Anion Transporter: Highly Cell-type-specific Expression of Distinct Polypeptides and Transcripts in Erythroid and Nonerythroid Cells

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ABSTRACT Affinity-purified antibodies and cDNA probes specific for the chicken erythrocyte anion transporter (also referred to as band 3) have been used to demonstrate that this protein is expressed in a highly cell-type-specific manner in the avian kidney. Indirect immunofluorescence analysis indicates that this polypeptide is present in only a small subset of total kidney cells and is predominantly localized to the proximal convoluted tubule of this organ. Chicken erythrocytes synthesize and accumulate two structurally and serologically related band 3 polypeptides. The polypeptide that accumulates in kidney membranes has an apparent molecular weight greater than either of its erythroid counterparts. This diversity is also reflected at the RNA level, as the single band 3 mRNA species detected during various stages of erythroid development is distinct in size from that found in kidney cells. Genomic DNA blot analysis suggests that both the erythroid and kidney band 3 RNAs arise from a single gene. Furthermore, of the adult tissues we have examined that are known to express ankyrin and spectrin polypeptides, only kidney accumulates detectable levels of the band 3 mRNA and polypeptide. These observations suggest that a subset of kidney cells use an anion transport mechanism analogous to that of erythrocytes and that band 3 is expressed in a noncoordinate manner with other components of the erythroid membrane skeleton in nonerythroid cells.

The anion transporter, also referred to as band 3, is the major integral membrane protein of mammalian erythrocytes, and is composed of a membrane domain and a cytoplasmic domain, each of which has a distinct function. The membrane-spanning domain mediates the anion transport property of band 3, which primarily exchanges internal HCO_3^- for external Cl^- (22). The cytoplasmic domain provides an attachment site for the erythrocyte membrane cytoskeleton through its interaction with ankyrin (3, 18) and possibly also protein 4.1 (30). In addition, hemoglobin (8) and the glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase compete for a common binding site on the intracellular domain (32, 45). Through anion transport and drug-binding studies molecules functionally analogous to mammalian erythrocyte band 3 have been identified in chicken erythrocytes (20). However, unlike the mammalian protein which migrates on SDS gels as a single diffuse band with an apparent molecular weight of 95,000, chicken band 3 is composed of two polypeptides with apparent molecular weights of ~100,000 and 105,000 (20). Furthermore, structural differences detected between human and chicken band 3 by one-dimensional peptide mapping are reflected in the loss of the glyceraldehyde-3-phosphate dehydrogenase binding site from the chicken molecule (20).

Physiological studies have demonstrated that anions are actively transported in a wide variety of tissues among higher vertebrates by an electrically silent Cl^--HCO_3^- exchange mechanism (12, 24, 38). However, the molecular details underlying this active transport process are understood only for the erythrocyte anion transporter. In this study, we have attempted to determine if molecules related to chicken erythrocyte band 3 exist in other avian tissues, and hence may be involved in the active transport of anions in cells other than erythrocytes.

Through the use of affinity-purified antibodies and a cDNA probe specific for chicken erythrocyte band 3, we demonstrate

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here that molecules related to band 3 are expressed in the avian kidney, but not in other adult nonerythroid tissues examined, some of which express nonerythroid forms of ankyrin (9, 27, 28), spectrin (4, 6, 13, 14, 33), and protein 4.1 (1, 15, 17). These probes have further revealed that band 3 diversity in erythrocytes and kidney occurs at both the protein and RNA levels, since the kidney band 3 RNA and band 3 polypeptide are different in size from their erythroid counterparts. Our data suggest that the distinct kidney and erythroid band 3 RNAs arise from a single gene. Furthermore, the highly tissue-specific expression of band 3 indicates that this gene is regulated noncoordinately with other genes for membrane cytoskeletal proteins (α-spectrin, β-spectrin, ankyrin, and protein 4.1) in a variety of nonerythroid cell types, suggesting that the membrane binding site(s) for these nonerythroid membrane skeleton polypeptides is not invariably band 3.

MATERIALS AND METHODS

Antiserum Production: Adult chicken erythrocyte plasma membranes were prepared as previously described (16), washed with H_2O to remove some peripheral membrane proteins (33), and the proteins were separated on several 12.5% SDS polyacrylamide slab gels. The band 3 region was cut from these gels and the gel slices were equilibrated in 125 mM Tris (pH 6.8), 1% β-mercaptoethanol and 0.1% NaN_3. The gel slices were then pooled, homogenized, brought to 1% in SDS, and re-electrophoresed on a 7.5-15% polyacrylamide gradient SDS gel. The center of the band 3 region was again excised from the gel, washed twice in 95% EtOH to remove acetic acid and once in phosphate-buffered saline (PBS). The gel slices were homogenized in PBS, emulsified with complete Freund's adjuvant, and injected subcutaneously into a New Zealand white rabbit in five locations. Booster injections containing complete Freund's adjuvant were administered 38 and 50 d after the initial injection. A final booster injection containing no Freund's adjuvant was given an additional 115 d later. Blood was collected 7 d after each booster injection. Antiserum from the bleed following the third booster injection was used for this study.

Gel Electrophoresis: SDS PAGE was performed according to the method of Laemmli (23) as previously modified and described (19).

Immunoblotting and Affinity Purification of Antibodies: Antibodies were affinity purified from nitrocellulose as described in detail elsewhere (29). Briefly, the protein from adult chicken erythrocyte plasma membranes was electrophoresed on a 7.5% SDS polyacrylamide slab gel and transferred to nitrocellulose essentially according to the method of Towbin et al. (39). Nitrocellulose filters were blocked for 1 h at 37°C in Tris-buffered saline (TBS)[1] that contained 0.25% gelatin. The filters were then incubated overnight with a 1:1,000 dilution of band 3 antisera in TBS containing 0.25% gelatin and washed subsequently in TBS that contained 0.05% Tween 20. Strips from these blots were incubated with a 1:1,000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 1 h and washed as above. The region corresponding to band 3 was localized by incubation in 0.02% (wt/vol) 4-chloro-1-naphthol in 6 ml methanol, 25 μl of 30% H_2O_2 and 94 ml TBS for 15 min. These strips were used as markers to cut the band 3 region from the remainder of the blot. Antibodies were eluted from these strips by incubation in 3 vol of 0.2 M glycine (pH 2.3) for 2 min on ice. The solution was neutralized by the addition of 1 vol of 1 M Tris base, and dialyzed against two changes of 200 vol of TBS. This antibody preparation was used directly for both immunoblotting and indirect immunofluorescence.

