MOLECULAR AND CELLULAR BIOLOGY, Oct. 1988, p. 4416–4424
0270-7306/88/104416-09$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 8, No. 10

Two Different mRNAs Are Transcribed from a Single Genomic Locus Encoding the Chicken Erythrocyte Anion Transport Proteins (Band 3)

HYEONG-REH CHOI KIM,[1] NELSON S. YEW,[1] WILHELM ANSORGE,[2] HARTMUT VOSS,[2] CHRISTIAN SCHWAGER,[2] BJÖRN VENNSTRÖM,[2] MARTIN ZENKE,[2] AND JAMES DOUGLAS ENGEL[1]*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208,[1] and European Molecular Biology Laboratory, Heidelberg 6400, Federal Republic of Germany[2]

Received 29 June 1988/Accepted 25 July 1988

The chicken erythrocyte anion transport protein (band 3 of the erythrocyte cytoskeleton) is a central component taking part in two widely divergent functions of erythroid cells; it is a primary determinant of cytoskeletal architecture and responsible for electroneutral Cl^-/HCO_3^- exchange across the plasma membrane. To analyze interesting aspects of the developmental regulation of this gene, we have cloned the cDNA and genomic counterparts of the erythroid-specific anion transport protein. We show that a single genetic locus for band 3 encodes two different erythroid cell-specific mRNAs, with different translational initiation sites, which predict polypeptides of sizes very close to those observed in vivo. In vitro translation and immune precipitation of synthetic mRNA derived from one putative fully encoding cDNA clone demonstrate that this clone gives rise to a protein which is identical in size and antigenicity to bona fide chicken erythroid band 3.

The anion transport protein (band 3) is the most abundant transmembrane protein present in mature erythrocytes (RBC) (15). It has two distinct cellular functions; it serves as a structural anchor for the network of cytoskeletal peripheral membrane proteins, and it simultaneously functions as an anion transporter in active erythroid cells (3, 5, 7, 16). These separate functions have been localized within the linear map of the band 3 polypeptide sequence; the former (cytoskeletal anchor) is provided by the N-terminal cytoplasmic region, while the latter (anion transport) resides in the C-terminal transmembrane domain. In humans, the cytoplasmic domain of band 3 has also been reported to provide binding sites for hemoglobin and glycolytic enzymes (31, 37, 40, 44). Band 3 has also been suggested as playing a role in elimination of aged or abnormal RBC by the immune system; clusters of band 3 with deoxygenized and denatured hemoglobin may provide binding sites for autoantibodies which, in turn, may serve as a signal for phagocytosis by circulating macrophages (18, 34, 35, 39, 40).

Cloned copies of band 3 mRNAs show long regions of sequence identity when the predicted translation products of human, mouse, and chicken proteins are compared (especially in the binding site for the cytoskeleton and transmembrane regions [5a, 11, 17a, 20, 29a]), indicating that band 3 function is highly conserved during vertebrate evolution. However, unlike the mammalian band 3, with a single apparent M_r of 95,000, avian band 3 appears as a doublet, with apparent M_rs of 100,000 and 105,000, upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (17). The patterns of these two avian band 3 species (after partial proteolysis) show significant similarity, suggesting that they are structurally highly related (17). In its structural role, band 3 forms part of the intrinsic attachment point of the RBC cytoskeleton to the plasma membrane through its association with ankyrin and band 4.1 (3, 5, 16). This cytoskeletal network is thought to be responsible for maintenance of the required RBC membrane properties of deformability and stability under mechanical stress (i.e., passage through blood capillaries). The fractional proportion of band 3 associated with the cytoskeleton changes during chicken embryonic development at the time when RBC switch from expression of embryonic to adult hemoglobins in primitive and definitive RBC, respectively (10).

The primary nonstructural role of band 3 in RBC function is that of an anion transporter, which exchanges HCO_3^- for Cl^- across the plasma membrane by electroneutral one-for-one exchange (7, 19). The anion exchange protein not only enhances the ability of blood to carry CO_2 and O_2 but also regulates intracellular pH in coordinate activity with the cytoplasmic enzyme carbonic anhydrase (36).

Recent evidence suggests that band 3 may also play a vital role in erythroid cellular differentiation, providing the proper intracellular pH for terminal erythroid cell maturation. The v-*erbA* oncogene, which blocks differentiation of erythroid precursor cells (transformed by temperature-sensitive mutants of avian erythroblastosis virus [4]), quite specifically suppresses transcription of the band 3 and carbonic anhydrase genes (45; M. Zenke and H. Beug, unpublished observations), even in the absence of a functional v-*erbB* gene product. The v-*erbA*-induced block in erythroid differentiation can be suppressed by extracellular alkalinization, which inactivates the biological activity of v-*erbA* (Zenke and Beug, unpublished), leading to abundant synthesis of band 3 (45). One of the major reasons for failure of v-*erbA*/ts S13- or v-*erbA*/ts v-*erbB*-transformed cells to differentiate is therefore likely to be due to the absence of anion transport between the cytoplasm and blood plasma, a function directly mediated by band 3 (45).

In this communication, we describe the isolation and characterization of avian RBC band 3 cDNA and genomic clones, which has allowed an analysis of the primary transcriptional unit of two band 3 mRNAs which have different translational initiation sites. We include avian band 3 cDNA sequences and deduced amino acid sequences which disagree with experiments recently reported by Cox and Lazarides (9). We have also examined the mRNA accumulation

* Corresponding author.

of band 3 during erythropoiesis by using one of the isolated cDNA clones as a probe in S1 nuclease analysis and at the translational level by using an anti-band 3 monoclonal antibody in immune precipitation experiments.

MATERIALS AND METHODS

Isolation of chicken band 3 clones. An erythroid λgt11 cDNA library (43) was screened with nick-translated murine band 3 cDNA (20) as described previously (14). By immunological screening, an erythroid λgt11 cDNA library (45) was screened essentially as described previously (42) by using a previously characterized monoclonal antibody raised against the chicken RBC band 3 protein (43). Positive cDNA clones were used for plaque hybridization screening of the same library and a second cDNA library was prepared by using RNA isolated from 10-day-old chicken embryos (45).

A chicken genomic library was prepared by the procedure described by Maniatis et al. (28). Chicken RBC genomic DNA was cleaved by partial digestion with restriction endonuclease *Mbo*I, and fragments of 18 to 22 kilobase pairs (kb) were purified by sucrose gradient centrifugation. The DNA was then ligated to either EMBL3 or EMBL4 arms (Promega Biotec) and packaged in vitro. Approximately 500,000 primary recombinant bacteriophages were screened by using chicken band 3 cDNA clones as previously described (12). Positively hybridizing recombinant phages were plaque purified, and phage DNA was prepared by using Lambda-Sorb (Promega Biotec).

DNA sequence determination. Sequence analysis of the pBIIIC1 cDNA clone was done by using fluorescently labeled oligonucleotide primers and automated sequence recording as previously described (2, 23). Partial analysis of the cDNA clones, as well as the sequencing of small segments of the genomic clones, was accomplished by M13 and double-stranded plasmid sequencing as previously described (8, 33).

