Tissue-specific Expression of Distinct Spectrin and Ankyrin Transcripts in Erythroid and Nonerythroid Cells

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ABSTRACT cDNA probes for three components of the erythroid membrane skeleton, α spectrin, β spectrin, and ankyrin, were obtained by using monospecific antibodies to screen a λgt11 expression vector library containing cDNA prepared from chicken erythroid poly(A)+ RNA. Each cDNA appears to hybridize to one gene type in the chicken genome. Qualitatively distinct RNA species in myogenic and erythroid cells are detected for β spectrin and ankyrin, while α spectrin exists as a single species of transcript in all tissues examined. This tissue-specific expression of RNAs is regulated quantitatively during myogenesis in vitro, since all three accumulate only upon myoblast fusion. Furthermore, RNAs for two of the three genes do not accumulate to detectable levels in chicken embryo fibroblasts, demonstrating that their accumulation can be noncoordinate. These observations suggest that independent gene regulation and tissue-specific production of heterogeneous transcripts from the β spectrin and ankyrin genes underlie the formation of distinct membrane skeletons in erythroid and muscle cells.

The membrane skeleton of mammalian erythrocytes lies in apposition to the cytoplasmic face of the plasma membrane, and influences cell shape, elasticity, and the lateral mobility of membrane components (reviewed in references 5, 9, and 14). The major component of the membrane skeleton, spectrin, is a heterodimeric protein containing an α-subunit (molecular weight 240,000) and a β-subunit (molecular weight 220,000). In addition to interacting with actin and protein 4.1, spectrin binds to the extrinsic membrane protein ankyrin (molecular weight ~210,000) (13, 18, 52, 53). The association of ankyrin with the transmembrane anion transporter (4, 30) and of protein 4.1 with the transmembrane protein glycoporphin (1), serves to anchor these and other skeleton proteins to the membrane. Many of the erythroid membrane-skeleton proteins, such as spectrin (6, 10, 21, 24, 45), ankyrin (15, 42), and protein 4.1 (2, 26, 29), have recently been found in avian and mammalian nonerythroid cells.

It is likely that the architecture and specialized function of membrane skeletons differ in erythroid and nonerythroid cells, and that this specialization has arisen through the tissue-specific, developmentally regulated expression of multiple variants for each of these polypeptides. In nonerythroid chicken tissues, spectrin heterodimers are composed of an α-subunit highly homologous to erythroid α spectrin (45), and a variable subunit. In most nonerythroid cell types this variable subunit is the γ-subunit (23; also referred to as fodrin, reference 36), which has a peptide map distinct from erythroid β-spectrin (22, 43). In skeletal and cardiac muscle, the variable subunit is highly homologous to the β-subunit of erythroid spectrin (41). The third known variable subunit, the TW260 polypeptide (22), is found only in intestinal epithelial cells. Certain neurons in the central nervous system co-express the β- and γ-subunits in distinct membrane domains (35), further suggesting that the variable subunits impart specialized properties to membrane skeletons.

Ankyrin also exists as multiple variants, since brain and erythrocyte ankyrin have distinct peptide maps (15), and the erythroid and myogenic ankyrin isoforms have different electrophoretic mobilities (42). Multiple tissue-specific variants of protein 4.1 have also been identified (29).

The molecular mechanisms by which membrane skeletons acquire distinct isoforms of their constituent polypeptides are currently unknown. To aid in elucidating the mechanisms by which distinct membrane skeleton isoforms are produced, we have isolated cDNA probes specific for chicken α spectrin, β spectrin, and ankyrin using antibodies to screen an expression vector library (55). These probes have enabled us to demonstrate that chicken myogenic cell transcripts for β spectrin and ankyrin, but not α spectrin, are different in size from their erythroid counterparts, and levels of all three myogenic transcripts increase upon myoblast fusion. The evidence suggests that this mRNA diversity arises from single genes for
each spectrin subunit and for ankyrin, and is regulated in a tissue-specific manner upon cell differentiation. Differential accumulation of these three RNA species in chicken embryo fibroblasts support the premise that the genes coding for these membrane skeleton proteins are independently regulated.

MATERIALS AND METHODS

Isolation of RNA from Nonerythroid Cells: Myogenic cells from chicken embryo thighs were prepared according to Gard and Lazarides (20) and were 95% free of fibroblasts. Myoblasts were harvested after 12 h of culture, a time at which <10% of the cells were fusng; early myotubes were obtained at 47 h after plating, when 80% of the cells had fused. Chicken embryo fibroblasts devoid of myogenic cells were prepared according to Gard et al. (19). Cultured cells were washed once with calcium-magnesium-free Earle’s balanced salt solution (EBSS), then lysed in a solution containing 5 M guanidinium thiocyanate, layered over a CsCl cushion, and centrifuged as previously described (11). Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography according to Aviv and Leder (3).

