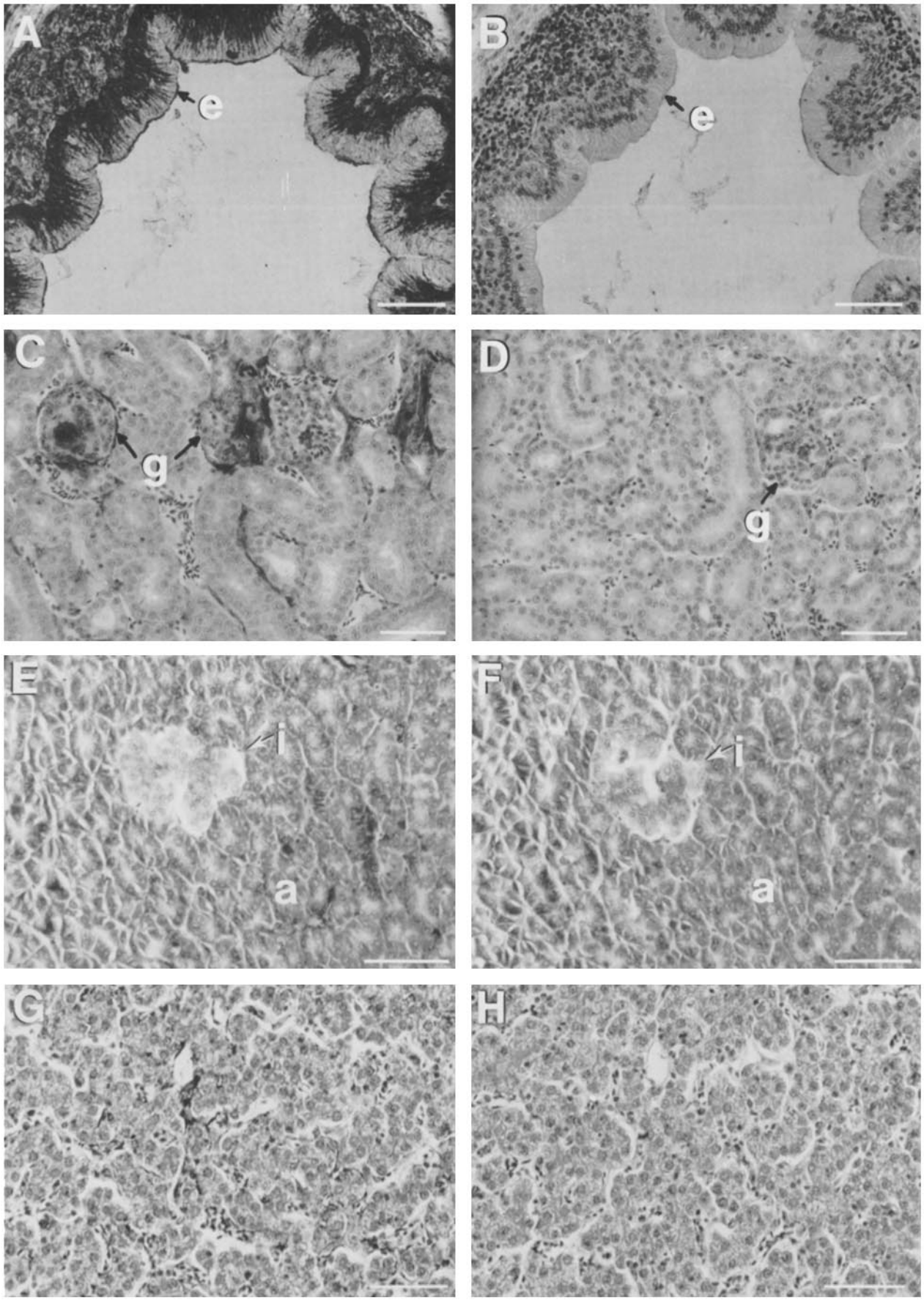


FIGURE 6 34-kD-protein content in cells of urinary tract, kidney, pancreas and liver. Paraffin embedded tissue sections were incubated with the rabbit anti-34-kD protein antibody (*A*, *C*, *E*, and *G*) or preimmune rabbit antibody (*B*, *D*, *F*, and *H*) and stained by the PAP technique. The transitional epithelial cells (e) of the urinary tract were intensely stained along their basal surface (*A*). Cells of the proximal and distal tubules of the kidney (*C*) were not stained although the endothelial cells of the glomerulus (g) were positively stained. Neither the acinar cells (a) or islet cells (*i*) of the pancreas (*E* and *F*) were detectably stained nor were the hepatocytes of the liver (*G* and *H*). Bars, 50 μm. (*A–D*) × 242. (*E–H*) × 300.