Immunoprecipitation: Erythroid cells from 10-d-old chicken embryos were isolated as previously described (16) and washed once in methionine-free minimal essential medium at room temperature. A 10% suspension of cells was incubated at 37°C in this medium, which contained 0.1 mCi/ml of [^{35}S]methionine (1,200–1,400 Ci/mmol, Amersham Corp., Arlington Heights, IL). At 10 or 30 min of incubation the cells were pelleted, washed once in methionine-free minimal essential medium, and then hypotonically lysed in 10 mM Tris (pH 7.5), 5 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged for 5 min in an Eppendorf centrifuge. The hypotonic pellet was resuspended in 150 mM NaCl, 0.1% (wt/vol) SDS, 1% (wt/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 20 mM Tris (pH 8), 5 mM EDTA and sonicated three times for 30 s each. Insoluble material was pelleted and discarded. Affinity-purified band 3 antibodies were added to the supernatant and incubated for 12 h at 4°C. Immune complexes were isolated with protein A-Sepharose beads and analyzed on a 12.5% SDS polyacrylamide gel. Fluorography was performed by incubation of the gel for 1 h in Enhance (New England Nuclear, Boston, MA) followed by a 1-h H_2O wash. The gel was dried and exposed to Kodak X-AR5 X-ray film for 36 h at −80°C.

Indirect Immunofluorescence: Tissue from a 3-mo-old chicken was frozen at −40°C in O.C.T. compound (Tissue Tek, Miles Scientific Div., Naperville, IL). 6-μm sections were cut with a cryotome, thawed onto glass coverslips, and fixed by incubation in 2% formaldehyde in TBS containing 0.5% Triton X-100 for 3 min at 22°C. The coverslips were then immersed in TBS that contained 0.5% Triton X-100 for 5 min before staining with affinity-purified band 3 antibodies for 1 h at 37°C. The coverslips were then washed with the same buffer as above and incubated with a 1:100 dilution of fluorescein-conjugated goat anti-rabbit IgG (Miles-Yeda, Israel) for 30 min at 37°C. Images were recorded through a 63x lens using a Leitz microscope equipped with epifluorescence optics.

Preparation of Purified Band 3 from Avian Erythrocytes: Adult chicken erythrocyte plasma membranes were prepared as previously described (16) and extracted with 0.1 N NaOH for 2 h at 0°C. The extract was centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant discarded. The pellet was washed once in 10 mM Tris (pH 8) and recentrifuged at 13,000 rpm for 15 min. This pellet was resuspended in 10 mM Tris (pH 8), 1 M KCl, 1% (wt/vol) Triton X-100, 5 mM EDTA, homogenized 10 times with a Dounce homogenizer, and incubated on ice for 4 h. The solution was centrifuged at 13,000 rpm for 15 min and the pellet discarded. The supernatant was then incubated with SM-2 Biobeads (Bio-Rad Laboratories, Richmond, CA) to remove the Triton X-100. This preparation was used for band 3 iodopeptide maps.

Preparation of Kidney Membranes: Kidneys from 3-mo-old chickens were perfused with TBS that contained 0.05% heparin. The kidneys were dissected free of surrounding blood vessels and homogenized 15 times in a Dounce homogenizer in 10 mM Tris (pH 7.5), 5 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was layered over a sucrose step gradient of 10% (wt/vol) and 40% (wt/vol) sucrose in the same buffer and centrifuged at 25,000 rpm for 6 h at 4°C in an SW41 rotor. The 10–40% sucrose interface was collected, brought up to 5 ml with lysis buffer, and centrifuged at 13,000 rpm in an SS-34 rotor for 1 h at 4°C. The resulting membrane pellet was used as a source of kidney membranes for immunoblotting.

Screening of a λgt11 cDNA Library: A previously characterized λgt11 cDNA library (25) was screened as described in detail elsewhere (25, 43). Overnight cultures of *Escherichia coli* strain Y1088 (44) were infected with recombinant λgt11 from the nonamplified M library (25) and 500,000 plaque-forming units were screened by filter hybridization as described (25) using the band 3 antibodies as a probe. The antibodies were preadsorbed with bacteria and phage and the antibody solution was diluted 1:1,500 before incubation with nitrocellulose filters for 2 h at 22°C. Filters were washed in TBS that contained 0.1% gelatin and 0.1% Tween 20 six times for 15 min each. ^{125}I-Protein A was added to 0.2–0.3 μCi/ml for 1 h at 22°C followed by six washes as above. Filters were air dried and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) overnight with an intensifying screen. All positive plaques were isolated and three of the initial 24 positives rescreened as positives.

Preparation of Fusion Protein: *E. coli* strain Y1089 (supF$^-$) were infected with the λgt11 recombinant clone with the largest cDNA insert. These cells were streaked on NZY plates that contained 50 μg/ml ampicillin and grown overnight at 32°C. Single colonies from these plates were replica plated on NZY plates that contained ampicillin and grown at 32°C and 42°C. Lysogens, which grew at 32°C and not at 42°C, were selected. One of these lysogens was grown up in 100 ml NZY media that contained ampicillin to an OD_{600} of ~0.5. The lysogens were then induced by incubation at 45°C for 20 min in a shaking H_2O bath. At this time, 5 mM IPTG (isopropyl-β-D-thiogalactopyranoside from Calbiochem-Behring Corp., San Diego, CA) was added to one half of the culture and the other half served as the control. Both were incubated for 1.5 h at 37°C. The cells were then pelleted by centrifugation. One-twenty-fifth of both the IPTG-treated and control samples were boiled directly in SDS sample buffer for analysis by immunoblotting. The remainder of each sample was made 5% (wt/vol) in ice cold TCA and incubated on ice for 30 min. The samples were spun for 5 min in an Eppendorf centrifuge and the pellet was washed two times in ice cold acetone and then lyophilized. The lyophilized pellets were boiled in 1 ml of SDS sample buffer and clarified by centrifugation. The supernatant of each sample was brought to 10 ml with 150 mM NaCl, 0.1% (wt/vol) SDS, 1% (wt/vol) Triton X-100, 1% (wt/vol) sodium deoxycho-

[1] *Abbreviations used in this paper:* IPTG, isopropyl-β-D-thiogalactopyranoside; TBS, Tris-buffered saline.

late, 20 mM Tris (pH 8), 5 mM EDTA to which ~1–2 μg of affinity-purified band 3 antibodies were added. Samples were incubated for 12 h at 4°C and immune complexes were isolated with protein A-Sepharose beads. One-twentieth of each sample was analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel which was silver stained; the remainder was used for two-dimensional peptide maps.