Construction of agt11 cDNA Libraries from Erythroid RNA: Erythroid cells from 14-15-d-old chicken embryos were obtained and washed as described by Granger et al. (28). Cells were lysed in 70-100 vol of the guanidinium thiocyanate buffer, homogenized 10 times in a tight-fitting glass Dounce homogenizer, forced twice through a 22-gauge needle to further shear DNA, then layered over a CsCl pad and centrifuged as described. Poly(A)-containing RNA was purified by three cycles of oligo(dT)-cellulose chromatography. Approximately 200 μg of poly(A)* RNA was layered over an 11.5 ml 4-20% sucrose gradient containing 70% formamide, 3 mM triethanolamine (pH 7.4), and 3 mM EDTA, and centrifuged in a Beckman SW41 rotor (Beckman Instruments, Palo Alto, CA) for 38 h at 39,000 rpm (20°C). Fractions of 400 μl were collected, and the RNA precipitated with 0.24 M NH₄Ac and 2 vol of ethanol at −80°C. The RNA was pelleted for 30 min at 12,500 g in a microfuge, washed twice with 70% ethanol, dried under N₂, and dissolved in 20 μl of glass distilled water. Subsequent RNA blotting experiments showed this method to be unreliable in terms of degradation, and for maximum reproducibility subsequent erythroid RNA was prepared by phenol extraction as described below (see Positive Hybrid Selected Translation).

A high activity rabbit reticulocyte lysate was prepared according to Jackson and Hunt (31), except that the final wash of reticulocytes substituted KCl for NaCl, and the reticulocytes were lysed in 1.5 vol of 33 mM KCl, 1.67 mM dithiothreitol instead of water. RNA fractionated by sucrose gradients was translated in the reticulocyte lysate (1 μl RNA in 10 μl lysate for 90 min at 30°C), and 10,000 cpm of trichloroacetic acid-insoluble [35S]methionine-labeled from each fraction was loaded on a 12.5% polyacrylamide SDS gel. Fluorography of the gel using Enhance (New England Nuclear, Boston, MA) revealed several fractions containing spectrin as the predominant translatable RNAs (Fig. 1).

Two libraries were prepared from the gradient-fractionated poly(A)* RNA. The L library was prepared from RNA in gradient fractions 6 to 10 (Fig. 1), and was enriched in cDNAs for large RNAs such as spectrin and ankyrin. The M library was prepared with cDNA for smaller RNAs (fractions 11 to 16 in Fig. 1) to enable screening for other membrane skeleton clones. cDNA was synthesized using oligo(dT)₁₅ as primer (16), in the presence of 1 mM methylmercury hydroxide. The second strand was synthesized with DNA polymerase I large fragment, then continued with reverse transcriptase. Double-stranded cDNA was digested with S1 nuclease, methylated with EcoRI methylase, and the ends were blunted with DNA polymerase I large fragment. EcoRI linkers (New England Biolabs, Beverly, MA) were phosphorylated and ligated onto the double-stranded DNA template. For restriction enzyme digestion with EcoRI, the cDNA was separated from linkers and enriched for larger cDNA’s by differential precipitation from 2 M NH₄Ac by the addition of 0.8 vol isopropanol for 30 min (20°C) followed by centrifugation. After repeating four times, isopropanol was lowered to 0.6 vol for three additional precipitations.

Agt11 DNA (55) was prepared by dialysis against formamide (51), the cos ends were ligated with T4 DNA ligase, and the DNA was digested with EcoRI. Calf intestinal alkaline phosphatase (CIAP) was heated to 62°C for 6 min to inactivate contaminating nucleases, then used to dephosphorylate DNA for 30 min at 37°C at a ratio of 0.015 U CIAP/μg DNA. The reaction was digested with proteinase K in SDS and the DNA was extracted with phenol and then precipitated with ethanol. The resulting vector was able to ligate with EcoRI-cut pBR322, and to form plaques that inactivated the lacZ gene (55) at an efficiency of 2 x 10⁸ plaques per μg vector, with 95% of the plaques containing pBR322 (as an insert/vector molar ratio of 1:1).

cDNA was ligated into EcoRI-digested agt11 at an estimated two- to fivelfold molar excess of cDNA. Infection efficiencies in Escherichia coli strain LE392

![Figure 1](https://example.com/image1.png)  
**FIGURE 1** Translation of aliquots of alternate sucrose gradient fractions displaying functions used to prepare the L (fractions 6-10) and M (fractions 11-16) cDNA libraries. Fraction numbers and the pelleted (P) material are noted at the top of the figure, with the direction of centrifugation from right to left.

were 5 x 10⁸ plaques per μg vector for L cDNA (75% contained insert), and 3 x 10⁸ plaques/μg vector for M cDNA (80% contained insert). For the L library 650,000 plaque-forming units, and for the M library 1.2 x 10⁸ plaque-forming units were used to prepare permanent amplified libraries by plate amplification in two E. coli strains, LE392 and Y1088.