S1 nuclease protection analysis. cDNA clone pBIIIC1 was digested with *Pvu*II and then end-labeled with polynucleotide kinase in the presence of [γ-^{32}P]ATP. The plasmid was then digested with *Sca*I at a unique site within the vector, 1,270 nucleotides (nt) from the 5′ end of the cDNA insert. The 1,721-base-pair (bp) singly end-labeled *Sca*I-*Pvu*II fragment was isolated and used as a probe for band 3 mRNA analysis. The probe was denatured for 10 min at 75°C, hybridization was carried out by continuing incubation at 50°C for 10 to 12 h under conditions previously described (8, 41), and the RNA-DNA hybrids were digested for 2 h at 30°C with 1,000 U of S1 nuclease (Bethesda Research Laboratories) per ml in 300 μl of a solution containing 280 mM NaCl, 50 mM sodium acetate (pH 4.6), 4.5 mM $ZnSO_4$, and 20 μg of (carrier) tRNA per ml. S1 nuclease-protected DNA was electrophoresed on 7 M urea–5% polyacrylamide gels.

In vitro transcription and translation. Synthetic RNA from cDNA clone pBIIIC1 was prepared by in vitro transcription by using SP6 polymerase as previously described (6, 22); a 5′-terminal cap structure was added to the synthetic mRNA by using guanylyltransferase (Bethesda Research Laboratories) by incubating for 30 min at 37°C in 50 mM Tris hydrochloride (pH 7.9), 1.5 mM $MgCl_2$, 6 mM KCl, 2.5 mM dithiothreitol, 100 μM GTP, 100 μM *S*-adenosylmethionine, and 1 U of RNasin per μl (22, 29). SP6 RNA was purified by digestion of the in vitro transcription products with RNase-free DNase I, phenol extraction, and ethanol precipitation. SP6 RNA and RBC poly(A)$^+$ RNA were translated in vitro by using a rabbit reticulocyte cell lysate (Promega Biotec) at 30°C for 90 min in the presence of [^{35}S]methionine.

Immunoprecipitation and gel electrophoresis. Radiolabeled, in vitro-translated band 3 proteins were immunoprecipitated by using a monoclonal antibody to band 3 protein and rabbit anti-mouse antibody fixed to protein A-Sepharose (42). The immunoprecipitated proteins were suspended in 10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, 1% SDS, 10% sucrose, and 40 mM dithiothreitol, incubated at 37°C for 5 min, and finally analyzed by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS (15, 24).

In vivo labeling of total RBC proteins was accomplished by isolation and in vitro culture of erythroid cells from 4.5-day-old embryos, 11-day-old embryos, and 11-day-old chicks in methionine-free Dulbecco modified Eagle medium, supplemented with [^{35}S]methionine, for 1 h at 37°C. RBC plasma membranes were purified from the in vivo-radiolabeled erythroid cells as previously described (43) and then immunoprecipitated in a solution containing 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 20 mM Tris hydrochloride (pH 8.0), and 5 mM EDTA by using the monoclonal antibody raised against band 3 protein.

RESULTS

Isolation of chicken erythroid band 3 cDNA and genomic clones. Two cDNA clones, λBIIIC1 and λBIIIC2, were isolated by immunological screening of a λgt11 expression library prepared from the RNA of chicken erythroblasts transformed by a new avian *erbB*-transducing virus (45) by using the monoclonal antibody raised against the chicken band 3 protein (43). cDNA clone λBIIIC3 was isolated from a chicken erythroid λgt11 cDNA library (43) by using a heterologous murine band 3 cDNA probe (20) essentially as previously described (14). Approximately 200 additional cDNA clones (data not shown) were isolated from the erythroblasts and a new chicken embryo library (45; see Materials and Methods); six of these clones (pBIIIC4 through pBIIIC9) were further analyzed by partial DNA sequencing. All of the cDNA clones were shown to overlap in restriction enzyme mapping and preliminary DNA sequencing experiments (Fig. 1A), and both λBIIIC1 and λBIIIC2 were found to also cross-hybridize to the murine band 3 cDNA clone (data not shown).

The 3-kbp *Eco*RI-subcloned (pBIIIC1) insert of λBIIIC1 was used to screen the primary λEMBL3 genomic library prepared from chicken RBC chromosomal DNA. Sixteen independent genomic recombinants were isolated and plaque purified; three of these recombinants (λBIIIG1, λBIIIG2, and λBIIIG3) were subsequently shown to hybridize to a fragment representing the 5′-most 150 bp of pBIIIC1. The 5′ end of the chicken erythroid band 3 genomic locus is depicted in Fig. 1B.

Characterization of chicken erythroid band 3 cDNA clones. We have sequenced the entire cDNA clone pBIIIC1, containing a recombinant segment of 2,968 bp, and approximately 500 bp at the 5′ ends of cDNA recombinants pBIIIC2 through pBIIIC9. pBIIIC4 through pBIIIC9 were all found to be identical in internal DNA sequence to pBIIIC1. The largest of these recombinants was pBIIIC4, which was found to be 63 bp longer than pBIIIC1 at the 5′ end; the compiled 3,031-bp sequence is presented in Fig. 2.

A short open reading frame (nt 26 to 40) precedes the major open reading frame in pBIIIC1, beginning at an ATG at position 77 to 79 and extending to a termination codon at position 2843 to 2845. The DNA sequence therefore predicts a polypeptide of 922 amino acids which has a predicted molecular weight of 102,329 before posttranslational modifi-