Two-Dimensional Peptide Maps: Two-dimensional peptide mapping was performed essentially according to the method of Elder et al. (11) with the following modifications. The band 3 immunoprecipitate from IPTG-treated lysogens, ~5 μg of erythrocyte band 3 (purified as described above), and ~1 μg β-galactosidase (Sigma Chemical Co., St. Louis, MO) were lyophilized and resuspended in 100 μl of 50 mM Tris (pH 8), and 1% SDS. These samples were iodinated with carrier-free ^{125}NaI (ICN K&K Laboratories Inc., Plainview, NY) using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IODO-GEN, Pierce Chemical Co., Rockford, IL), and electrophoresed on a 7.5% SDS polyacrylamide gel. Each of the three fusion peptides in the immunoprecipitate that were detected on the immunoblot of the IPTG-treated lysogens (see Fig. 5, lane *2*), the 100,000- and 105,000-mol-wt band 3 polypeptides and β-galactosidase, were excised from the gel. The gel slices of the three fusion peptides were equilibrated in Laemmli SDS sample buffer and re-electrophoresed on a 7.5% SDS polyacrylamide gel and again excised to insure the homogeneity of the sample. The gel slices were washed extensively in 0.5 M potassium iodide and equilibrated in 0.1 M ammonium bicarbonate. Samples were proteolyzed with TPCK-trypsin (Worthington Biochemical Corp., Freehold, NJ) for 24 h at 37°C. The peptides were spotted on thin layer cellulose plates (Eastman 13255, Eastman Kodak Co.) and separated in the first dimension by high voltage electrophoresis in 15% acetic acid and 5% formic acid and in the second dimension by chromatography in butanol:pyridine:acetic acid:H_2O (40.6:27:8.2:32.7). Autoradiography was performed for 2–3 d with intensifying screens using Kodak XAR-5 X-ray film.

Subcloning λgt11 Inserts into pBR322: cDNA inserts were obtained from recombinant λgt11 clones by digestion with *EcoRI*. Restriction fragments were isolated from low-melting point agarose gels (42), and ligated into the *EcoRI* site of pBR322. Recombinant plasmids were then used to transform *E. coli* strain HB101.

Restriction Endonuclease Mapping: Restriction endonuclease sites were determined by either single or double digests with various restriction endonucleases, and resolution of the resulting digestion products by electrophoresis on agarose gels.

Isolation of RNA: Kidneys from 3-mo-old chicks were perfused with TBS that contained 0.05% heparin and dissected. Chicken embryo erythroid cells were isolated as previously described (16). Cells or tissue sections were homogenized in a solution that contained 5 M guanidinium isothiocyanate, 50 mM Tris (pH 7.5), 50 mM EDTA, 5% β-mercaptoethanol, and 3% sodium lauryl sarcosine using a Dounce homogenizer. Homogenates were layered over a cushion of 5.7 M CsCl and centrifuged as previously described (7). Alternatively, erythroid RNA was obtained by extraction with phenol of postmitochondrial supernatants of erythroid cells from 14-d-old chicken embryos (35). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography essentially as described by Aviv and Leder (2).

DNA and RNA Blotting and Hybridization: Chicken liver DNA (kindly provided by Dr. Y. Capetanaki, California Institute of Technology) was digested with various restriction endonucleases, fractionated by electrophoresis on 0.9% agarose gels, and transferred to nitrocellulose (36). RNA electrophoresis and blotting was performed as previously described (7). ^{32}P-labeled nick-translated probes (34) were hybridized to nitrocellulose filters as described elsewhere (7).

RESULTS

Characterization of Band 3 Antibodies by Immunoblotting and Immunoprecipitation

Previous studies have shown that avian erythrocytes contain two polypeptides which may function as their anion transporter (20). The existence of these two polypeptides in avian erythroid membranes at steady state is shown in Fig. 1, lane *M*. Occasionally, both the 100,000- and 105,000-mol-wt bands are resolved on SDS gels as doublets (see Fig. 3, lanes *1*); since the basis for this apparent heterogeneity is unclear, we will hereafter refer to these bands as 100-kD and 105-kD polypeptides. To investigate the relationship between these polypeptides, antibodies were raised in rabbits against SDS gel–purified band 3 from chicken erythrocyte plasma membranes. As part of our characterization of this antiserum, we investigated the *de novo* synthesis of newly synthesized polypeptides that cross-react with these antibodies by labeling cells with [^{35}S]methionine, followed by immunoprecipitation, SDS gel electrophoresis, and fluorography. This revealed two immunoreactive [^{35}S]methionine-labeled species (Fig. 2, lane *2*) which co-migrate on SDS gels with the band 3 molecules present in a [^{35}S]methionine-labeled, hypotonic insoluble fraction (Fig. 2, lane *1*). Similar immunoprecipitates performed with preimmune sera were entirely negative (data not shown). The basis for the observed difference in relative abundance of the higher molecular weight polypeptide vs. the lower molecular weight polypeptide after [^{35}S]methionine labeling, as compared with steady-state levels, has not yet been determined. The antigenic relatedness of the band 3 molecules at steady state was determined by affinity-purification of antibodies (29) from the individual polypeptides in the following manner. Proteins from erythrocyte ghost membranes were separated on SDS polyacrylamide slab gels, transferred to nitrocellulose, and probed with whole band 3 antiserum. Strips from adjacent lanes on these blots were probed with peroxidase-conjugated goat anti-rabbit-IgG to localize the band 3 polypeptides. These strips were then used as markers to cut out the region from the remainder of the blot corresponding to the 100-kD polypeptide, the 105-kD polypeptide, or both. The antibodies were eluted from the nitrocellulose as described in Materials and Methods and used to reprobe

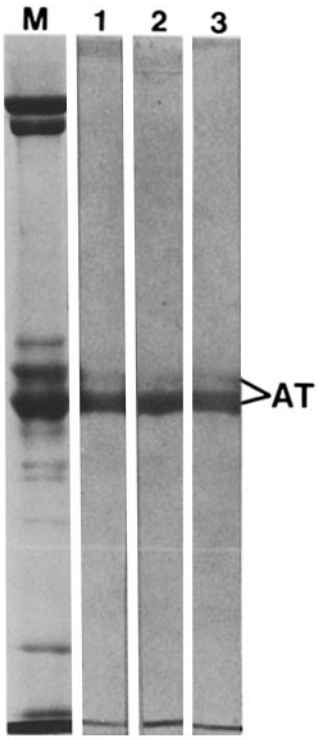

FIGURE 1 Characterization of affinity-purified band 3 antibodies. Adult chicken erythrocyte plasma membrane proteins were separated by electrophoresis on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose. Strips from this blot were probed with antibodies affinity purified (as described in the text) from (lane *1*) total band 3, (lane *2*) the 105-kD band 3 polypeptide, or (lane *3*) the 100-kD band 3 polypeptide, and the bands were visualized by immunoperoxidase staining. Lane *M* is a comparable loading of chicken erythrocyte plasma membranes stained with Coomassie Blue. *AT*, Anion transporter polypeptides (also referred to as band 3).