**Screening the agt11 Library:** Overnight cultures (200 μl) of E. coli strain Y1088 (56) or LE392 were infected with 50,000 plaque-forming units of recombinant agt11 from the amplified L library, diluted with 7 ml L-top agar, and plated on 150 mm L-plates (containing ampicillin for Y1088) at 42°C. Nitrocellulose filters (Millipore Corp., Bedford, MA, type HA, 0.45 μm) were impregnated (for Y1088 only) with 10 mM isopropyl-β-d-thiogalactopyranoside (IPTG, from Calbiochem-Behring Corp., La Jolla, CA) for 1 min, followed by air drying. At the earliest sign of plaque formation (3-4 h), the filters were placed onto the top agar and the plates cultured upside-down at 37°C for 10-12 h. The filters were then 45° rotated with a 50 μl filter tip, and washed with TBS (150 mM NaCl, 15 mM Tris.Cl pH 7.5, 1 mM EDTA, 5 mM NaN₃) for 15 min in a plastic tray to remove bacterial debris. The filters were then incubated with 40 μl filter of G buffer (TBS with 0.1% gelatin) at 37°C for 2-4 h with continual rocking. The filters were then incubated at 37°C for an additional 2 h with G buffer supplemented with 0.1% Tween 20 (GT buffer, reference 29).

Polyonal antibodies monospecific for α spectrin (45), β spectrin (41), and ankyrin (designated “goblin” in birds) (42) have been previously characterized. The goblin antibodies (42) react with the same chicken polypeptide (37) recognized by antibodies against human ankyrin. For initial screenings, all three antisera were mixed together. Antibodies were preadsorbed against bacteria and phage by mixing 100 μl of each antiserum with 100 μl overnight culture of Y1088 or LE293 in 10 ml G buffer for 30 min at 20°C. The bacteria were then removed by centrifugation and the supernatant was poured onto a damp L-plate containing 50,000 plaque-forming units of agt11 with no cDNA insert. After 30 min at 20°C the antibody solution was diluted in GT buffer to give an antibody dilution of 1:1,500, then incubated with the nitrocellulose filters (25-50 μl filter) for 2 h at 22°C. Unbound antibody was removed by six washes with GT buffer for 15 min each. [35S]labeled protein A was added to 0.2-0.3 μCi/ml for 1-2 h (20°C), followed by six washes with GT buffer as above. After air drying and exposure to Kodak X-AR5 X-ray film overnight, positive plaques were recovered. All positive plaques were re-screened with separate antisera until homogeneous phage populations were obtained (Fig. 2). Of the 500,000 plaques screened, 9 of 20 initial positives rescreened as positives, with four for ankyrin, three for β spectrin, and two for α spectrin. α spectrin clones were obtained only from agt11-infected Y1088, whereas β spectrin and ankyrin clones were obtained with either strain of E. coli. This result demonstrates the utility of using Y1088 for screening, since this strain carries a plasmid that represses expression of foreign genes (which may be detrimental to host cell growth) until induction with IPTG (56). Strong positive signals correlated well with larger cDNA inserts.

**Subcloning agt11 Inserts into pBR322:** cDNA inserts were
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Figure 2 Autoradiography of a typical nitrocellulose filter replica of plated recombinant αgt11, upon final rescreening with spectrin or ankyrin antisera and antibody localization with [3H]-protein A.

obtained from recombinant αgt11 by digestion with EcoRI. Restriction fragments were isolated following electrophoresis in low melting point agarose gels (54), and ligated into the EcoRI site of pBR322. Recombinant plasmids were then used to transform E. coli HB101.

Positive Hybrid-selected Translation: Identification of clones by positive hybrid-selected translation required modification of previous methods (46). Recombinant pBR322 were linearized by restriction at sites outside the inserts, phenol extracted, and 10 μg was bound to nitrocellulose (11). Alternatively, Pall Biodyne Pall A 1.2-μm nylon filters were cut into 3 x 3-mm squares, and 15 μg of linearized plasmid in 5 μl of 10 mM Tris, pH 7.5, 0.1 mM EDTA was spotted onto the filter and air dried. The filter was placed on a Whatman 3MM filter (Whatman Chemical Separation, Inc., Clifton, NJ) saturated with 1.5 M NaCl, 0.5 N NaOH for 5 min, then on a filter saturated with 3 M NaAc, pH 6, for 5 min. The wet filter (covered by a thin layer of 3 M NaAc) was air dried, covered by a thin layer of 3 M NaAc, pH 6, for 5 min. The wet filter (covered by a thin layer of 3 M NaAc) was then added to the eluate to 0.5 M and 0.2%, respectively, followed by purification of poly(A)-negative RNA from the column with 200 ng of ssDNA gradient-purified RNA mixture.

Erythroid RNA for hybrid-selected translation was obtained by extraction with phenol (49) of postmitochondrial supernatants of 14-d chick embryo erythroid cells. Spectrin and ankyrin RNA prepared by this method, unlike lamp at 400°C (pH 6.4), 0.6 M NaCl, 2 mM EDTA, 50 μg/ml poly(A), 100 ng/ml tRNA at sucrose gradient-purified globin mRNA. After hybridization, the filters were washed as previously described (11), except that SSPE was substituted for SSC.