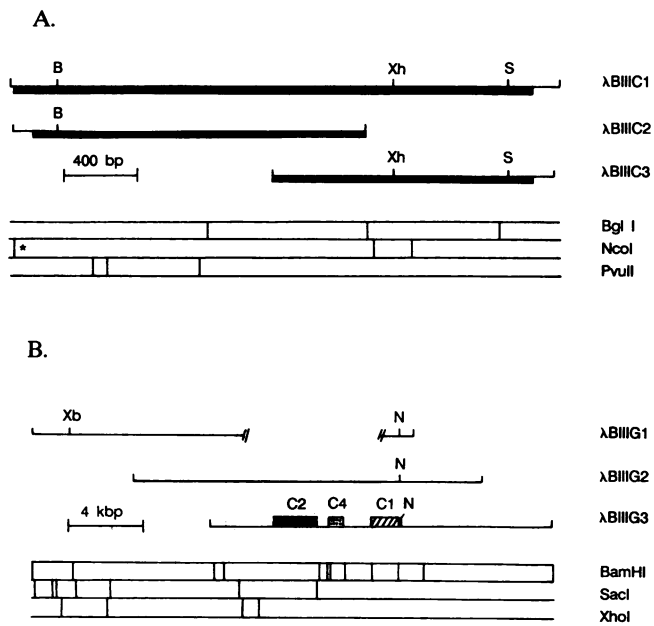

FIG. 1. Restriction maps of the chicken erythroid band 3 cDNA and chromosomal recombinants. (A) Restriction enzyme maps of the three isolated band 3 cDNA clones. An abbreviated restriction enzyme map is depicted below the drawings, showing the position and size of the three cDNA clones. Unique sites present in the cDNA clones are shown in relative position within the sequence: B, *Bam*HI; Xh, *Xho*I; S, *Sac*I. (Note that the 5'-most *Nco*I site [*] is present in the recombinant insert of λBIIIC1 but not in λBIIIC2.) Solid boxes indicate the predicted coding sequences of the two band 3 proteins (Fig. 3). (B) Relative position of restriction enzyme sites within the band 3 genomic locus and abbreviated restriction maps of the three 5'-most chicken genomic recombinants. (The broken line indicates a region deleted in genomic clone λBIIIG1.) Unique sites present within the genomic locus, and their relative positions, are shown: Xb, *Xba*I; N, *Not*I. The dotted box above the line denoting the relative position and size of λBIIIG3 represents the fragment which hybridizes to the pBIIIC4-specific oligonucleotide, the solid box represents the position of hybridization to the pBIIIC2-specific probe, and the hatched box represents the position of hybridization to the pBIIIC1-specific probe (see Results and Fig. 7).

cation. The sequences surrounding the ATG for this long open reading frame correspond well to the consensus sequence thought to function as a translational initiation site in eucaryotic mRNA (21). The predicted pI of the large pBIIIC1 translation product is 6.55, in good agreement with the value, experimentally determined by Jay (17), of 5.8 to 6.5 for the chicken band 3 protein. The identity between the murine and chicken band 3 sequences is 60.4% as predicted from conceptual translation of the pBIIIC1 cDNA clone (Fig. 3).

In addition to the sequences presented in Fig. 2, we have sequenced both the 5' and 3' ends of pBIIIC2 through pBIIIC9. The only disparity found between all of the independent cDNA clones described in this communication is a sequence mismatch of 15 bp at the 5' end of pBIIIC2 and all the others (C2, Fig. 2). Since an in-frame termination codon in pBIIIC2 occupies the same relative sequence position of the AUG initiation codon of pBIIIC1 (Fig. 2; C2, nt 77 to 79), pBIIIC2 is predicted to initiate translation of an 889-amino-acid polypeptide which starts precisely 33 amino acids downstream of the pBIIIC1 initiation codon (in the same reading frame), giving rise to an N-terminally foreshortened protein (identical in internal sequence to the C1 protein) of M_r 98,777.

This result is clearly in disagreement with the chicken erythroid anion transport protein cDNA sequence and polypeptide sequence (predicted by conceptual translation of the cDNA clone pCHB3-1) previously described by Cox and Lazarides (9). The band 3 protein which these authors deduce from pCHB3-1 DNA sequence begins translation at amino acid 79 of the pBIIIC1 sequence (Fig. 2; 3-1), predicting a polypeptide of 844 amino acids with a molecular mass of 93,784 (erroneously reported as 109,000 [9]) and a pI of 9.83; both the molecular weight and pI of this predicted protein substantially differ from values in the established literature (17). Although internal sequences within cDNA clone pCHB3-1 match the DNA sequence of pBIIIC1 with long regions of identity (including, however, a number of base sequence and several frameshift discrepancies), sequences 5' to position 267 of pBIIIC1 are not related to pCHB3-1 (Fig. 2). This observation left open the possibility that multiple band 3 mRNAs are present in chicken erythroid cells and that pBIIIC1, pBIIIC2, and pCHB3-1 cDNAs (9) all represent genuine band 3 mRNAs.

Expression of anion transport protein mRNAs. To distinguish whether or not multiple erythroid band 3 mRNAs exist in chicken erythroid cells, S1 nuclease protection analysis was performed. Total RBC RNA isolated from anemic hens was hybridized to a 1,721-nt probe from pBIIIC1 (end labeled at the *Pvu*II site within the cDNA) and then digested with S1 nuclease (41). If the cDNA clones all represent bona fide band 3 mRNAs, fragments of 451, 423, and 248 nt would be expected after hybridization and S1 nuclease digestion by erythroid band 3 mRNAs representative of pBIIIC1, pBIIIC2, and pCHB3-1, respectively.

As shown in Fig. 4 (lane 5), only the 451- and 423-nt fragments were detected in adult RBC RNA; no mRNA corresponding to pCHB3-1 (9) was detected. To ensure that the lack of the 248-nt fragment corresponding to this clone was not due to S1 nuclease digestion conditions, we lowered the S1 nuclease concentration to 300 U/ml and performed the digestion at room temperature (compared to our standard condition of 1,000 U of S1 nuclease per ml at 30°C); under these conditions, a 248-nt fragment was still not detected (data not shown). Although we cannot, from these data alone, rule out the possibility that pCHB3-1 represents a genuine band 3 transcript expressed at extremely low levels, we tentatively conclude that this clone (9) was generated by artifactual ligation of two unrelated cDNA products into one isolated recombinant.

We have not examined pBIIIC1-C9 to specifically address the question of whether or not these recombinants contain a band 3 mRNA which is reported to lack one membrane-spanning exon (9). Recombinants pBIIIC1 through pBIIIC3 all contain this exon by direct DNA sequence analysis (data not shown). Since the experiment reporting the missing exon was technically flawed (only protection fragments corresponding to either full-length deletion recombinant probe or the band 3 sequence reported here were visualized in that experiment [9]), we cannot comment on the possibility that such an mRNA does or does not exist in chicken RBC mRNA, although we find no evidence for the existence of that species of band 3 mRNA in the experiments reported here.

To study the expression of band 3 mRNA synthesis during embryonic development, S1 nuclease protection analysis was performed with total RNA isolated from 4.5-day-old embryonic RBC (primitive erythroid cells), 11-day-old em-