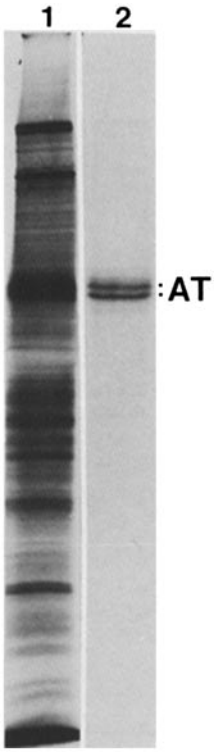

FIGURE 2 Immunoprecipitation of band 3 from chicken erythroid cells. 10-d-old embryonic erythroid cells were incubated in methionine-free minimal essential medium that contained 0.1 mCi/ml of [^{35}S]methionine for 30 min at 37°C, and then hypotonically lysed. The band 3 immunoprecipitated from the hypotonically insoluble fraction was analyzed by electrophoresis on a 12.5% SDS polyacrylamide gel. Lane *1*, Total [^{35}S]methionine-labeled protein from the hypotonically insoluble fraction; lane *2*, band 3 immunoprecipitate from the hypotonically insoluble fraction. *AT*, Anion transporter.

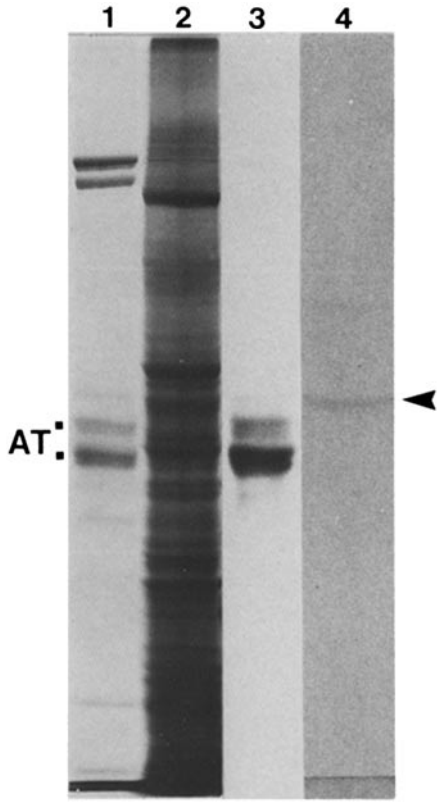

FIGURE 3 Immunoblot analysis of erythrocyte and kidney membranes with band 3 antibodies. Chicken erythrocyte plasma membranes (lane *1*) and purified chicken kidney membranes (lane *2*) were subjected to electrophoresis on a 7.5% SDS polyacrylamide gel and stained with Coomassie Blue. A duplicate unstained gel was transferred to nitrocellulose and probed with affinity-purified band 3 antibodies, and the immunoperoxidase staining patterns are shown for erythrocyte plasma membranes (lane *3*) and kidney membranes (lane *4*). Arrowhead marks the band 3–related polypeptide observed in kidney membranes. An additional faint band in the blot of the kidney membranes is due to reaction with the second antibody alone. *AT*, anion transporter.

blots of erythrocyte ghost membranes. As shown in Fig. 1, antibodies affinity purified from the 105-kD polypeptide (lane *2*) and the 100-kD polypeptide (lane *3*) recognize both molecular weight species and the immunostaining pattern for each is identical to that obtained with antibodies eluted from total band 3 (lane *1*). These results indicate that at least some of the antigenic determinants recognized by these antibodies are shared by the 100-kD and 105-kD polypeptides. This conclusion is further substantiated by two-dimensional peptide maps, presented below. Since the immunostaining patterns observed in Fig. 1 are the same regardless of the source of antibody, all subsequent experiments were performed with antibodies affinity purified from total band 3.

Identification and Localization of Band 3–related Polypeptides in Kidney

Although physiological studies have established that anions are actively transported in a variety of nonerythroid tissues (12, 24, 38), the molecular basis of this facilitated transport has remained obscure. To determine whether band 3–related polypeptides may be present, and hence potentially involved in these transport processes in nonerythroid cells, an immunoblotting analysis was undertaken using the band 3 antibodies as a probe. Examination of adult skeletal muscle, spinal cord, cerebellum, lens, and retina by this technique revealed these tissues to be negative for band 3 expression (data not shown). However, a band 3–related polypeptide was detected in kidney membranes (Fig. 3, lane *4*) isolated from discontinuous sucrose gradients as described in Materials and Methods. This polypeptide migrates slightly slower on SDS gels than the 105-kD band 3 polypeptide from erythrocytes and has an approximate molecular weight of 115,000. The signal obtained on the immunoblot of kidney membranes suggests that this polypeptide comprises a minor percentage of total membrane protein.

Indirect immunofluorescent staining of frozen sections of adult kidney has demonstrated that the band 3–related polypeptide is present in detectable amounts in 1% or less of total kidney cells. Although the highly cell-type–specific expression of this polypeptide is seen to occur randomly throughout the various regions of the kidney, it is primarily localized in the columnar type epithelium of the proximal convoluted tubule (Fig. 4, *A–D*). Even in this region of the kidney, adjacent cells may be either positive or negative for band 3 fluorescence, as illustrated in Fig. 4, *B* and *D*. Furthermore, based solely upon the levels of fluorescence intensity, the amount of band 3 present in these kidney epithelial cells on a per cell basis is much less than that found in erythrocytes (Fig. 4, *D* and *E*).

Isolation and Characterization of Band 3 cDNA Clones

The band 3 antibodies were used to screen λgt11 expression vector library M (25), constructed from gradient-fractionated poly(A)$^+$ RNA from chicken embryo erythroid cells. The purpose was to obtain cDNA probes that might reveal the molecular basis of band 3 diversity in erythroid cells, and to verify by a different experimental approach the existence of band 3 in kidney. Three putative band 3 cDNA clones were