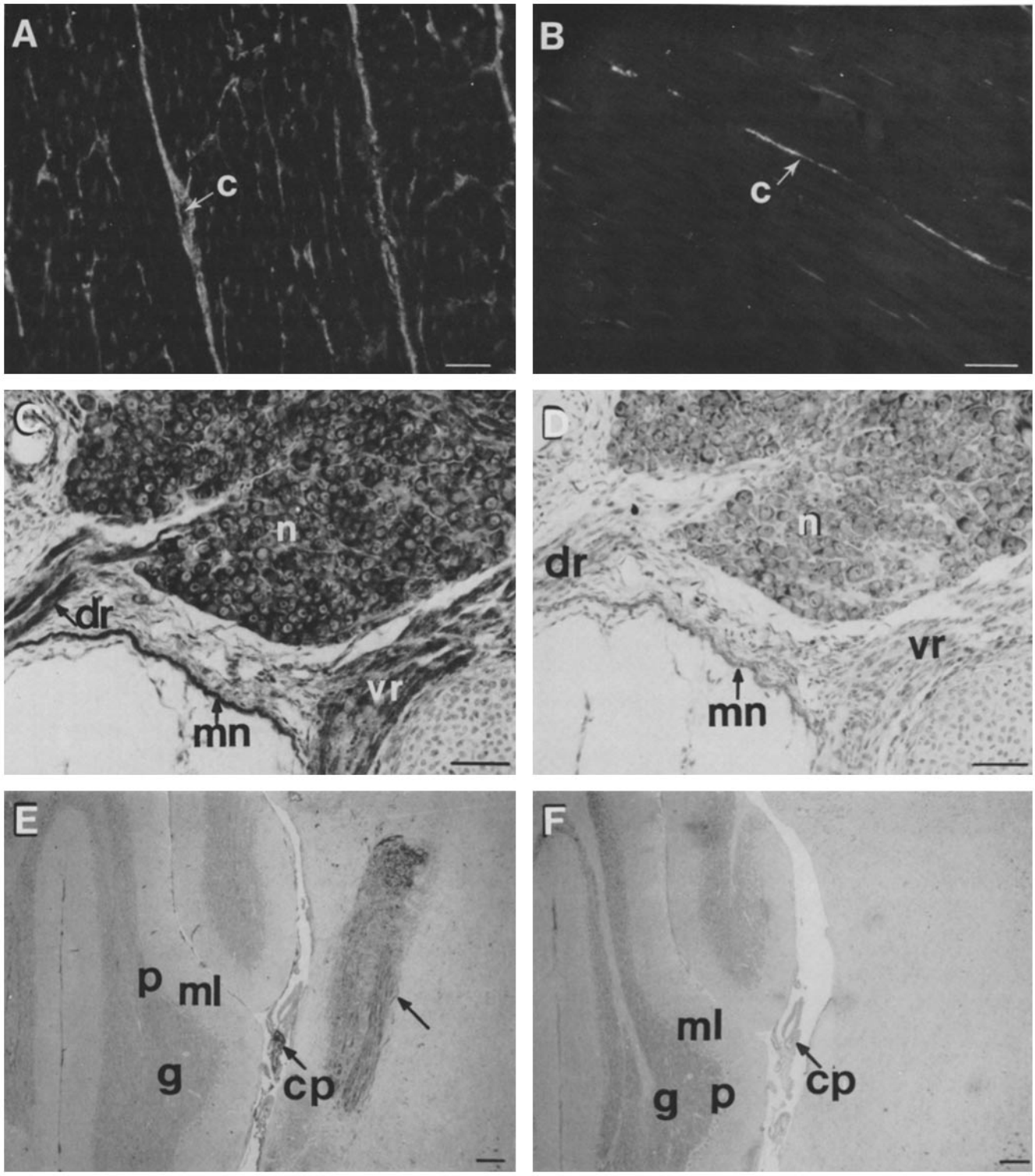


FIGURE 7 Localization of the 34-kD protein in muscle and nerve tissue by immunofluorescence or PAP staining techniques. Frozen sections of adult cardiac muscle (*A*) or breast skeletal muscle (*B*) were stained with the monoclonal anti-34-kD protein antibody and fluorescent rabbit anti-mouse IgG. Paraffin sections of 13-d-old embryonic dorsal root ganglion (*C* and *D*) and saggital sections of adult cerebellum and brain stem (*E* and *F*) were incubated with rabbit antibodies to the 34-kD protein (*C* and *E*) or preimmune antibodies (*D* and *F*) and visualized by the PAP method. In both muscle sections, only the vascularized connective tissue (*c*) was stained; the cardiac muscle tissue and skeletal muscle tissue were unstained. In the embryonic dorsal root ganglion, large ganglion neurons (*n*) and fibers of the dorsal root (*dr*) and ventral root (*vr*) were intensely stained. Membranes enclosing the spinal cord (*mn*) were also positively stained. In adult cerebellum, the granule cells (*g*), Purkinje cells (*p*), and fibers of the molecular layer (*ml*) were not stained. However, one region of the brainstem (arrow) stained intensely. In addition, parts of the choroid plexus (*cp*) were stained by the anti-34-kD protein antibodies. (*A* and *B*) Bar, 50 μm. × 177. (*C* and *D*) Bar, 50 μm. × 221. (*E* and *F*) Bar, 200 μm. × 28.