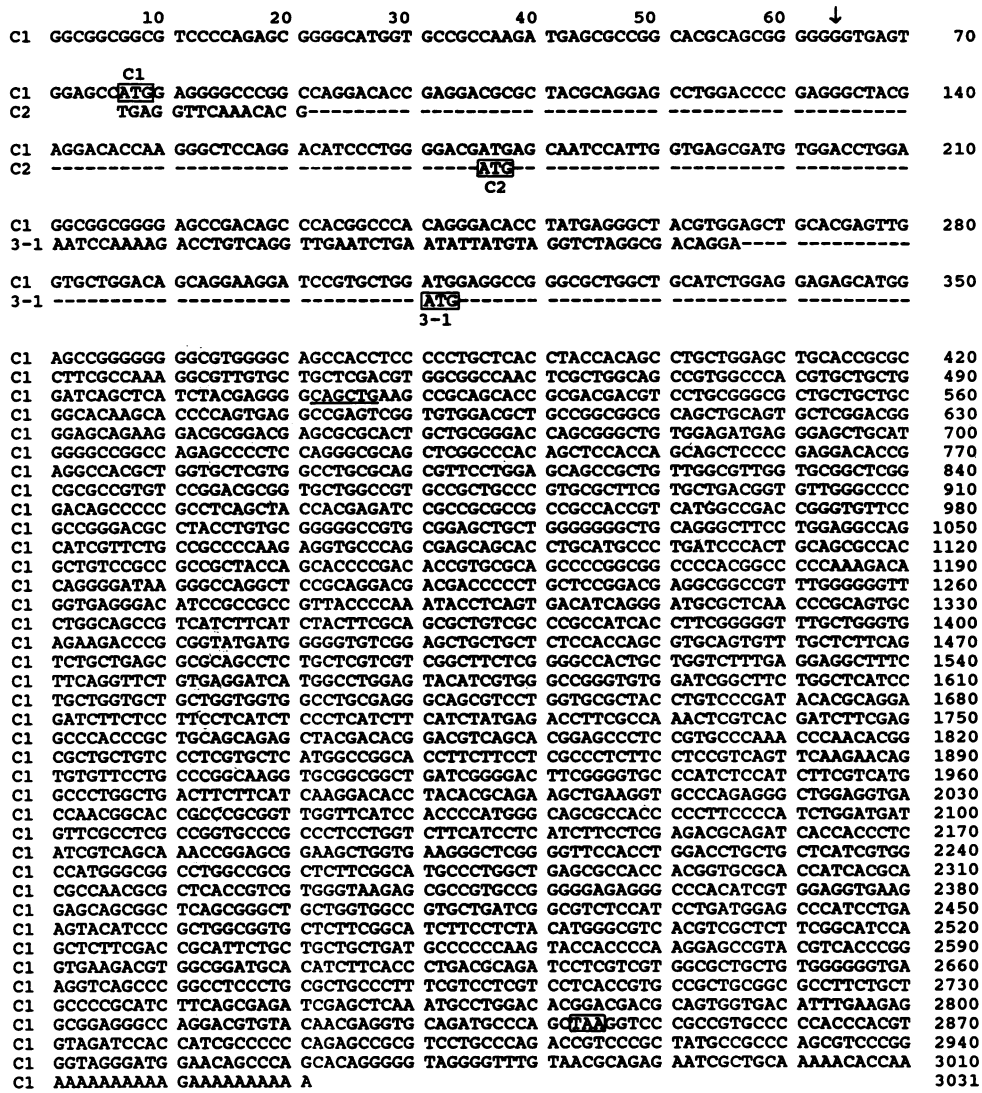

FIG. 2. DNA sequence of chicken erythroid band 3 cDNAs. The compiled DNA sequence of several overlapping band 3 cDNA clones (see Results) is shown, in comparison to the 5′ ends of recombinants pBIIIC2 and pCHB3-1 (9). The AUG corresponding to translation initiation (boxed) indicates the predicted translational start site for band 3 polypeptides corresponding to cDNA clones pBIIIC1 (nt 77 to 79), pBIIIC2 (nt 176 to 178), and pCHB3-1 (nt 311 to 313). Nucleotide 1 corresponds to the first nucleotide of recombinant λBIIIC4, a 5′ extension of λBIIIC1 (Fig. 1; see Results). An arrow indicates the position of the 5′-most nucleotide of λBIIIC1. Where pBIIIC1 through pBIIIC9 or pCHB3-1 are identical, only the pBIIIC1 sequence or dashes are shown. The 5′-most 661 bp of pCHB3-1 (9) do not match pBIIIC1, whereas the 5′-most 15 bp of pBIIIC2 are not identical. pBIIIC1 (Fig. 1) and pCHB3-1 (9) also differ after nt 267 by multiple point and frameshift discrepancies (not shown) which change the predicted polypeptide sequence (Fig. 3). The *Pvu*II site used in S1 nuclease mRNA protection (Fig. 4) is underlined (nt 512 to 517).

bryonic RBC (90 to 95% immature definitive erythroid cells), and anemic adult hen RBC (mature definitive cells). As shown in Fig. 4, the two band 3 mRNAs represented by cDNA clones pBIIIC1 and pBIIIC2 were expressed in both primitive and definitive cells in roughly equal quantities at all developmental stages. Since the library from which pCHB3-1 was isolated was prepared by using RBC RNA isolated from 15-day-old chick embryos (9), it is equally clear that this mRNA is not present in either late embryonic definitive (11-day-old embryonic RBC) or mature definitive (anemic adult hen RBC) erythroid cells.

To determine whether or not the band 3 cDNA clones encode mRNAs which are erythroid specific, we performed RNA blot analysis of poly(A)$^+$ mRNAs isolated from various chicken tissues and cell lines. As shown in Fig. 5, no hybridization was detected to equal amounts of RNA from a transformed erythroid progenitor (HD6) cell line, a lymphoid (MSB-1) cell line, or perfused adult chicken kidney or liver cells (1, 4). Thus the recombinant segment of pBIIIC1 hybridized only to mRNA(s) prepared from RBC of an anemic adult hen by using standard stringency blot washing conditions (equivalent to 10 mM monovalent cation at 50°C), revealing that this recombinant indeed represents chicken erythroid-specific band 3 mRNA(s). We cannot knowledgeably comment on the origin of the less intense 5-kb band visualized in this RNA blot. It may represent a genuine band 3 transcript, although we feel that it may equally likely represent an aldehyde cross-linking artifact, an artifact fre-