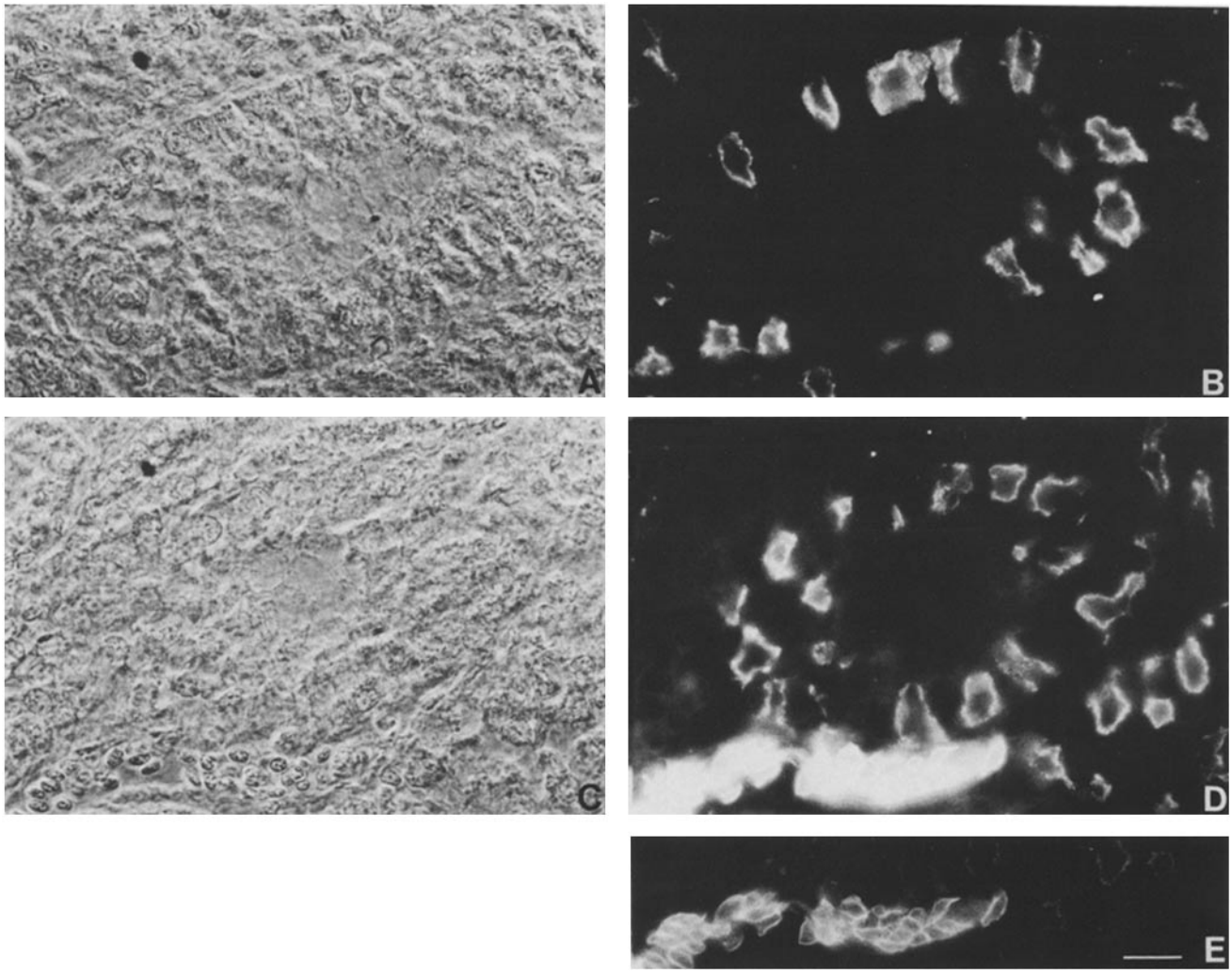

FIGURE 4 Indirect immunofluorescence staining of frozen sections of adult kidney. Frozen sections of adult kidney were stained with affinity-purified band 3 antibodies. (*A* and *C*) The corresponding phase-contrast images of *B* and *D*, respectively. The intensely fluorescent cells in the lower corner of *D* are erythrocytes. (*E*) A shorter exposure of the lower left hand corner of *D* demonstrating the different levels of band 3 fluorescence in kidney epithelial cells and erythrocytes. Bar, 12 μm.

selected as described in Materials and Methods. The largest of these λ clones, λ3(9-1), was shown to contain cDNA sequences that code for band 3 by comparing the two-dimensional peptide map of its β-galactosidase–band 3 fusion protein with the peptide maps of the 100-kD and 105-kD band 3 polypeptides from erythrocytes. The fusion protein was produced in bacterial cells lysogenically infected with λ3(9-1) as described in Materials and Methods. An immunoblot of the total protein from IPTG-treated and control cells probed with band 3 antibodies revealed three immunoreactive polypeptides in the IPTG-treated cells (Fig. 5, lane *2*) that were absent in the controls (Fig. 5, lane *1*). The largest of these three polypeptides has an approximate molecular weight of 160,000, of which ~40,000 is encoded by the cDNA insert. Band 3 immunoprecipitates were prepared from IPTG-treated and control cells identical to those used in Fig. 5. A portion of these immunoprecipitates was electrophoresed on an SDS polyacrylamide gel and silver stained. The same polypeptides detected on the immunoblot (Fig. 5) were also present in the immunoprecipitate from IPTG-treated cells whereas they were absent from the control cells (data not shown). The remainder of the immunoprecipitate from IPTG-treated cells, β-galactosidase prepared from *E. coli*, and native band 3 purified from erythrocytes (as described in Materials and

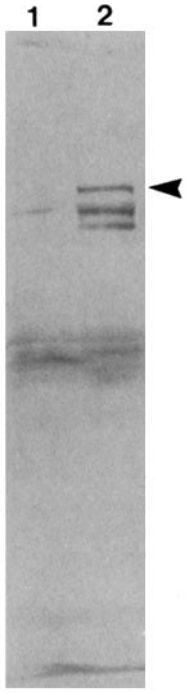

FIGURE 5 Immunoblot of lysogens with affinity-purified band 3 antibody. Total protein from bacterial cells lysogenically infected with λ3(9-1), which were grown in the absence (lane *1*) or presence (lane *2*) of 5 mM IPTG, was electrophoresed on a 7.5% SDS polyacrylamide gel, and transferred to nitrocellulose. The blot was incubated with affinity-purified band 3 antibodies and the immunoperoxidase staining pattern is shown. The arrowhead marks the largest of the β-galactosidase–band 3 fusion proteins whose peptide map is shown in Fig. 6*D*.

Methods) were iodinated and then electrophoresed separately on an SDS polyacrylamide gel. The regions of the gel corresponding to the three immunoreactive fusion peptides, β-galactosidase and the 100-kD and 105-kD erythrocyte band 3 polypeptides were excised and the polypeptides subjected to two-dimensional peptide mapping (11).