tively. In all three organs, only the connective tissue stained brightly.

Changes in 34-kD Protein Distribution during Differentiation and Maturation

In a number of tissues the expression of the 34-kD protein varied during the development of the chicken. Notable examples are the decrease in the amount of the 34-kD protein during maturation of the luminal epithelial cells of the proventriculus (Fig. 5, *A–D*) and intestine (Fig. 4, *B* and *C*) and the appearance of the 34-kD protein during the differentiation of epithelial and connective tissue of skin (Fig. 3, *A–D*) and connective tissue of the gastrointestinal tract (Figs. 4, *B* and *C* and 5, *A–D*).

Fig. 8 summarizes changes in the expression of the 34-kD protein in embryonic and adult chickens as mesenchymal cells differentiate first into chondroblasts and then into chondrocytes. Embryonic mesenchymal cells do not express the 34-kD protein (Figs. 3, *A* and *B*, 4*C*, 5, *A* and *B*, and 8, *A* and *B*) but this protein was detected in chondroblasts of the perichondrium in both embryonic and adult cartilage (Fig. 8, *A–D*). The 34-kD protein was not detectable in embryonic chondrocytes (Fig. 8, *A* and *B*). In contrast, the chondrocytes present in cartilage of the adult trachea (Figs. 4*A* and 8, *C*