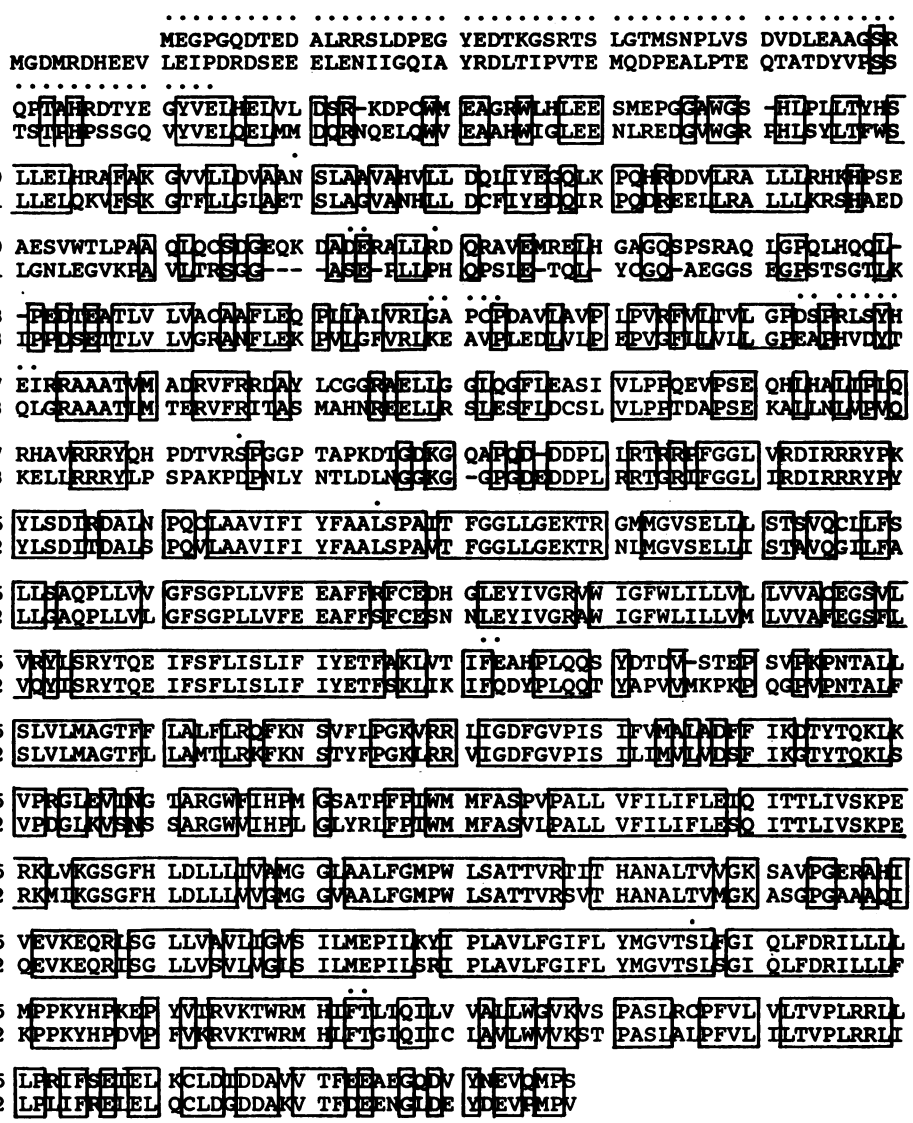

FIG. 3. Amino acid sequence comparison between the murine (lower line) and chicken (upper line) erythroid band 3 proteins. The predicted amino acid sequence of the chicken erythroid band 3 cDNA clone (pBIIIC1) is compared with the deduced mouse band 3 sequence (20) and aligned to maximize identical sequences (boxed). Amino acids are designated by the single-letter code. Amino acid sequence predictions which differ between the two avian RBC band 3 cDNA clones pBIIIC1 and pCHB3-1 (9) are highlighted by a dot above the predicted sequence of pBIIIC1.

quently visualized in this type of gel analysis system (13). Although we detected only erythroid-specific transcripts under these conditions, others have reported the existence of band 3-like molecules in both mammalian and chicken kidney cells (38).

pBIIIC1 encodes a protein indistinguishable from erythroid band 3. We next performed an experiment designed to ascertain whether or not pBIIIC1 encodes a full-length band 3-coding sequence. If so, one would anticipate that in vitro translation products of total RBC poly(A)$^+$ RNA or of in vitro transcripts of pBIIIC1 should be precipitable with the anti-band 3 monoclonal antibody and that these two immune precipitation products should be the same in size. The resulting data from this experiment are shown in Fig. 6A, in which the polypeptide encoded by pBIIIC1 is indeed the same size as the in vitro translation product of RBC poly(A)$^+$ RNA; additionally, these in vitro-translated band 3 polypeptides migrate as the same apparent size as in vivo labeled band 3 protein (17). (In the previous paper, pCHB3-1 [9] was not shown to encode a full-length band 3 polypeptide; only an internally truncated in vitro transcript was used in the characterization of in vitro translation products, once again underscoring the possibility that pCHB3-1 represents a cloning artifact.)

In a parallel series of experiments, we also examined the expression of band 3 protein during development (Fig. 6B). The absolute levels of band 3 protein did not change dramatically; however, band 3 protein seemed to be associated, at later times in development, with other cytoskeletal proteins strongly enough for them to be coprecipitated as a complex with band 3. These data agree well with the previous observations of Cox et al. (10), who showed that the portion of band 3 associated with the cytoskeleton changes during chicken embryonic development. We cannot rule out the possibility that this association is due to adventitious binding to mature cytoskeletal components because of slight differ-

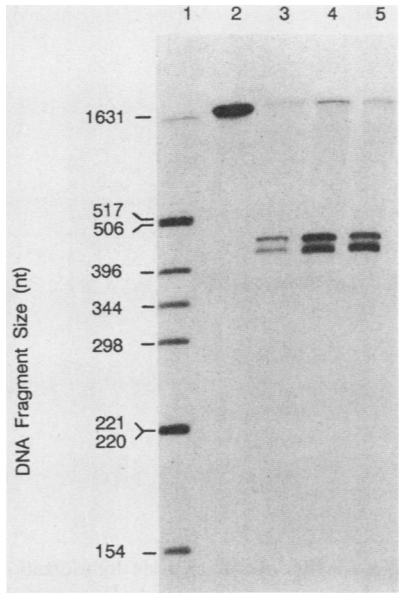

FIG. 4. S1 nuclease protection of band 3 mRNAs during chicken erythroid development. Five micrograms of erythroid total cellular RNA isolated from 4.5-day-old embryos (lane 3), 11-day-old embryos (lane 4), or anemic adult hens (lane 5) was analyzed by S1 nuclease protection by using a 1,721-bp *Sac*I-*Pvu*II fragment of pBIIIC1 singly end labeled at the *Pvu*II site (underlined in Fig. 2). Lane 2 shows the undigested S1 nuclease protection probe. DNA size markers (pBR322 digested with *Hin*fI and end labeled with [γ-^{32}P]ATP) were electrophoresed in parallel (lane 1).

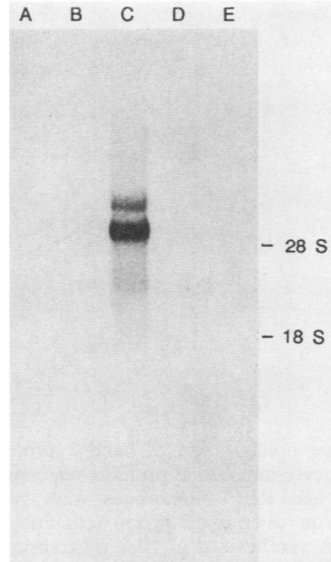

FIG. 5. Tissue-specific expression of RBC anion transport protein mRNA. Two micrograms of poly(A)$^+$ RNA were electrophoresed on a 1.2% HCHO agarose gel (25), transferred to nitrocellulose, and probed with the nick-translated 3-kbp insert of cDNA clone pBIIIC1. Exposure time was 3 h with an intensifying screen. The blot was stripped and then rehybridized with a nick-translated chicken rRNA genomic clone (30) for internal size standardization. Lane 1, Perfused liver RNA; lane 2, MSB-1 RNA; lane 3, anemic adult reticulocyte RNA; lane 4, HD6 RNA; lane 5, perfused kidney RNA. MSB-1 cells are a chick splenic lymphoma cell line (1), while HD6 cells are an erythroblast cell line transformed by *ts*167AEV (4).

ences in immunoprecipitation conditions or that the extent of band 3 association with cytoskeletal protein(s) varies during RBC maturation and that the RBC used both here and in the previous report were simply at different stages of erythroid cellular maturation.