The initial feature to be noted from these peptide maps (Fig. 6) is that the 100-kD band 3 polypeptide (*B*) and the 105-kD band 3 polypeptide (*A*) yield very similar maps. Most of the major peptides are in common, with the exception of the two peptides (denoted by large arrowheads in *A*) which are unique to the 105-kD polypeptide. The structural similar-

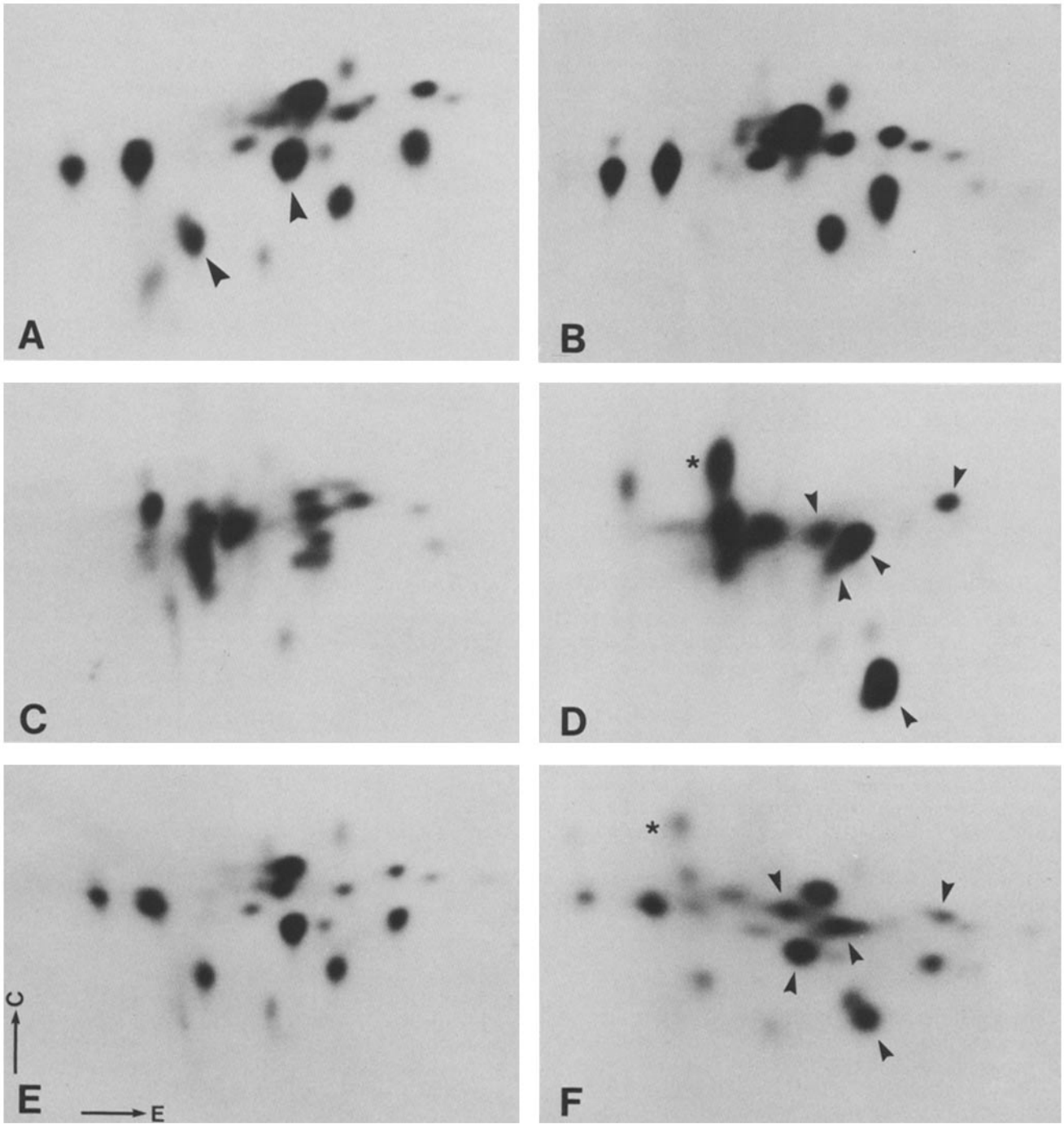

FIGURE 6 Two-dimensional tryptic peptide map comparisons. The largest β-galactosidase-band 3 fusion protein, purified erythrocyte band 3, and β-galactosidase were iodinated and digested with TPCK-trypsin as described in the Materials and Methods. These tryptic digests were separated by high voltage electrophoresis in the first dimension (*E*) and ascending chromatography in the second dimension (*C*). The samples are as follows: (*A*) 105-kD erythrocyte band 3 polypeptide; (*B*) 100-kD erythrocyte band 3 polypeptide; (*C*) β-galactosidase; (*D*) β-galactosidase-band 3 fusion protein; (*E*) 105-kD band 3 polypeptide; (*F*) mixture of 105-kD band 3 polypeptide and β-galactosidase-band 3 fusion protein. Large arrowheads in *A* mark those peptides in the 105-kD band 3 polypeptide not found in the 100-kD band 3 polypeptide. Small arrowheads in *D* and *F* mark those peptides that are found in the fusion protein and not in β-galactosidase, and in addition, co-migrate with peptides from the 105-kD band 3 polypeptide upon co-electrophoresis.

ity of these two polypeptides is consistent with their antigenic relatedness (see Fig. 1). With regard to determining the identity of the cDNA clone, the peptide map of the largest β-galactosidase–band 3 fusion protein contains several peptides (denoted by small arrowheads in *D*) that are not present in the map of β-galactosidase alone (*C*). To determine if the peptides unique to the fusion protein arose from sequences homologous to the band 3 gene, a peptide map of a mixture of the fusion protein and the 105-kD band 3 polypeptide (*F*) was compared to the map of the 105-kD band 3 polypeptide alone (*E*). As seen in *E* and *F*, four peptides from the fusion protein are absent from the map of the 105-kD band 3 polypeptide alone. Three of these peptides are derived from β-galactosidase (*C*) while the fourth (marked with an asterisk in *D* and *F*) is probably derived from the fusion region of β-galactosidase and band 3 since it is not observed in either of the maps alone. Five peptides observed in the peptide maps of the fusion protein (*D*) and the native band 3 (*E*), co-migrate when band 3 and fusion protein peptides are co-electrophoresed (small arrowhead in *D* and *F*). The co-migration of these peptides indicates that the λ clone 3(9-1) contains sequences homologous to the band 3 gene. The peptide maps of the two lower molecular weight fusion proteins were subsets of the peptide map shown in *D* and probably resulted from proteolysis of the higher molecular weight species (data not shown).

The 1.3 kb insert from this λ clone was isolated by digestion with *EcoRI* and subcloned into pBR322. A map of the restriction endonuclease sites within λ3(9-1) is shown in Fig. 7.

Hybridization of p3(9-1) cDNA to Genomic DNA

Genomic blot analysis of chicken genomic DNA by hybridization with p3(9-1) cDNA suggests that this cDNA hybridizes to a single gene type (Fig. 8). Briefly, genomic DNA was digested with restriction endonucleases, bound to nitrocellulose, and hybridized with ^{32}P-labeled p3(9-1) probe prepared by nick translation of the cDNA. As shown in Fig. 8, the cDNA probe hybridizes with a single band in DNA restricted with *EcoRI* (lane *R*) and *HindIII* (lane *H*). DNA digested with *BamHI* (lane *B*) reveals two bands with which the cDNA hybridizes, as predicted by the restriction map. The additional faint band detected in the *BamHI* digest (marked by arrowhead in Fig. 8) could arise from a *BamHI* site within an intervening sequence or from restriction site polymorphism.