Identification and Characterization of Spectrin and Ankyrin Polyribosomes: Three 5-6 mo old white Leghorn chickens were made anemic by injection into breast muscle of 0.5 ml 2.5% phenylhydrazine on days 1, 2, 4, and 5. On day 7 each was injected with 2 ml of 16% methyl hematin intravenously, then injected intramuscularly with 3 ml Nembutal. Birds were bled from the jugular vein into 1 liter of NKM (130 mM NaCl, 5 mM KCl, 7.5 mM MgCl₂, 0.1 mM EGTA, 10 mM triethanolamine, pH 7.4, 20 μM cycloheximide, 20 μM emetine) kept at −2°C, which was then filtered through cheesecloth. Erythroid cells were pelleted five times in a GSA rotor at 2,000 rpm (5 min) followed by removal of the buffy coat after centrifugation. Washed erythroid cells were lysed in 5.5 vol of 20 mM KCl, 4 mM MgAc, 2 mM EGTA, 10 mM triethanolamine (pH 7.4), 2 mM dithiothreitol, 20 μM emetine, 20 μM cycloheximide, 0.1 mM leupeptin, 50 μg/ml heparin, 200 μg/ml RNasin (Promega Biotech, Madison, WI). Lysates were centrifuged in an SS34 rotor for 10 min at 8,000 rpm (0°C). 2.5 M KCl was added to the supernatant to a final concentration of 145 mM and 10% NP40 was added to 0.1% (vol/vol). The supernatant was layered over 9 ml cushions of 50% sucrose (preliminary in lysis buffer containing 50 mM KCl) and centrifuged in a Beckman 60 Ti rotor (Beckman Instruments, Palo Alto, CA) at 35,000 rpm for 3 h at 4°C. Pelleted polyribosomes were suspended in 125 mM KCl, 4 mM MgAc, 2 mM EGTA, 10 mM triethanolamine (pH 7.4), 5% sucrose, 2 mM dithiothreitol, 40 μg/ml RNasin, 2 mg/ml heparin, 20 μM emetine, 0.1 mM leupeptin. 1 mM Na₃C₅O₄. Insoluble material was removed by centrifugation, and the polyribosomes were frozen in liquid nitrogen.

Antisera for α spectrin, β spectrin, and ankyrin (1 ml each) were pooled, and the IgG purified on a 5-ml column of protein(A)-sepharose (34), and concentrated by negative pressure dialysis against 150 mM NaCl, 20 mM NaPO₄, pH 7.6, to 40 mg/ml protein. Purified IgG contained no ribonuclease activity as determined by in vitro translation and RNA blot analysis of RNA preincubated with IgG.

Polyribosomes were thawed, diluted to 18 ml with column buffer (polyribosome buffer replacing KCl with NaCl), and 4 mg of purified IgG was added to 26 μg U of polyribosomes. After gentle inversion for 1 h at 4°C, 0.2 g protein(A)-sepharose was hydrated and added for an additional hour. The protein(A)-sepharose was washed three times with 50-ml column buffer by centrifugation, then poured into a glass column and washed with 100 ml additional buffer at a flow rate of 1.5 ml/min. Polyribosomal RNA was released with 20 mM EDTA, 20 mM triethanolamine (pH 7.4); NaCl and SDS were then added to the eluate to 0.5 M and 0.2%, respectively, followed by purification of poly(A)-specific polyribosomal RNA on 0.1 g oligo(DT)-cellulose. Poly(A)⁺ RNA was eluted from the column with 10 μg/ml RNA, and precipitated from 0.24 M NH₄Ac with 2 vol of ethanol at −80°C. Aliquots of the RNA were used for in vitro translation and RNA blot analysis.

Restriction Endonuclease Mapping: The restriction endonuclease sites of cDNA subcloned into pBR322 were determined by digestion with one or several enzymes, resolution of restriction fragments on agarose gels, and assignment of sites relative to known sites in pBR322 or established sites in the DNA insert.

DNA and RNA Blotting and Hybridization: Chicken liver DNA (kindly provided by Dr. Y. Capetanaki, Caltech) was digested with restriction endonucleases, separated on 0.9% agarose gels, and transferred to nitrocellulose (30). RNA electrophoresis and blotting was as described by Capetanaki et al. (11).

cDNA inserts from pBR322 were excised with EcoRI, isolated from low melting point agarose gels (54), and used to prepare [3H]-labeled nick-translated probes (47), with specific activities generally 1-10 x 10⁶ cpm/μg DNA. The pSC5 chicken vimentin cDNA (11) was nick-translated and used to detect vimentin sequences. Probes were hybridized to nitrocellulose filters, and the filters washed as previously described (11), except that SSPE was substituted for SSC.

RESULTS

Identification and Characterization of Spectrin and Ankyrin cDNA Clones

An expression vector library was constructed in αgt11 (55) using as a template gradient-fractionated chicken erythrocyte poly(A)⁺ RNA enriched in high molecular weight RNA. Putative α spectrin, β spectrin, and ankyrin cDNA clones were identified by several rounds of screening with monospecific
antisera for α spectrin, β spectrin, or ankyrin (see Materials and Methods and Fig. 2). Inserts were isolated by digestion with EcoRI and subcloned into pBR322. Identification of these cDNA clones was confirmed by positive hybrid-selected translation. Poly(A)^+ RNA from embryonic chicken erythroid cells was hybridized with filter-bound recombinant pBR322, and the hybridized RNA was eluted and translated in vitro.