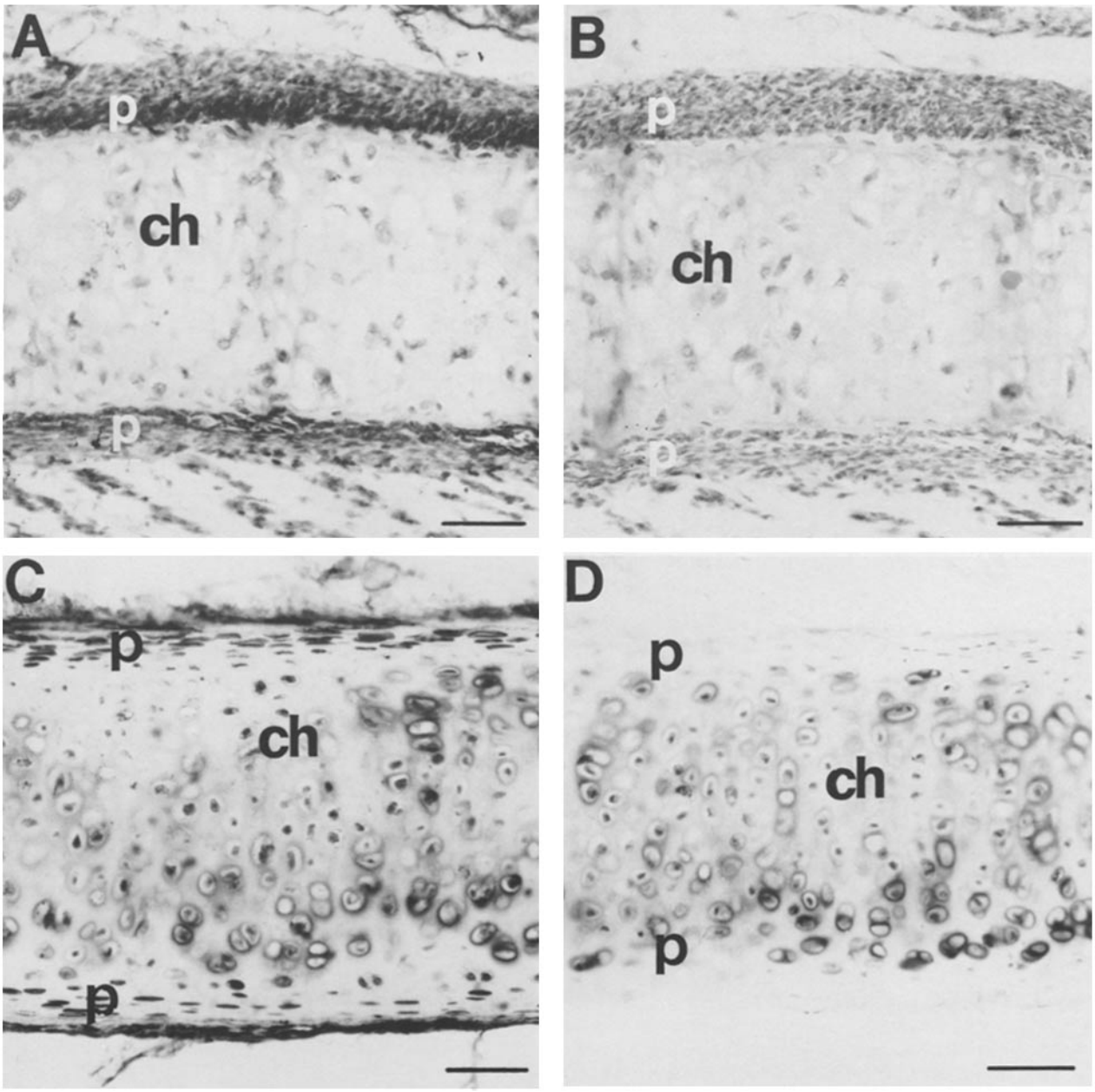


FIGURE 8 Specific staining of the 34-kD protein in adult and embryonic cartilage. Staining with rabbit antibodies to the 34-kD protein (*A* and *C*); preimmune antibodies (*B* and *D*) visualized by the PAP technique. Embryonic cartilage (*A* and *B*) showing specific staining of perichondrium (*p*). Embryonic chondrocytes (*ch*) are unstained. Adult tracheal cartilage (*C* and *D*). Chondroblasts of the perichondrium (*p*) were intensely stained. Adult chondrocytes (*ch*) were specifically stained; they contained brown reaction product, in contrast to chondrocytes in the control section which displayed only the blue hematoxylin stain. Bars, 50 μm. × 266.

and *D*) and tongue (not shown) stained brightly with the anti-34-kD protein antibody.

