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Expression of cytoskeletal protein 4.1 during avian erythroid cellular maturation

(monoclonal antibody/cDNA clone/cytoskeletal assembly)

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ABSTRACT **We have isolated a cDNA clone encoding part of protein 4.1, an integral component of the erythrocyte cytoskeleton. The recombinant was isolated by immunological screening of a chicken erythroid λgt11 cDNA library using a monoclonal antibody directed against protein 4.1. DNA blot analysis shows that the gene is present as a single copy per haploid chicken genome, while RNA blot analysis reveals the presence of a single mRNA of 7 kilobases in reticulocytes. Message of the same size (in reduced amounts) is also present in an erythroleukemic cell line transformed by avian erythroblastosis virus and is also present in vastly reduced quantities in nonerythroid hemopoietic cells. Immunoblotting and immunofluorescence experiments show that a subset of the chicken 4.1 variant proteins is preferentially expressed during *in vitro* differentiation of chicken erythroleukemic cells. These data indicate that the gene is both actively transcribed and translated during early erythroid cellular maturation.**

Protein 4.1 is a major component of the erythrocyte cytoskeleton, a network of proteins underlying the plasma membrane that is essential for maintaining the integrity and deformability of the highly dynamic mature erythrocyte (1). Protein 4.1 forms a complex with spectrin and actin, enhancing and stabilizing their interaction (2, 3). Protein 4.1 may also function in anchoring the network to the membrane by binding to the integral membrane protein glycophorin, with a secondary binding site to band 3, the anion transport protein (4, 5).

Chicken protein 4.1 is actually composed of an electrophoretically diverse family of closely related proteins. Four variants of 77, 87, 100, and 115 kDa are the most abundant species in erythrocytes, with larger molecular mass variants (145, 150, 160, and 175 kDa) present in much lower amounts. The variants are differentially phosphorylated but appear to be highly related on the basis of comparison of two-dimensional peptide maps of different purified protein 4.1 species (6).

We have used a chicken erythroleukemic cell line transformed by a temperature-sensitive (ts) mutant of avian erythroblastosis virus (ts34 AEV; see ref. 7) to examine the temporal expression, during *in vitro* differentiation, of protein 4.1. AEV has been shown to block precursor cells at the erythroid colony-forming unit stage in the temporal development of the chicken erythroid lineage (8). When shifted to the nonpermissive temperature (42°C), ts34AEV-transformed cells terminally differentiate, acquiring hemoglobin, erythroid cell-surface antigens, and a morphology clearly resembling that of mature erythrocytes (7). The isolation of a cDNA clone to protein 4.1 has allowed a preliminary investigation of the transcriptional and translational activation of this protein in both normal mature erythrocytes and transformed erythroleukemic cells.

MATERIALS AND METHODS

Protein 4.1 Monoclonal Antibody Isolation and Screening. Erythrocyte membranes were isolated from an adult chicken and purified as described (9), except that the lysed cells were gently sonicated in an ultrasonic cleaner bath (two times, 30 sec each) to disrupt the plasma membrane. The released membrane fragments containing the assembled membrane skeleton, which was left after pelleting the nuclei and debris, were then isolated by banding at the interface of a 28–50% sucrose step gradient (10). The membranes were solubilized in 0.1% $NaDodSO_4$, placed in a boiling water bath for 2 min, emulsified 1:1 (vol/vol) with complete Freund's adjuvant, and 150 μg of total membrane protein was injected into mice. Booster injections were given on days 14 and 28, and on day 32 the animals were sacrificed and the spleens were removed.

Spleen cells from an immunized animal were fused with SP2/0 myeloma cells (33) using polyethylene glycol, and hybridoma lines were selected in hypoxanthine/aminopterin/thymidine medium (11). Wells containing fusion hybrids were screened for antibody production using total erythrocyte membrane protein fixed to plastic dishes in a solid-phase radioimmunoassay (12). Culture supernatants from antibody-producing wells were then incubated with immunoblots of total erythrocyte membrane proteins fractionated on a $NaDodSO_4$/10% polyacrylamide gel, followed by reaction with ^{125}I-labeled rabbit anti-mouse IgG. This identified the particular band(s) recognized by each hybridoma (see *Results*). Positive wells were cloned by limiting dilution (11), and these clones were then expanded into 4-liter cultures. IgG from fusion cell-culture supernatants was precipitated with 50% ammonium sulfate and finally purified on a Protein A-Sepharose CL-4B column (Pharmacia).