The major translation products of the selected RNAs co-migrate on SDS polyacrylamide gels with ankyrin and the spectrin subunits, and react specifically with the ankyrin and spectrin antibodies used to isolate the recombinant λgt11 (Fig. 3). The translation product of RNA which hybridized to the putative ankyrin cDNA (pA1-4 ankyrin) (lane 3) co-migrates with the ankyrin/α spectrin band of erythroid poly(A)^+ RNA (lane 2), and immunoprecipitation with anti-ankyrin antisera of both translation reactions yields ankyrin (lanes 6 and 7). Similarly, pA2-2 α spectrin is confirmed as an α spectrin cDNA, as it selects mRNA encoding a protein (lane 4) which co-migrates with the ankyrin/α spectrin band in a total poly(A)^+ RNA translation (lane 2), and which is identified as α spectrin by immunoprecipitation (lanes 8 and 9). Finally, hybrid-selected translation with the pA4-1 β spectrin cDNA yields a translation product (Fig. 3, lane 5) which is identified by immunoprecipitation as β spectrin (Fig. 3, lanes 10 and 11). Since each of these antisera cross-reacts solely with the polypeptide used as immunogen, the immunoprecipitation data demonstrate that all three cDNAs hybrid-select distinct mRNAs.

Antiserum specific for the intermediate filament protein vimentin was included in the immunoprecipitation from hybrid-selected translations as an internal control to demonstrate that the ankyrin and spectrin signals were enhanced above that of other RNAs, such as vimentin, which is approximately tenfold more abundant than spectrin or ankyrin (quantitation not shown). Although vimentin is readily identified by immunoprecipitation of 2 μl of reticulocyte lysate after translation of erythroid poly(A)^+ RNA (lane 13), no vimentin is obtained by immunoprecipitation of 5 μl of lysate in lanes 6, 8, or 10, demonstrating the specificity of the cDNA probes for hybrid-selecting ankyrin or spectrin mRNAs. Many [35S]methionine-labeled bands are evident in Fig. 3, lanes 3–5, migrating below the indicated ankyrin and spectrin bands. The major bands at the bottom of the gel represent endogenous bands (lane 1). Most of the minor higher molecular weight bands are present in all hybrid-selected translations and are therefore due in part to nonspecific mRNA binding. Some minor bands represent α and β spectrin degradation products, as demonstrated by the recovery of these minor bands by immunoprecipitation (Fig. 3, lanes 8 and 10).

A second line of evidence supporting the designation of pA2-2α spectrin, pA4-1β spectrin, and pA1-4 ankyrin as spectrin and ankyrin clones was obtained by the analysis of immunoprecipitated polynucleosomes. Antisera for these three proteins were mixed, and used to immunoprecipitate polynucleosomes obtained from erythroid cells of anemic chickens. The polynucleosomal poly(A)^+ RNA contains RNA sequences that hybridize to the pA2-2α spectrin (Fig. 7A, lane 2) and pA4-1β spectrin (Fig. 7B, lane 2) cDNA probes (pA1-4 ankyrin not determined); no hybridization is observed when a vimentin cDNA is used as probe (Fig. 7D, lane J). Significantly, poly(A)^+ RNA from these polynucleosomes directs the in vitro translation solely of α spectrin, β spectrin, and ankyrin (Fig. 4, lane 2 and confirmed by immunoprecipitations, not shown).

The spectrin and ankyrin cDNA clones were further characterized by size and restriction sites. The insert in the pA1-4 ankyrin cDNA is 2.8 kb in size, the pA4-1 β spectrin insert is 1.8 kb, and the pA2-2 α spectrin insert is 0.6 kb, as determined by migration in agarose gels relative to known restriction fragments of λ and pBR322 DNA. These inserts represent,
FIGURE 4 Immunoprecipitated spectrin and ankyrin polyribosomes contain translatable RNA for spectrin and ankyrin. Lane 1, [35S]methionine-labeled in vitro translation products of the pooled gradient-fractionated RNA used to prepare the L cDNA library. This provided markers for ankyrin (A), α spectrin (a), and β spectrin (β). Lane 2, in vitro translation products of poly(A)+ RNA prepared from polyribosomes that had been immunoprecipitated with ankyrin and spectrin antibodies reveals spectrin and ankyrin (also by immunoprecipitations not shown), but no contaminating globin. Lane 3, translation products of poly(A)+ RNA from chicken embryo erythroid cells, with large globin band evident. Lane 4, endogenous translation products without added RNA. 5 μl of each translation reaction was applied to an SDS 10–20% exponential polyacrylamide gel that was then processed for fluorography.

that the weak bands represent a second homologous gene is rendered less likely, as previously argued for the Drosophila myosin heavy chain gene (47).

Distinct Spectrin and Ankyrin Transcripts in Erythroid and None erythroid Cells

To investigate the qualitative and quantitative nature of homologous RNAs in various cell types, poly(A)+ RNA was respectively, 32, 25, and 8% of the lengths of the erythroid mRNAs to which the probes hybridize (Fig. 7). Restriction maps for the three cDNAs are shown in Fig. 5.