Localization of the 34-kD Protein in Lymphoid Organs

A subpopulation of the cells present in spleen, bone marrow, bursa, and thymus were positive for anti-34-kD protein antibody staining (Fig. 9). In the spleen the small lymphocytes and erythrocytes were not stained with the antibodies but positive staining was observed in a small number of cells (Fig. 9*A*). On the basis of their large size and lightly stained nucleoli in PAP-stained sections these cells were identified as macrophages. In addition, the capsule enclosing the spleen, which is composed primarily of connective tissue, was observed to stain brightly with the anti-34-kD protein antibody (Fig. 9*A*).

The 34-kD protein content of the bursa (Fig. 9, *D* and *E*) was also investigated. The columnar epithelial cells lining the lumen of the bursa (not shown) expressed little or no 34-kD protein, except for the epithelial tuft cells of the bursal follicles, which were observed to stain with the anti-34-kD protein antibody. None of the lymphocytes present in the bursa contained the 34-kD protein. The stromal elements, however, including the connective tissue framework surrounding the follicles and the fine network of capillaries separating the bursal cortex and medulla were intensely stained (Fig. 9, *D* and *E*). Endodermal support cells in the bursal medulla, which were identified by virtue of their dendritic extensions visible under high magnification, were also positively stained with the anti-34-kD protein antibody (Fig. 9, *D* and *E*).

Additional experiments were carried out to analyze the cells of the lymphatic and blood systems for their 34-kD protein content. In many adult and embryonic tissues, erythrocytes and lymphocytes that were present within blood vessels were not detectably stained with the anti-34-kD protein antibody. Blood platelets were also found to be devoid of the 34-kD protein. An analysis of bone marrow from a 1-wk-old chicken (Fig. 9, *B* and *C*) established that a subpopulation of the bone marrow cells were positive for the 34-kD protein. On the basis of their morphology the positively stained cells were determined to be of the granulocytic lineage and also included precursors to monocytes and macrophages. The cells of the erythroid series were negative.

A subpopulation of cells present in both the medulla and cortex of the adult thymus was also stained by the antibodies to the 34-kD protein (Fig. 9, *F* and *G*). These large cells contained prominent nucleoli but were distinguished from blast cells by their dendritic extensions and the absence of mitotic figures. On this basis these cells were identified as epithelial support cells. The reticular epithelial cells, which form the septa of the thymus, were also positive for the 34-kD protein (not shown). Small lymphocytes were unstained with the anti-34-kD protein antibody.