Mapping the 5′ end of the chicken erythroid band 3 gene. As shown in Fig. 2, the 5′ end of pBIIIC2 does not match the pBIIIC1 sequence in the first 15 nucleotides. After this point, the two cDNAs become perfectly matched, and as shown in Fig. 4, both sequences were approximately equally represented in mRNA isolated from RBC. This implies one of three possibilities: that pBIIIC1 and pBIIIC2 represent differentially spliced mRNAs of a single band 3 primary transcript, that the two mRNAs are transcribed from different promoters within the same gene, or that the two mRNAs are transcribed from two different genes.

In efforts to further characterize the transcriptional nature of these two mRNAs, oligonucleotides which are unique and complementary to the 5′ ends of each of the mRNAs were synthesized, radiolabeled, and then used to probe identical blots of band 3 genomic subclones digested with several restriction enzymes (Fig. 7). The probes represented anti-mRNA sense oligonucleotides corresponding to the 5′ ends of pBIIIC1 (3′-ACTCACCTCGGTACCTCC-5′; nt 66 to 83 [Fig. 2]), pBIIIC4 (3′-CCGCCGCCGCAGGGGTC-5′; nt 1 to 17 [Fig. 2]), and pBIIIC2 (3′-CTCCAAGTTTGTGCGTCC TG-5′; nt 78 to 97 of C2 [Fig. 2]). The pBIIIC4-specific oligomer hybridizes to an 0.8-kbp *Bam*HI fragment (Fig. 1B, dotted box on the line depicting λBIIIG3), while the pBIIIC1 oligonucleotide hybridizes to a 1.5-kb *Bam*HI fragment (Fig. 1B, hatched box), both within a *Sac*I-*Not*I fragment of λBIIIG3. The pBIIIC2-specific oligomer hybridizes to a 2.2-kb *Apa*I-*Sac*I fragment of λBIIIG3 (Fig. 1B, solid box). Thus, the two mRNAs represented by cDNA clones pBIIIC1 and pBIIIC2 clearly arise by transcription within a single genetic locus.

DISCUSSION

In this paper, we report the isolation and initial characterization of cDNA and genomic clones encoding the chicken RBC anion transport proteins (band 3). Nucleotide sequence analysis of cDNA clones and S1 nuclease protection analysis suggest that two chicken erythroid band 3 polypeptides are produced by different mRNAs with different translational start sites. We have assigned the Met codon at position 77 to 79 (Fig. 2) as the translational start site of an mRNA corresponding to pBIIIC1, since it is the only in-frame ATG downstream of a prior in-frame stop codon which can encode a protein of approximately the expected size. We have also assigned the second Met codon (numerically equivalent to nt 176 to 178 of pBIIIC1 [Fig. 2]) as the translational start site of an mRNA represented by pBIIIC2, since it is the first in-frame ATG downstream of a stop codon (corresponding to nt 77 to 79 of pBIIIC1) in this cDNA clone (Fig. 2, C2).

The difference between the pBIIIC1 and pBIIIC2 cDNA clones occurs at a position which is very close to the predicted translational initiation sites of the two mRNAs (Fig. 2). The first ATG encountered in recombinant pBIIIC2 is numerically equivalent to nt 176 to 178 of pBIIIC1, predicting that a polypeptide synthesized from a pBIIIC2 mRNA would initiate translation precisely 33 amino acids downstream of the translational initiation site of pBIIIC1 in the same reading frame. This implies that two chicken erythroid band 3 polypeptides (with predicted molecular

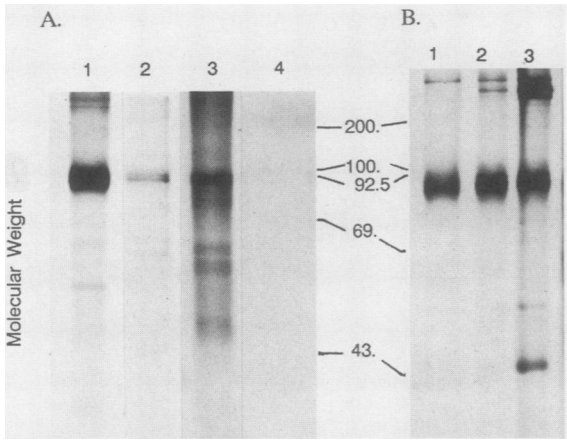

FIG. 6. Immune precipitation of band 3 protein. (A) Anemic adult hen reticulocyte membrane proteins were pulse-labeled with [^{35}S]methionine, and RBC membranes were purified (lane 1). Poly(A)$^+$ RNA from anemic adult hen reticulocytes (lane 2) and sense strand RNA synthesized in vitro by transcription of band 3 cDNA clone pBIIIC1 capped by guanylyltransferase (lane 3) or uncapped (lane 4) were translated in vitro in the presence of [^{35}S]methionine. All four samples were then immune precipitated by using the monoclonal antibody raised against chicken RBC band 3 protein (43) and rabbit anti-mouse antibody fixed to protein A-Sepharose. The immunoprecipitated proteins were then electrophoresed on a 10% polyacrylamide–0.1% SDS gel. Molecular weight markers ($\times 10^3$) (^{14}C-labeled protein standards; Amersham Corp.) were run in a parallel lane of the same gel. (B) Expression of the chicken anion transport protein during development. Circulating erythroid cells from 4.5-day-old embryos (lane 1), 11-day-old embryos (lane 2), and anemic adult hens (lane 3) were pulse-labeled with [^{35}S]methionine. RBC membranes were purified from the cells and then immunoprecipitated and electrophoresed as described above.

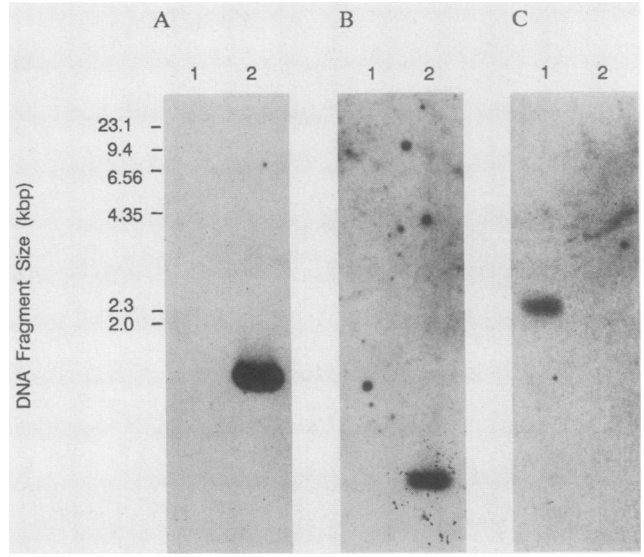

FIG. 7. cDNA-specific oligonucleotide hybridization to band 3 genomic clones. A genomic subclone of λBIIIG3 was digested with *Apa*I and *Sst*I (lane 1), while a nonoverlapping subclone of the same parental genomic recombinant was digested with *Bam*HI (lane 2); the DNA samples were then electrophoresed on a neutral 1.0% agarose gel and blotted simultaneously to nitrocellulose filters. Panel A shows a filter probed with pBIIIC1-specific oligomer, panel B shows an identical filter probed with the pBIIIC4-specific oligomer, and panel C shows a third filter probed with the pBIIIC2-specific oligomer (see Results). DNA size markers (λ*c*I857*S*am7 digested with *Hin*dIII) were electrophoresed in a parallel lane of the same gel. The position of the hybridizing bands are shown in relation to the band 3 genomic locus (within λBIIIG3) in Fig. 1; the dotted box indicates the position of hybridization of the pBIIIC4-specific oligonucleotide, the hatched box indicates the position of DNA fragment hybridization to the pBIIIC1-specific oligomer, and the solid box shows the position of the DNA fragment detected by pBIIIC2-specific oligomer hybridization.

weights of approximately 102,000 and 99,000) are produced by different mRNAs with different translational start sites; this is an attractive possibility, since these are very close to the sizes for the band 3 proteins determined by in vivo labeling (105,000 and 100,000 [17]).