Hybridization of Spectrin and Ankyrin Probes to Genomic DNA

The pA2-2 α spectrin and pA4-1 β spectrin cDNAs each appear to hybridize to a distinct single gene type, as determined by hybridizing 32P-labeled cDNA probes to filter-bound chicken genomic DNA restricted with BamHI, EcoRI, or HindIII (Fig. 6). The pA2-2 α spectrin (Fig. 6A) and pA4-1 β spectrin (Fig. 6B) probes each hybridize to a single band from DNA digested with EcoRI (lane R) and to two bands from DNA digested with HindIII (lane H), which has a known restriction site within each of the two cDNAs (Fig. 5). The generation of two BamHI fragments which hybridize with pA2-2 α spectrin (Fig. 6A, lane B), and two distinct bands which hybridize with pA4-1 β spectrin (Fig. 6B, lane B) is not predicted based on restriction maps of these probes which show no BamHI sites (Fig. 5), but could arise through BamHI sites within intervening sequences. No additional bands homologous to the pA2-2 α spectrin and pA4-1 β spectrin probes were obtained by lowering the criterion of hybridization and filter washes by 12–15°C (data not shown).

Southern blot analysis with the pA4-1 ankyrin cDNA suggests that this cDNA also recognizes a single distinct gene type (Fig. 6C). One strong and one faint band were obtained after digestion with BamHI, EcoRI, and HindIII (Fig. 6C), although none of these enzymes have restriction sites within the cDNA (Fig. 5). Since reducing the criterion of hybridization and filter washes by 15°C did not increase the relative intensity of the weak bands (data not shown), the possibility

FIGURE 5 Maps of restriction endonuclease sites within each cDNA.

FIGURE 6 Genomic DNA blot analysis with cDNA probes for α spectrin, β spectrin, and ankyrin. Chicken liver genomic DNA was digested with BamHI (B), EcoRI (R), or HindIII (H) prior to electrophoresis, blotting, and hybridization to nick-translated 32P-labeled pA2-2 α spectrin (A), pA4-1 β spectrin (B), or pA1-4 ankyrin (C). Size markers (in kilobases) denoted in A and by dots in B and C are several of the restriction fragments of HindIII digested λ DNA.
separated on formaldehyde-agarose gels, blotted onto nitrocellulose, and hybridized with nick-translated 32P-labeled spectrin and ankyrin cDNAs. A single α spectrin transcript of 8 kb (Fig. 7A, lane 1), a single β spectrin transcript of 7.1 kb (Fig. 7B, lane 1), and a single major ankyrin transcript of 8.8 kb (Fig. 7C, lanes 1 and 2) are present in poly(A)+ RNA of 14-d-old chicken embryo erythroid cells. Some smaller RNAs are detected as minor bands by the ankyrin probe (Fig. 7C, lane 1). All three major RNAs are of sufficient size to encode chicken α spectrin, β spectrin, and ankyrin.

It has previously been demonstrated that cultured myogenic cells switch from an αγ spectrin to an αβ spectrin phenotype (40). Here we show that spectrin and ankyrin RNAs accumulate during myogenesis in vitro. Although some α spectrin is synthesized in myoblasts (40), the level of spectrin and ankyrin RNAs in myoblasts 12 h after plating is below the level of detection, as judged by RNA blot analysis (Fig. 7, A and B, lane 5, Fig. 7C, lane 4). By 47 h after plating, 80% of the cells have fused, and mRNA for α spectrin (Fig. 7A, lane 4), β spectrin (Fig. 7B, lane 4), and ankyrin (Fig. 7C, lane 3) are detected. As a positive control, we show that the myoblast RNA contains sequences that hybridize to a vimentin probe (Fig. 7B, lane 4).

Surprisingly, the myotube form of β spectrin (Fig. 7B, lane 4) is ~0.7 kb larger than its erythroid counterpart (Fig. 7B, lanes 1 and 2) (7.8 vs. 7.1 kb). This size difference is confirmed when the erythroid and myoblast RNAs are co-electrophoresed (Fig. 7B, lane 3). The converse size disparity is obtained by comparing erythroid and myotube RNAs homologous to the ankyrin cDNA probe, as the myotube ankyrin RNA (Fig. 7C, lane 3) is 5.4 kb smaller (8.8 vs. 3.4 kb) than the erythroid form of ankyrin RNA (Fig. 7C, lanes 1 and 2). α spectrin RNAs in both myogenic cells (Fig. 7A, lane 4), and erythroid cells (Fig. 7A, lanes 1 and 2) are similar in size as evidenced by their mobility in formaldehyde-agarose gels with an apparent size of 8 kb, and by the generation of only a single α spectrin band when the two RNAs are mixed prior to electrophoresis and blotting (Fig. 7A, lane 3). No spectrin or ankyrin transcripts were detected in erythroid, myoblast, or myotube poly(A)+ RNA (data not shown).

The unexpectedly small size of the myotube form of ankyrin raises reasonable concern that the observed band is an artifactual signal. This possibility is unlikely since the myotube ankyrin band does not co-migrate with ribosomal RNA or with any prominent band stained by ethidium bromide, nor is it obtained with total rather than poly(A)+ RNA (data not shown). Further, its presence is not a result of culturing cells in vitro, since the 3.4-kb ankyrin transcript is detected at comparable levels in poly(A)+ RNA from chick cardiac muscle, but from no other tissue examined (data not shown).