Erythroid λgt11 Library Construction and Screening. Double-stranded cDNA was synthesized from reticulocyte poly(A)$^+$ RNA of an anemic adult hen. The first strand was synthesized with reverse transcriptase using oligo(dT) as primer (13); the second strand was generated by the procedure described by Gubler and Hoffman using RNase H and DNA polymerase I (14). The cDNA was methylated with *Eco*RI methylase, the ends were then filled with DNA polymerase I, and *Eco*RI linkers were ligated as described (15). The cDNA was fractionated on a 1-ml Sepharose 4B column; cDNAs >500 base pairs (bp) were pooled and ligated to λgt11 arms. After *in vitro* packaging, the library contained a total of 2.6×10^6 plaque-forming units, of which 50% were independent recombinants. The library was amplified once on Y1090 (r^-m^+; Promega Biotec, Madison, WI).

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Abbreviations: ts, temperature sensitive; AEV, avian erythroblastosis virus.

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Screening of the library with the protein 4.1 monoclonal antibody was performed essentially as described (16), except that ^{125}I-labeled rabbit antibody to mouse IgG was used as the radiolabeled probe. Positive clones were picked and plaque-purified by two successive rounds of screening as described (17).

Induction of HD3 Cell Differentiation. Approximately 10^7 HD3 cells were resuspended in 5 ml of differentiation medium (7). This particular subline has been selected to undergo terminal differentiation in a majority of the cells (J.D.E., unpublished observations). The cultures were grown at 42°C, 0.5% CO_2 for 52, 76, or 100 hr. Cells were counted and then washed one time with 4°C phosphate-buffered saline (PBS; 150 mM NaCl/10 mM $NaPO_4$, pH 7.5) containing fresh 0.5 mM phenylmethylsulfonyl fluoride. Cells ($3–7 \times 10^6$) were resuspended in 100 μl of PBS and 100 μl of 2× $NaDodSO_4$ sample buffer [250 mM Tris·HCl, pH 6.8/2 mM EDTA/2% $NaDodSO_4$/20% (wt/vol) sucrose/80 mM dithiothreitol] was added. The samples were immediately placed in a boiling water bath for 5 min, and then stored at −20°C until gel electrophoresis.

Immunofluorescence of Erythroid Cells. Cells were fixed for 15 min in 3.7% (wt/vol) paraformaldehyde (solubilized by alkaline pH treatment at 60°C, followed by neutralization) in PBS at room temperature. They were rinsed once with PBS, treated with acetone/water (1:1) at 0°C for 3 min, undiluted acetone at −20°C for 5 min, then once again with acetone/water (1:1) at 0°C for 3 min. Immunofluorescence was carried out as described by Beug *et al.* (18).

RESULTS

Isolation of a Monoclonal Antibody to Protein 4.1. We injected total erythrocyte membrane protein into mice to elicit antibody response to the several membrane-associated proteins that make up the erythroid cytoskeleton. Hybridomas were prepared from the spleen cells of the immunized mice by fusion to SP2/0 myeloma cells. The protein recognized by each antibody-producing cell line was tentatively identified on the basis of band(s) detected on immunoblots of total erythrocyte membrane protein fractionated by gel electrophoresis (6, 9, 19). Monoclonal antibodies recognizing band 3, β-spectrin, and actin were generated in this way (data not shown). One hybridoma antibody reacted on immunoblots with the unique pattern characteristic of chicken protein 4.1 (ref. 6; see Fig. 4, lane F). This antibody was used in subsequent studies as the monoclonal antibody directed against protein 4.1 (below).

Isolation of a Protein 4.1 cDNA Clone. We used the isolated monoclonal antibody to protein 4.1 to screen the λgt11 expression library (≈8×10^5 total plaque-forming units screened) and isolated three positive plaques. One clone, 4.1A, was shown to be a bona fide 4.1-coding cDNA clone by comparing the predicted amino acid sequence derived from the 4.1A cDNA sequence to the amino acid sequence of a 10-kDa α-chymotryptic peptide of human protein 4.1 (Fig. 1; refs. 22 and 32). There is very high homology (>90%) between the human and predicted chicken amino acid sequences in this fragment.