The predicted amino acid sequence of the larger band 3 protein (Fig. 3) shows significant identity to the amino acid sequence predicted from conceptual translation of murine erythroid band 3 cDNA; the similarity is striking, especially in the transmembrane-spanning regions (the anion transport functional domain) between human, mouse, and chicken erythroid band 3 and human nonerythroid band 3 proteins (5a, 11, 20, 29a). Although the cytoplasmic domain is only poorly conserved among these, the tentative ankyrin-binding site is very highly conserved (11, 17a, 20). As in the human and mouse erythroid band 3 proteins, there is a highly conserved region, rich in tryptophan residues, N terminal to the tentative ankyrin-binding site (residues 79, 85, and 98; Fig. 3); there is also a region rich in proline which is C terminal to the ankyrin-binding site within the cytoplasmic domain (residues 149, 166, 176, 214, and 221; Fig. 3), although the absolute positions of the proline residues are not well conserved between chicken and mammalian band 3 proteins.

It has been proposed that the cytoplasmic domain of human band 3 forms a homodimer with the pH-dependent, reversible hinge in the proline-rich region (26) and that the dimers may form tetramers at the plasma membrane surface (27, 32). Although it is not known whether chicken band 3 forms a homodimer or heterodimer, it seems likely that both of the chicken erythroid band 3 polypeptides are involved in the anion transport function, since both are labeled with [$^3H_2$]DIDS (4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonic acid), a compound which inhibits band 3-mediated anion transport (17). The N terminus of the predicted chicken RBC band 3 protein is 10 amino acids (pBIIIC1) or 43 amino acids (pBIIIC2) shorter in homology alignment than the predicted mouse and human erythroid band 3 proteins (Fig. 3). This agrees well with the lack of binding of glyceraldehyde-3-phosphate dehydrogenase to the chicken anion transport protein (17), whose binding site is in the first 11 residues of human band 3 (39).

DNA filter hybridization analysis of band 3 genomic clones probed with pBIIIC1, pBIIIC2, or pBIIIC4 cDNA-specific oligonucleotides indicates that these two mRNAs are transcribed from the same gene (Fig. 7), although only one cDNA clone (pBIIIC2) encoding the smaller predicted polypeptide has been isolated. Identification of the true 5′ terminus of the chicken erythroid band 3 gene will provide conclusive information on the mechanism of transcription and splicing within this genetic locus. If two mRNAs share the first exon, it is likely that they are differentially spliced versions of a single band 3 primary transcript; if they remain diverse at the 5′ ends, it suggests that they are transcribed from two different promoters within the same locus.

The 3′ ends of the pBIIIC1 and pBIIIC3 cDNA clones are virtually identical (pBIIIC3 has an A residue at position

3021 [Fig. 2]), but no consensus polyadenylation signal (AAUAAA) is found in either clone. We are presently unsure whether the polyadenylate residues at the 3' ends of clones C1 and C3 represent the actual 3' processing and polyadenylation sequences or whether the oligo(dT) primer used in first-strand cDNA synthesis initiated reverse transcription at an internal oligo(rA) sequence within band 3 mRNA. Since the size of the largest cDNA clone that we have sequenced is 3 kbp, and yet the size of the most intensely hybridizing band on RNA blot analysis is 4.4 kb, a combination of 5'- and 3'-untranslated segments of the mRNAs should account for the missing 1.4 kb. This clearly leaves open the possibility that the 3'-untranslated region extends beyond the oligo(A) segment present in the cDNA clones. Final resolution of this question will await further characterization of the genomic clones and complete mRNA analysis.

ACKNOWLEDGMENTS

We thank K.-L. Ngai (Northwestern University Biotechnology Facility) and B. Sproat and P. Neuner (European Molecular Biology Laboratory [EMBL]) for preparation of oligonucleotides, R. Kopito and H. Lodish (Massachusetts Institute of Technology) for the gift of the murine band 3 cDNA clone, K. Nördström (EMBL) for isolation and partial sequence analysis of several additional cDNA clones, and H. Beug (EMBL) for advice and continuing interest.

This work was supported by Public Health Service grants from the National Institutes of Health.

LITERATURE CITED

1. **Akiyama, Y., and S. Kato.** 1974. Two cell lines from lymphomas of Marek's disease. Biken J. **17**:105–116.
2. **Ansorge, W., B. Sproat, J. Stegemann, C. Schwager, and M. Zenke.** 1987. Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis. Nucleic Acids Res. **15**:4593–4602.
3. **Bennett, V., and P. J. Stenbuck.** 1979. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. Nature (London) **280**:468–473.
4. **Beug, H., S. Palmeiri, C. Freudenstein, H. Zentgraf, and T. Graf.** 1982. Hormone-dependent terminal differentiation *in vitro* of chicken erythroleukemia cells transformed by ts mutants of avian erythroblastosis virus. Cell **38**:907–919.
5. **Branton, D., C. M. Cohen, and J. Tyler.** 1981. Interaction of cytoskeletal proteins on human erythrocyte membrane. Cell **24**: 24–32.

5a. **Brock, C. J., M. J. A. Tanner, and C. Kempf.** 1983. The human erythrocyte anion transport protein. Partial amino acid sequence and a possible molecular mechanism for anion exchange. Biochem. J. **213**:577–586.