Band 3 mRNA Expression during Erythroid Development

The expression of band 3 mRNA during erythroid development was investigated by RNA blot analysis. Poly(A)$^+$ RNA was prepared from erythroid cells isolated from 4-d-, 10-d-, and 15-d-old chicken embryos. This RNA was fractionated by electrophoresis on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to nick-translated p3(9-1) cDNA. This probe detects a major 4.4-kb mRNA species at all three stages of erythroid development (Fig. 9). However, as erythroid development proceeds (5) by switching from predominantly primitive series erythroid cells (day 4), to mixed primitive and definitive cell lineages (day 10), to predominantly definitive cell lineages (day 15), the abundance of this transcript increases (lanes *1–3*), especially between day 4 and 10. Several minor RNA species are also detected in erythroid cells (Fig. 9, lanes *2* and *3*) that appear to accumulate during development in a manner similar to the 4.4-kb transcript.

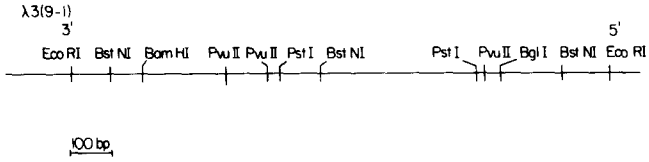

FIGURE 7 Map of restriction endonuclease sites within λ3(9-1) cDNA insert.

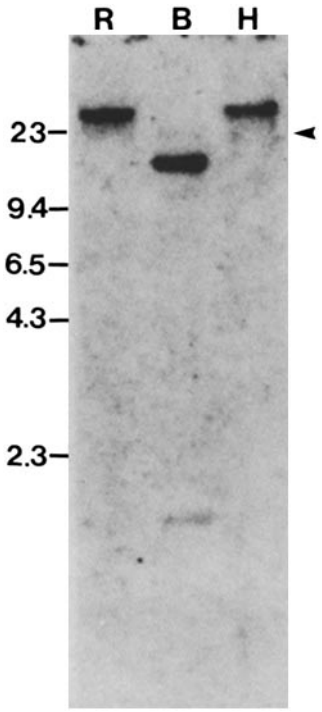

FIGURE 8 Genomic DNA blot analysis with p3(9-1) cDNA probe. Chicken liver DNA, digested with *EcoRI* (*R*), *BamHI* (*B*), and *HindIII* (*H*), was fractionated by electrophoresis on agarose gels, transferred to nitrocellulose, and hybridized to nick-translated ^{32}P-labeled p3(9-1) cDNA. Size markers (in kilobases) are denoted to the left.

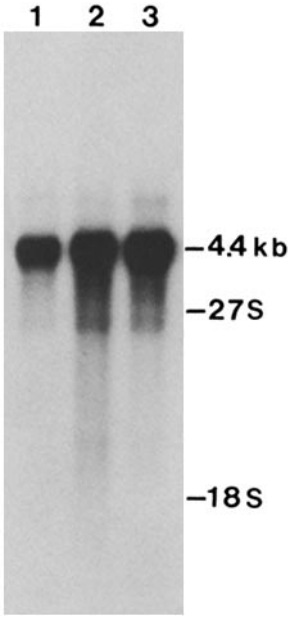

FIGURE 9 Expression of band 3 mRNA during erythroid development. 3 μg of poly(A)$^+$ RNA isolated from 4- (lane *1*), 10- (lane *2*), and 15-d-old (lane *3*) chicken embryo erythroid cells was fractionated on a 1.3% formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to nick-translated ^{32}P-labeled p3(9-1) cDNA. The position of 27S and 18S rRNA was determined by ethidium bromide–staining 7 μg total chicken erythroid RNA from the adjacent lane.

Band 3 mRNA Expression in Kidney

The p3(9-1) cDNA has been used to confirm by an inde-

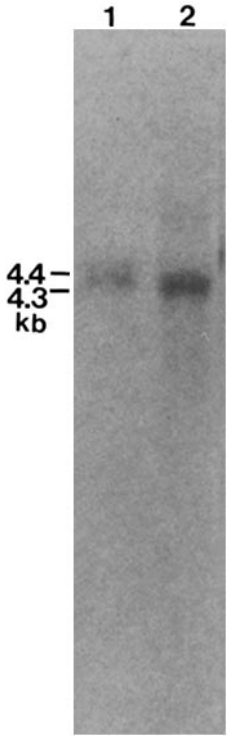

FIGURE 10 Comparison of band 3 mRNA expression in erythrocytes and kidney. An RNA blot similar to that described in Fig. 9 was performed with 60 ng poly(A)$^+$ erythroid RNA obtained from the postmitochondrial supernatant of erythroid cells from 14-d-old chicken embryos (lane *1*) and 3 μg poly(A)$^+$ kidney RNA (lane *2*). Length determinations for the erythroid and kidney band 3 mRNAs were made by comparison to the ethidium bromide–stained 18S and 27S rRNA from the adjacent lane.

pendent method the existence of band 3 in kidney and to begin investigating the molecular basis for the different sizes of the erythroid vs. kidney polypeptides. Poly(A)$^+$ RNA was isolated from a 3-mo-old chicken kidney after perfusion to remove erythrocytes. When this RNA was blotted onto nitrocellulose and probed with nick-translated p3(9-1) cDNA, a slightly smaller transcript was observed in kidney (Fig. 10, lane *2*) than in erythroid cell RNA (4.3 kb vs. 4.4 kb) (Fig. 10, lane *1*). Preliminary co-electrophoresis experiments of these two RNA samples corroborates this observed size difference (data not shown). The abundance of the band 3 mRNA in kidney is severalfold less than that found in erythrocytes since it requires ~50 times more kidney RNA to obtain a signal on blots comparable to that seen with erythroid RNA, in accordance with what is observed at the protein level.