Spectrin and ankyrin mRNAs are more abundant in erythroid cells than in intestine, cerebellum, liver, kidney, or cardiac muscle (data not shown). Among nonerythroid tissues surveyed, cultured myotubes appear to be a major source of these transcripts. From separate RNA blots we find that there is 5–10-fold more α spectrin, β spectrin, and ankyrin RNA in...
erythroid cells than in cultured myotubes. However, precise quantitation is not meaningful since the RNAs were necessarily prepared by different methods (see Materials and Methods).

**Noncoordinate Expression of α Spectrin, β Spectrin, and Ankyrin Transcripts**

As both erythroid and myogenic cells express some form of α spectrin, β spectrin, and ankyrin RNA, we wished to determine whether the co-expression of RNAs homologous to the three cDNA probes was obligatory in any cell type known to express any of these polypeptides, or alternatively, whether these RNAs can accumulate independently. We investigated the existence of spectrin and ankyrin RNAs in cultured chicken embryo fibroblasts since it was previously observed that α spectrin and γ spectrin polypeptides are present in these cells (23), but not β spectrin or ankyrin (Nelson, W. J., and E. Lazarides, unpublished results). RNA blot analysis reveals that poly(A)+ RNA from chicken embryo fibroblasts contains α spectrin RNA (Fig. 8A, lane 2) indistinguishable from erythroid α spectrin (Fig. 8A, lane 1), but no RNA which hybridizes to the β spectrin (Fig. 8B, lane 2) or ankyrin cDNA probes (Fig. 8C, lane 2). Accumulation of the α spectrin, β spectrin, and ankyrin mRNAs homologous to these probes therefore must be regulated independently in nonerythroid cells.

**DISCUSSION**

We report here the isolation of cDNA probes specific for three components of the membrane skeleton of erythroid cells, α spectrin, β spectrin, and ankyrin. Isoforms of these membrane skeleton components have been detected in nonerythroid cells (see Introduction), but little is known about the molecular mechanisms responsible for generating antigenically related isoforms, or whether spectrin and ankyrin-like molecules are co-expressed. As discussed below, heterogeneity in RNA transcripts exists for some of these polypeptides, and may reflect an even greater diversity in membrane skeleton proteins than had been expected. Furthermore, these probes have established that spectrin and ankyrin transcripts are developmentally regulated during myogenesis in vitro, and that spectrin and ankyrin transcripts are not co-expressed in chicken embryo fibroblasts. Taken together, these results provide the initial evidence that the expression of nonerythroid membrane skeleton transcripts can be regulated noncoordinately and that β spectrin and ankyrin may be encoded by tissue-specific transcripts.

**Heterogeneity in Spectrin Transcripts**

The pA4-1 β spectrin probe hybridizes to RNAs in cells known (41) to contain β spectrin (erythroid and myogenic cells), but not to RNAs in cells known (23) to contain predominantly γ spectrin (e.g., chicken embryo fibroblasts) or the TW260 polypeptide (intestine, data not shown). Hence, the cDNA demonstrates specificity for β spectrin transcripts and the lack of homology with RNAs for other variable subunits that bind α spectrin. The absence of an RNA in fibroblasts or intestine which hybridizes to the β spectrin cDNA further suggests that the γ spectrin and TW260 subunits, with their distinct peptide maps (22), are transcribed from separate genes distinct from the β spectrin gene.

The unexpected result obtained on RNA blots with the β spectrin probe demonstrates that the β spectrin transcript in myotubes is larger than the erythroid β spectrin mRNA. If myogenic and erythroid β spectrins are identical polypeptides encoded by distinct mRNAs, the observed differences in transcript size is significant in that it reflects unsuspected differences in tissue-specific spectrin mRNA expression. On the other hand, it is possible that the differences in transcript size also reflect differences within the coding regions and subtle differences consequently may exist between the erythroid and myobrist β spectrin polypeptides. Therefore, cells may be able to express β spectrin, γ spectrin, or the TW260 polypeptide differentially to impart specialized properties to membrane skeletons, and they may also be able to make subtle tissue-specific alterations within these classes of polypeptides. That variants of β spectrin exist is supported by the apparent presence in erythroid and myogenic cells of an immunologically-related variant, β'-spectrin (40, 41). It is unclear whether the observed myobrist β spectrin mRNA codes for this variant, however, since cultured myotubes have been reported to synthesize both β and β'-spectrin (40).

DNA blot analysis suggests that the cDNA probes for α spectrin, β spectrin, and ankyrin each hybridize to a unique gene type in the chicken genome, although the copy number of each gene has not been determined. We cannot exclude the possibility that the observed genomic restriction fragments homologous to the cDNA probes are produced from more than one gene type, and hence further study will be required to fully define the organization of the genes encoding spectrin and ankyrin.

If chicken erythroid and myogenic cells do in fact generate β spectrin mRNA diversity from a single gene, several mechanisms could be involved. Such mechanisms include the use of differential transcriptional initiation sites, differential termination of transcription or 3' post-transcriptional processing, or differential splicing. The generation of β spectrin mRNA heterogeneity through such mechanisms has precedent within other myogenic cell structural proteins. Although transcript diversity in myogenic cells can arise through differential expression of multigene families (reviewed in reference 17), the production of multiple mRNAs from single genes is evident for Drosophila myosin heavy chain (48), chicken
myosin light chain (39), and other Drosophila myofibrillar proteins (32).