Expression of 34-kD Protein by Cells in Culture

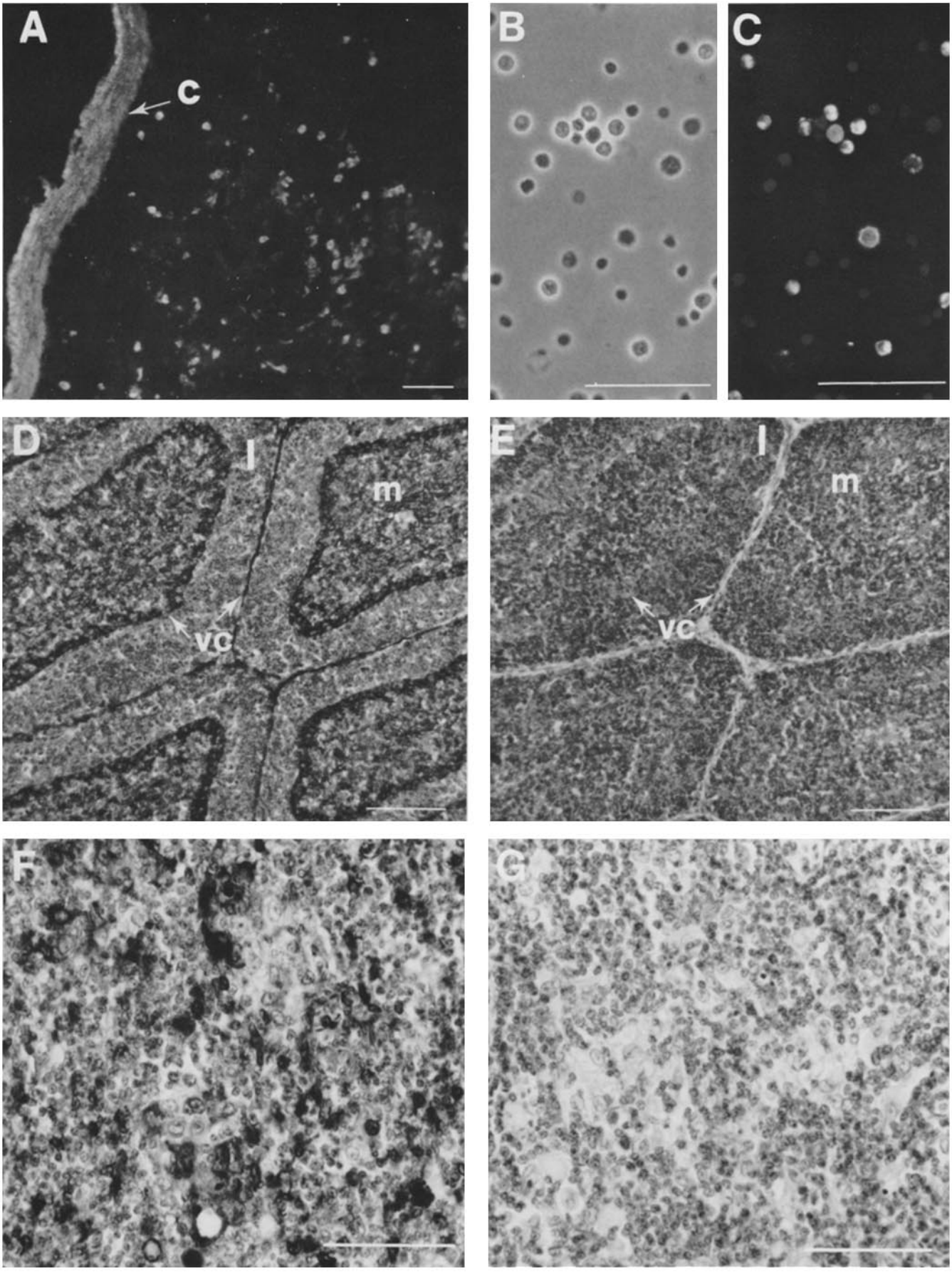
A summary of the 34-kD protein distribution in additional tissues and cells is outlined in Table I along with a summary of its content in a range of cells in culture, including primary cells and established cell lines. Except for neuronal cells, the 34-kD tyrosine kinase substrate was detected using the rabbit anti-34-kD protein antibody in all of the tissue culture cells studied, including those of rodent and human origin. For example, non-neuronal cells from chicken retina, believed to be of glial origin, and fused myotubes or myoblasts in culture (see also reference 37) both stained brightly with the anti-34-kD protein monoclonal antibody. This observation was unexpected since muscle and glial cells in vivo did not contain detectable amounts of this protein and it suggests that the 34-kD protein expression is turned on when these cells are grown in tissue culture. Normal neuronal cells in culture do not express the 34-kD protein, however retinal cells transformed by Rous sarcoma virus and several Rous sarcoma virus–transformed cerebellar cell lines (38) were found to contain significant quantities of this protein. It appears, in the chicken retinal cell system, that expression of $pp60^{src}$ results in the appearance of the 34-kD protein early in transformation (39).