DISCUSSION

We describe in this report that chicken erythrocyte band 3 is expressed in a highly tissue- and cell-type–specific manner in the avian kidney. cDNA clones and affinity-purified band 3 antibodies have enabled us to demonstrate the expression of a kidney-specific band 3 transcript and polypeptide, each of which differs in size from their erythroid counterparts. Indirect immunofluorescence microscopy indicates that this kidney-specific polypeptide is restricted to a small subset of kidney cells. The region of the kidney to which this band 3–related polypeptide is predominantly localized is the columnar type epithelium of the proximal convoluted tubule of cortical (reptilian) nephrons. These nephrons differ from the medullary (mammalian) nephrons in that they lack structures analogous to the loop of Henle. Previous studies on the mammalian kidney have established that the transport of HCO_3^- in the region of the proximal convoluted tubule plays an important role in the acidification of the kidney fluid (31). It has been postulated (40) that this HCO_3^- transport is mediated by an electroneutral HCO_3^--Cl^- exchange mechanism in the basolateral membrane of the epithelia, which is similar to that described for the erythrocyte anion transporter. Although we have not as yet demonstrated that the kidney band 3 polypeptide possesses a functional anion transport domain, it is tempting to speculate that this molecule may be involved in the exchange of internal HCO_3^- for external Cl^- in the avian proximal tubule. The highly localized expression of band 3 in kidney is consistent with this molecule performing a specialized function unique to this subset of the kidney epithelial cells. Whether in fact this function is an anion transport activity awaits further analysis.

Band 3 Diversity in Erythrocytes and Kidney

Immunological studies have indicated that avian erythrocyte band 3 is composed of two antigenically related polypeptides 100-kD and 105-kD in agreement with previous observations (20). Results obtained in this study by two-dimensional peptide mapping have further shown that these two polypeptides structurally are very similar. To investigate the origin of the diversity of these related band 3 polypeptides, embryonic erythroid cells were pulse-labeled with [^{35}S]methionine for periods of time as short as 10 min. Band 3 immunoprecipitates from these pulse-labeled cells have revealed two immunoreactive band 3 polypeptides. Whether these two polypeptides arise from two very similar size erythroid band 3 transcripts that we have not resolved by RNA blotting techniques, or from posttranslational processing of a single translation product remains unclear. Additional analysis will be required to distinguish between these alternative mechanisms. The diversity observed in both the newly synthesized and steady-state levels of band 3 may result in the generation of functionally distinct band 3 molecules. Previous investigators have demonstrated that human erythrocyte band 3 is present in ~10^6 copies/cell (37) of which only 15% bind the membrane cytoskeleton (3). It is possible that one of the avian erythrocyte band 3 variants contains an additional region in its cytoplasmic domain enabling it to interact with the membrane cytoskeleton, while the remainder of the band 3 population is unable to bind. Functional variability of this type has been observed between the cytoplasmic domains of human and avian erythrocyte band 3, since the latter apparently lacks the binding site for glyceraldehyde-3-phosphate dehydrogenase (20).

In addition to the diversity seen in the 100-kD and 105-kD erythrocyte band 3 polypeptides, the kidney-specific band 3 variant exhibits an apparent molecular weight of 115,000, ~10,000 more than its erythroid counterparts. At present, it is unknown whether the diversity observed between kidney and erythrocyte band 3 molecules resides in the cytoplasmic or transmembrane domain. RNA blotting analysis has indicated that the kidney-specific band 3 transcript is ~100 bp smaller than its erythroid counterpart. This result was somewhat unexpected since the protein data suggested that additional coding sequences would be required to generate the larger kidney band 3 polypeptide, assuming that differences in migration are not due to glycosylation. However, the 4.3-kb kidney band 3 mRNA is of more than sufficient length to encode the 115,000-mol-wt polypeptide.

Southern analysis of chicken genomic DNA strongly suggests that these multiple mRNAs arise from a single gene type. The possibility that more than one gene type gives rise

to the erythrocyte and kidney band 3 mRNAs has not been ruled out, and further analysis will be required to determine the organization of the band 3 gene in the chicken genome. However, if kidney and erythrocyte band 3 do in fact arise from a single gene, several mechanisms may be involved in generating the observed RNA diversity, including differential transcriptional initiation or termination, and differential splicing. Interestingly, variants of human erythrocyte band 3 that have an extra peptide in the cytoplasmic domain, which may be generated from alternative splice sites within the coding region of the gene, have been identified in a subset of the human population (26).

From the immunoblotting analysis described in this study we have shown that antibodies affinity-purified from avian erythrocyte band 3 cross-react with a highly cell-type–specific polypeptide in kidney. All other adult tissues examined by this technique were negative for band 3 expression. Recent results of other investigators (10, 21) suggest that polypeptides immunologically related to mammalian band 3 are expressed in a variety of nucleated somatic cells. The basis of this apparent discrepancy between these results is at this time unclear. However, in our studies it is worth noting that several of the tissues which were negative for band 3 expression by immunological criteria, including cerebellum, retina, and lens, also contained no detectable band 3 mRNA when examined by RNA blotting techniques (data not shown).

RNA blotting has demonstrated that the abundance of band 3 mRNA increases as erythroid development proceeds from day 4 to day 10, through a switch from primarily primitive series erythroid cells (day 4) to mixed primitive and definitive series cells (day 10) (5). During a similar time period, a sixfold reduction is observed in the incorporation of newly synthesized band 3 into chicken embryo erythroid membranes (41). Although these data are not directly comparable since the RNA loadings have not been normalized on a per cell basis, taken together they raise the intriguing possibility that the expression of erythrocyte band 3 is controlled at the translational or posttranslational level.

Noncoordinate Expression of Band 3 with Other Components of the Erythroid Membrane Skeleton

Many of the erythroid membrane cytoskeletal proteins, including spectrin (4, 6, 13, 14, 33), ankyrin (9, 27, 28), and protein 4.1 (1, 15, 17) have recently been found in avian and mammalian nonerythroid cells. These observations have led to the hypothesis that proteins related to the erythrocyte anion transporter may provide the membrane receptor for these proteins in nonerythroid cells, thereby stabilizing this membrane cytoskeletal complex. The highly tissue-specific expression of band 3 demonstrated in this report suggests that the cytoplasmic domain of this molecule provides the membrane attachment site for the cytoskeleton only in erythrocytes and possibly kidney. Whether in fact the subset of kidney cells that express band 3 also express ankyrin, spectrin, or protein 4.1 remains to be investigated. However, our data demonstrate that adult muscle and brain, which are known to express ankyrin (9, 27, 28), contain no detectable levels of band 3–related polypeptides or transcripts. This highly noncoordinate expression of band 3 with other proteins of the erythrocyte membrane cytoskeleton implies that molecules distinct from band 3 mediate the membrane attachment of the cytoskeleton in a variety of nonerythroid cells, if in fact an interaction of this type occurs at all. Furthermore, recent studies have demonstrated that the accumulation of α-spectrin, β-spectrin, and ankyrin mRNA is regulated noncoordinately in chicken embryo fibroblasts but accumulate concurrently during terminal differentiation of skeletal muscle cells (25). These results suggest that there is a great deal of flexibility in the assembly of the membrane cytoskeleton in nonerythroid cells, possibly to accommodate the specialized functions of specific cell types.

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