In contrast to the observed pattern of β spectrin RNAs, α spectrin RNAs in myogenic and erythroid cells are indistinguishable. Since α spectrin has not been shown to have isoforms in different chicken tissues (45), the conservation of α spectrin transcripts and the evidence for a single chicken α spectrin gene support the notion that this protein is invariant in chicken tissues.

Heterogeneity in Ankyrin Transcripts

The large disparity in size between the 3.4-kb myogenic ankyrin transcript and the 8.8-kb erythroid ankyrin transcript suggests a molecular basis for the biogenesis of different ankyrin-like polypeptides in these cell types (42). Previous studies have indicated that chicken myogenic cells express an antigenically-related ankyrin-like polypeptide with an apparent molecular weight of 235,000 compared with a molecular weight of 260,000 for the erythroid form of this polypeptide (42). The 8.8-kb transcript detected with the pA1-4 ankyrin cDNA in erythroid cells is of sufficient length to encode the 260,000-mol wt polypeptide detected in erythroid cells. However, the 3.4-kb transcript detected in myogenic cells would have a maximum coding potential corresponding to a polypeptide of ~125,000 molecular weight; therefore it is difficult to reconcile the existence of a small transcript with the presence of a polypeptide which exceeds the coding capacity of the transcript. One possibility is that the observed myotube ankyrin-like polypeptide (M, 235,000; 42) is encoded by a transcript of comparable size to the erythroid ankyrin transcript, but that these two transcripts have undergone sufficient evolutionary divergence so that the erythroid cDNA does not hybridize to this hypothetical myotube transcript in the region represented in the pA1-4 ankyrin cDNA. Some divergence between erythroid and myogenic ankyrins is expected due to the different apparent molecular weights of the polypeptides in the two cell types (42). The observed 3.4-kb myotube ankyrin-like transcript may reflect a less divergent ankyrin-like RNA sequence which is either not a functional mRNA, or which encodes a polypeptide smaller than erythroid ankyrin, and not recognized by the erythroid ankyrin antisem. Indeed, homology at the nucleic acid level within a limited region need not be reflected directly in the degree of antigenic relatedness between two polypeptides. A second explanation for the observed data is that the 3.4-kb transcript may encode the observed myotube ankyrin-like polypeptide (M, 235,000), but that the size of this polypeptide is grossly overestimated by SDS PAGE. A large disparity between molecular weight and electrophoretic mobility has been shown recently to be the case for one of the neurofilament proteins (33), and it has been previously observed that ankyrin indeed exhibits anomalous mobility in different electrophoretic systems (28, 42).

Developmental Regulation of Spectrin and Ankyrin Transcripts

RNAs homologous to erythroid α spectrin, β spectrin, and ankyrin accumulate during myogenesis in vitro. These results indicate that the greater synthesis of α and β spectrins (40) and ankyrin (Nelson, W. J., and E. Lazarides, unpublished results) in myogenic cells upon myoblast fusion is controlled primarily at the level of RNA accumulation. The observed size differences between the respective myogenic and erythroid RNAs for β spectrin and ankyrin imply tissue-specific developmental controls for the expression of these two membrane skeleton components. Other systems displaying developmental regulation of these polypeptides could now be studied on the RNA level to help elucidate developmental control of expression. For example, it will be of interest in the future to determine whether developmentally regulated β spectrin (35) and ankyrin (Nelson, W. J., and E. Lazarides, [42a]) in neurons are translated from the erythroid or myotube forms of β spectrin and ankyrin RNAs, or from other neuron-specific RNAs. Furthermore, the differences in the tissue-specific isoforms of these large structural proteins (see Introduction) may be more readily ascertained through analysis of their RNAs than through protein chemistry.

Noncoordinate Regulation of Spectrin and Ankyrin Transcripts

Our demonstration that chicken embryo fibroblasts express α spectrin RNA but no detectable β spectrin or ankyrin RNA suggests two proposals. First, nonerythroid membrane skeletons need not contain an isoform of each erythroid membrane skeleton polypeptide, since by RNA blot analysis and polypeptide analysis (Nelson, W. J., and E. Lazarides, unpublished) we detect no ankyrin in chicken embryo fibroblasts. Therefore, although these cells express γ spectrin as well as α spectrin (23), the high affinity membrane binding site for fibroblast spectrin, if it exists, is most likely distinct from erythroid ankyrin. Second, our data demonstrate that the accumulation of α spectrin, β spectrin, and ankyrin mRNA is regulated noncoordinately, which may enable a greater degree of variation in tailoring the expression of the polypeptides to suit the requirements of a specific cell type. A greater understanding of the regulation of membrane skeleton genes and membrane skeleton assembly (37, 38) will be among the benefits obtained by the molecular dissection of hereditary hemolytic anemias in humans (25, 44) and mice (8).

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Note Added in Proof: The observation that erythroid cells contain an 8.8 kb transcript and myotubes contain a 3.4 kb transcript, both of which hybridize to the pA4-1 ankyrin cDNA (see Fig. 7), has been substantiated with another ankyrin cDNA.

REFERENCES