Protein 4.1 Gene Copy Number. A genomic DNA blot (using the 4.1A cDNA clone as probe) is shown in Fig. 2*A*. The 4.1A recombinant 1.9-kbp insert was nick-translated and hybridized to a blot of chicken erythrocyte chromosomal DNA cleaved with *Eco*RI, *Hin*dIII, or *Pvu* II (Fig. 2*A*). As evident from the autoradiogram, multiple chromosomal bands are detected with the 4.1A cDNA probe.

To distinguish whether multiple band 4.1 genes exist that give rise to these hybridizing genomic fragments (and might then be expected to encode different protein 4.1 variants), or whether these Southern blot signals are derived from a single protein 4.1 gene, we probed an identical blot with a subfragment of clone 4.1A. If the hybridization pattern shown in Fig. 2*A* is due to detection of multiple genomic loci, hybridization to a smaller fragment would be expected to result in a pattern identical to that seen with the larger probe. Alternatively, if the hybridization detected in Fig. 2*A* is due to detection of multiple exons of a single gene, hybridization of an identical filter to a much smaller probe would result in many fewer bands, and these would be a subset of those detected with the entire 4.1A coding sequence. As shown (Fig. 2*B*), hybridization of a 520-bp (*Rsa* I/*Pvu* II) coding fragment of 4.1A detects only a subset of the bands detected with the 1.9-kbp probe. These data are therefore consistent with the conclusion (but do not prove the contention) that a single chromosomal 4.1 gene encodes the multiplicity of erythroid protein 4.1 variants (see below).

Expression of Protein 4.1 During Erythroid Cellular Differentiation. Blot analysis shows that the protein 4.1 mRNA appears as a single band of ≈7 kilobases (kb) in RNAs isolated from the cytoplasm of reticulocytes (Fig. 3, lane A). mRNA of the same size (but in reduced amounts) is also present in HD6 cells (lane B); this cell line represents erythroblasts transformed by a ts mutant (ts167) of AEV, but unlike HD3 cells, it cannot be induced for terminal erythroid differentiation (7). No hybridization to mRNA from MSB1 cells (a chicken splenic lymphoma cell line; ref. 27) is apparent (lane C), but on much longer exposure, a faint band of identical size to the erythroid protein 4.1 mRNA can be seen, indicating the presence of a very low level of 4.1-like message in this cell type (overexposure not shown).

We examined the accumulation of the protein 4.1 variants during the differentiation of an inducible ts34AEV-transformed erythroid cell line. HD3 cells (7) were induced to differentiate by shifting to the nonpermissive temperature in the presence of chicken erythropoietin. Cells were harvested at different times after shift to 42°C and lysed directly in

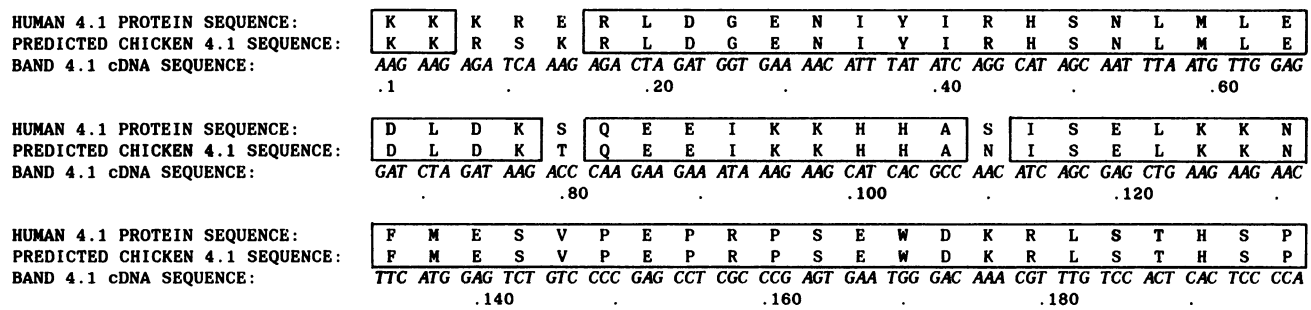

FIG. 1. Predicted partial amino acid sequence of the chicken band 4.1 protein. Alignment of the deduced amino acid sequence derived from the chicken protein 4.1 cDNA clone to part of the human band 4.1 protein sequence is shown. Boxed regions indicate sequence identity. DNA sequencing was accomplished by direct dideoxynucleotide chain termination (20) from alkali-denatured pGEM plasmid subclones (Promega Biotec) of 4.1A using synthetic primers complementary to the T7 and SP6 promoters (21). Amino acids are identified by the single-letter code.