DISCUSSION

The present study has established that the 34-kD tyrosine kinase substrate is present in a wide range of tissues in the embryonic and adult chicken (summarized in Table II). Although the 34-kD protein was not present in all cells, it was found in many cell types of the body including fibroblasts, epithelial cells, endothelial cells of blood vessels, and cells of cartilage. It was not detected in any of the three types of muscle nor, with one exception, in adult nervous tissue. In some instances the distribution was dynamic, the 34-kD protein being present during specific differentiation or maturation states of a cell and absent during others.

Although the detailed tissue specificity of the 34-kD protein was not easily categorized and did not compellingly suggest a particular function for the protein, the distribution observed does allow several significant conclusions to be drawn. One conclusion is that the function of the 34-kD protein in normal cells is more related to differentiation events than to those of the cell cycle. The identification of tissues that did not contain the 34-kD protein suggested that it is not an essential component of the metabolic processes of all cells. Moreover, its presence is not an absolute requirement for the processes of cell growth and division, secretion, protein synthesis, transport within a cell and across membranes, or formation of cell-cell junctions since many cells that undergo these processes do not contain detectable amounts of the 34-kD protein. The 34-kD protein was undetectable in some embryonic tissues such as retina at ages when cell growth and division are known to be occurring. Moreover, in various tissues, some cells that exhibited mitotic figures after histological processing were devoid of the 34-kD protein. Many cells that are actively involved in secretion such as those of the liver, adrenals, kidney, and pancreas do not contain detectable amounts of the 34-kD protein. In addition, cells that are known to form intercellular junctions and adhere tightly to each other, including lens epithelia and a variety of glandular epithelia, do not synthesize this protein.

When evaluating the functional significance of the 34-kD protein distribution, an important consideration was whether the methods used were capable of detecting the protein in all of the tissues examined. It is necessary that the 34-kD protein be well preserved during the fixation and processing of each of the tissues studied. This appeared to be the case, since two very different procedures, immunofluorescence staining of frozen tissue sections, and PAP staining of fixed paraffin-embedded tissue sections, gave virtually identical results. Significantly, those tissues that contained the 34-kD protein, as determined by immunoblotting of boiled SDS extracts, were always found to be positive in the staining procedures. One


A
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B
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TABLE II

Summary of the Tissue Distribution of the 34-kD Tyrosine Kinase Substrate

	34-kD protein present*	34-kD protein absent*
Epithelial cells:	Gastrointestinal tract: esophagus, proventriculus, gizzard, intestine	Liver
	Respiratory tract: trachea; 1°, 2°, 3° bronchi	Kidney
	Urinary tract	Pancreas
	Skin	Adrenal gland
		Adult sperm and lens
Connective tissue:	Fibroblasts	Embryonic mesenchyme
	Adult chondrocytes	
	Chondroblasts	Embryonic chondrocytes
	Perichondrium	
	Adipose tissue	
Blood and lymphoid systems:	Endothelial cells	Erythrocytes
	Stromal elements of newborn chick bursa and adult thymus	Adult platelets
		B and T lymphocytes
	Macrophages and capsule of adult spleen	
	Subpopulation of bone marrow cells from newborn chick	
Muscle:	—	Cardiac, skeletal, smooth
Neural tissue:	Embryonic dorsal root ganglion	Cerebellum
	Localized region of brainstem	Retina

* The 34-kD protein content was determined for both 13-d-old embryonic and adult tissues except as specifically indicated.

possibility that cannot yet be excluded is that tissues that appeared to lack the 34-kD protein might contain other forms of this protein that are not recognized by the monoclonal and polyclonal antibodies used in this study. It is also possible that in cells where it is not present the function of the 34-kD protein is performed by a different protein altogether.