6. **Brush, D., J. B. Dodgson, O.-R. Choi, P. W. Stevens, and J. D. Engel.** 1985. Replacement variant histone genes contain intervening sequences. Mol. Cell. Biol. **5**:1307–1317.
7. **Cabantchik, Z. I., P. A. Knauf, and A. Rothstein.** 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. Biochim. Biophys. Acta **515**:239–302.
8. **Choi, O.-R., and J. D. Engel.** 1986. A 3' enhancer is required for temporal and tissue-specific transcriptional activation of the chicken adult β-globin gene. Nature (London) **323**:731–734.
9. **Cox, J. V., and E. Lazarides.** 1988. Alternative primary structures in the transmembrane domain of the chicken erythroid anion transporter. Mol. Cell. Biol. **8**:1327–1335.
10. **Cox, J. V., J. H. Stack, and E. Lazarides.** 1987. Erythroid anion transporter assembly is mediated by a developmentally regulated recruitment onto a preassembled membrane cytoskeleton. J. Cell Biol. **105**:1405–1416.
11. **Demuth, D. R., L. L. Showe, M. Ballentine, A. Palumbo, J. J. Fraser, L. Cioe, G. Rovera, and P. J. Curtis.** 1986. Cloning and structural characterization of a human nonerythroid band 3-like protein. EMBO J. **5**:1205–1214.
12. **Dodgson, J. B., J. Strommer, and J. D. Engel.** 1979. Isolation of the chicken β-globin gene and a linked embryonic β-like globin gene from a chicken DNA recombinant library. Cell **17**:879–887.
13. **Dolan, M., J. B. Dodgson, and J. D. Engel.** 1983. Analysis of the adult chicken β-globin gene. J. Biol. Chem. **258**:3983–3990.
14. **Engel, J. D., and J. B. Dodgson.** 1981. Histone genes are clustered but not tandemly repeated in the chicken genome. Proc. Natl. Acad. Sci. USA **78**:2856–2860.
15. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
16. **Hargreaves, W. R., K. N. Giedd, A. Verklieji, and D. Branton.** 1980. Reassociation of ankyrin with band 3 in erythrocyte membranes and lipid vesicles. J. Biol. Chem. **255**:11965–11972.
17. **Jay, D. G.** 1983. Characterization of the chicken erythrocyte anion exchange protein. J. Biol. Chem. **258**:9431–9436.

17a. **Kaul, R. K., S. N. P. Murthy, A. G. Reddy, T. L. Steck, and H. Kohler.** 1983. Amino acid sequence of the N^{α}-terminal 201 residues of human erythrocyte membrane band 3. J. Biol. Chem. **258**:7981–7990.

18. **Kay, M. M. B.** 1975. Mechanism of removal of senescent cells by human macrophages *in situ*. Proc. Natl. Acad. Sci. USA **72**: 3521–3525.
19. **Knauf, P. A.** 1979. Erythrocyte anion exchange and the band 3 protein. Transport kinetics and molecular structure. Curr. Top. Membr. Transp. **12**:249–363.
20. **Kopito, R. R., and H. F. Lodish.** 1985. Primary structure and transmembrane orientation of the murine anion exchange protein. Nature (London) **316**:234–238.
21. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
22. **Krieg, P. A., and D. A. Melton.** 1984. Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. Nucleic Acids Res. **12**:7057–7070.
23. **Kristensen, T., H. Voss, and W. Ansorge.** 1987. A simple and rapid preparation of M13 sequencing templates for manual and automated dideoxy sequencing. Nucleic Acids Res. **15**:5507–5516.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
26. **Low, P. S., M. A. Westfall, D. P. Allen, and K. C. Appel.** 1984. Characterization of the reverse conformational equilibrium of the cytoplasmic domain of erythrocyte membrane band 3. J. Biol. Chem. **259**:13070–13076.
27. **Macara, T. G., and L. C. Cantley.** 1983. The structure and function of band 3, p. 41–87. *In* E. Elson, W. Frazier, and L. Glaser (ed.), Cell membranes: methods and reviews, vol. 1. Plenum Publishing Corp., New York.
28. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687–701.
29. **Martin, S., E. Paoletti, and B. Moss.** 1975. Purification of mRNA guanylytransferase and mRNA (guanine-7-)methyltransferase from vaccinia virions. J. Biol. Chem. **250**:9322–9329.

29a. **Mawby, W. J., and J. B. C. Findlay.** 1983. Characterization and partial sequence of di-iodosulphophenyl isothiocyanate-binding protein from human erythrocyte anion transport protein. Biochem. J. **205**:465–475.

30. **McClements, W., and A. M. Skalka.** 1977. Analysis of chicken ribosomal RNA genes and construction of lambda hybrids containing gene fragments. Science **196**:195–197.
31. **Murthy, S. N. P., T. Liu, R. K. Kaul, H. Kohler, and T. L. Steck.** 1981. The aldolase-binding site of the human erythrocyte membrane is at the NH_2 terminus of band 3. J. Biol. Chem. **256**: 11203–11208.

32. **Pappert, G., and D. Schubert.** 1983. The state of association of band 3 protein of the human erythrocyte membrane in solutions of nonionic detergents. Biochim. Biophys. Acta **730**:32–40.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
34. **Schluter, K., and D. Drenckhahn.** 1986. Co-clustering of denatured hemoglobin with band 3: its role in binding of autoantibodies against band 3 to abnormal and aged erythrocyte. Proc. Natl. Acad. Sci. USA **83**:6137–6141.
35. **Shaw, G. M., D. Aminoff, S. P. Balcerzak, and A. F. LoBulgio.** 1980. Clustered IgG on human red blood cell membranes may promote human lymphocyte antibody-dependent cell-mediated cytotoxicity. J. Immunol. **125**:501–507.
36. **Thews, G.** 1980. Atemtransport und Säure-base status des blutes, p. 537–571. *In* R. F. Schmidt and G. Thews (ed.), Physiologie des Menschen. Springer-Verlag, Heidelberg, Federal Republic of Germany.
37. **Tsai, I.-H., S. N. P. Murthy, and T. L. Steck.** 1982. Effect of red cell membrane binding on the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. **257**:1438–1442.
38. **Wagner, S., R. Vogel, R. Lietzke, R. Koob, and D. Drenckhahn.** 1987. Immunochemical characterization of a band 3-like anion exchanger in collecting duct of human kidney. Am. J. Physiol. **253**:F213–F221.
39. **Walder, J. A., R. Chatterjee, T. L. Steck, P. S. Low, G. F. Musco, E. T. Kaiser, P. H. Rogers, and A. Arnone.** 1984. The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. J. Biol. Chem. **259**:10238–10246.
40. **Waugh, S. M., and P. S. Low.** 1985. Hemochrome binding to band 3: nucleation of Heinz bodies on the erythrocyte membrane. Biochemistry **24**:34–39.
41. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.
42. **Yamamoto, M., N. S. Yew, M. Federspiel, J. B. Dodgson, N. Hayashi, and J. D. Engel.** 1985. Isolation of recombinant cDNAs encoding chicken erythroid δ-aminolevulinate synthase. Proc. Natl. Acad. Sci. USA **82**:3702–3706.
43. **Yew, N. S., H.-R. Choi, J. L. Gallarda, and J. D. Engel.** 1987. Expression of cytoskeletal protein 4.1 during avian erythroid cellular maturation. Proc. Natl. Acad. Sci. USA **84**:1035–1039.
44. **Yu, J., and T. L. Steck.** 1975. Associations of band 3, the predominant polypeptide of the human erythrocyte membranes. J. Biol. Chem. **250**:9176–9184.
45. **Zenke, M., P. Kahn, C. Disela, B. Vennstrom, A. Leutz, K. Keegan, M. J. Hayman, H.-R. Choi, N. S. Yew, J. D. Engel, and H. Beug.** 1988. v-*erbA* specifically suppresses transcription of the avian erythrocyte anion transporter (band 3) gene. Cell **52**:107–119.