The presence of the 34-kD protein in a variety of different tissues of the chicken allows additional conclusions to be drawn. While the expression of this protein was not strictly correlated with cell division, many of the quiescent cells that contained significant quantities of 34-kD protein retain the capacity to divide or migrate, often in response to injury. This is true of the fibroblasts and epithelial cells of the skin and is also believed to be the case for the endothelial cells of the blood vessels at least during the development of the embryo (40). Some cells, such as hepatocytes, that respond to injury by cell division and migration did not express the 34-kD protein. It would be useful to ascertain whether the 34-kD protein appears when liver cells are activated to divide and migrate during regeneration.

A particularly interesting aspect of the 34-kD protein distribution is its transient expression during the differentiation and maturation of particular cell types. This was exhibited dramatically in the epithelial cells of the skin, but was also observed in epithelial cells of the gastrointestinal tract, in fibroblasts of connective tissue and during the development of perichondrium and maturation of chondroblasts. In light of these observations, it might be informative to examine the localization of the 34-kD protein during the induction and differentiation events of early embryogenesis.

It would also be useful to determine the level at which expression of the 34-kD protein is controlled. The fact that some cells are completely devoid of this protein while other cell types contain high levels of the 34-kD protein makes it likely that its expression is regulated in part at the transcriptional level. The observation that expression of the 34-kD protein is turned on when neural retinal cells in culture are transformed by Rous sarcoma virus (39) should make it possible to develop a tissue culture system for studying the mechanisms involved in regulating 34-kD protein expression.

The differential expression of the 34-kD protein during development, the localization of this protein at the inner surface of the plasma membrane and the involvement of the 34-kD protein in the cells' response to growth factors or viral transformation, taken together raise the possibility that this protein may be involved in signaling at the cell surface. Further experiments are required to define clearly the function of the 34-kD protein, but it is intriguing to speculate that this protein might be involved in the transmission of differentiation or maturation signals across the plasma membrane. The phosphorylation of this protein at serine and tyrosine residues could alter its activity in normal cells during their differentiation and it would be worthwhile to determine the phosphorylation state of the 34-kD protein in various tissues at different stages of maturation. The present studies on its distribution provide a basis for this determination. They also support the idea that the activity of the 34-kD protein is regulated not only by phosphorylation but also by controlling its level of expression.

We gratefully acknowledge the excellent technical assistance of Ms. Mary Jo Przyborski and Ms. Mary Fedor and thank Mr. Steven Chin for histological services as well as many helpful discussions. We also wish to thank Dr. Malcolm Moore of the Sloan-Kettering Institute for his valuable assistance in the histological analysis of the lymphoid organs.

This work was supported by U.S. Public Health Service grants, AI-

FIGURE 9 34-kD protein localization in lymphoid organs. Frozen sections of adult spleen (*A*), cells isolated from leg bone marrow (*B* and *C*); and paraffin sections of bursa (*D* and *E*) or thymus (*F* and *G*) were incubated with monoclonal anti-34-kD protein antibodies (*A* and *C*) rabbit antibodies to the 34-kD protein (*D* and *F*) or preimmune rabbit antibodies (*E* and *G*). The presence of 34-kD protein was visualized by fluorescein-antibodies to mouse IgG (*A* and *C*), by the PAP technique (*D–G*), or by phase illumination (*B*). The capsule (c) of the spleen as well as isolated cells throughout the spleen were brightly stained as shown in *A*. The vast majority of small splenocytes were not stained. A subpopulation of cells from bone marrow (*C*) was positively stained. Endodermal support cells in the bursal medulla (*m*) were also intensely stained with the anti-34-kD protein antibody as were cells of vascularized connective tissue (*vc*) surrounding the bursal follicles and separating the bursal medulla and cortex. The quiescent lymphocytes (*l*) present in the cortex of the bursa were not stained. The small lymphocytes present in the thymus (*F* and *G*) were also unstained, however, a subpopulation of larger cells was intensely stained. Bars, 50 μm. (*A*) × 190. (*D* and *E*) × 294. (*B, C, F* and *C*) × 470.

11378 and AM-04256. M. E. Greenberg was a Biomedical Fellow of the Revson Foundation.

Received for publication 13 July 1983, and in revised form 20 October 1983.

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