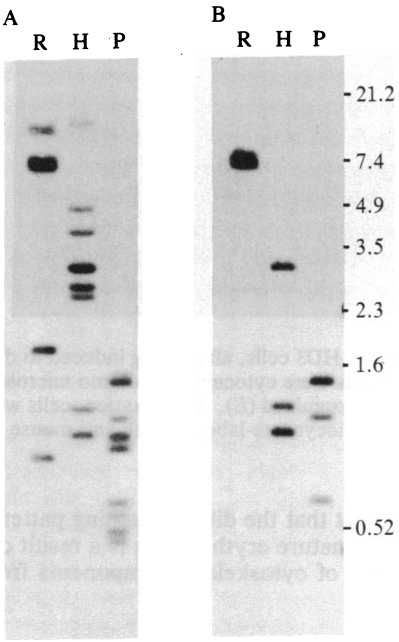

FIG. 2. Genomic DNA blot analysis with the 4.1 cDNA clone. Chicken chromosomal DNA (10 μg) was digested with *Eco*RI (lane R), *Hin*dIII (lane H), or *Pvu* II (lane P), electrophoresed on a 0.9% agarose gel, and transferred to nitrocellulose (23). Identical blots were then probed with the nick-translated 1.9-kbp insert of recombinant 4.1A (*A*) or a 520-bp *Rsa* I/*Pvu* II fragment of 4.1A (*B*). DNA size markers are shown on the right in kbp.

$NaDodSO_4$ gel sample buffer. The proteins were electrophoresed, blotted to nitrocellulose, and then probed with the anti-protein 4.1 antibody. Fig. 4 shows that in unshifted cells there is a low level of expression of the two smaller (77 and 87 kDa) variants and the 150-kDa variant (appearing as a doublet only in the undifferentiated cells). After shifting to 42°C, the 77- and 87-kDa variants predominate; an additional band of unknown, possibly artifactual, origin (≈55 kDa) is also recognized by the antibody (lanes B–D). The same pattern persists throughout the time course of differentiation.

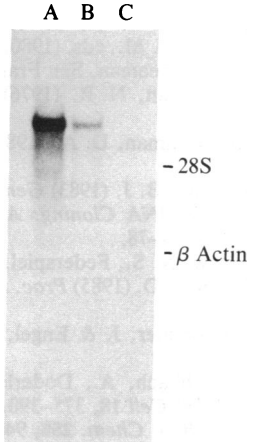

FIG. 3. Expression of protein 4.1 mRNA. Four micrograms of poly(A)$^+$ RNA isolated from anemic adult reticulocytes (lane A), HD6 cells (lane B), or MSB-1 cells (lane C) was electrophoresed on a 1.2% formaldehyde agarose gel (24), transferred to nitrocellulose, and probed with the nick-translated 1.9-kbp insert of recombinant 4.1A. Exposure time was 9 hr with an intensifying screen. The blot was then reprobed with a subclone of λGd1, a chicken ribosomal gene recombinant (25), and a β-actin cDNA clone (26) for internal RNA size standardization.

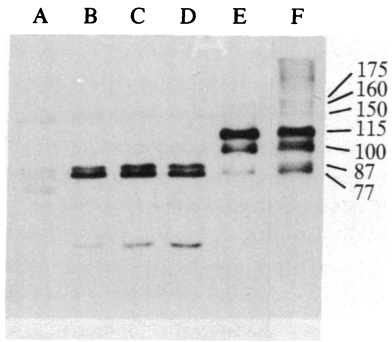

FIG. 4. Expression of the 4.1 variants during *in vitro* differentiation of HD3 cells. HD3 cells (7) were induced for erythroid differentiation by incubation at the (tsAEV) virally nonpermissive temperature (42°C). Cells were collected at 0 (lane A), 52 (lane B), 76 (lane C), and 100 (lane D) hr after induction and lysed directly in $NaDodSO_4$ gel sample buffer. The proteins were electrophoresed on an 8.3% polyacrylamide/0.1% $NaDodSO_4$ gel, electrophoretically blotted to nitrocellulose, and probed with the anti-protein 4.1 monoclonal antibody followed by reaction with ^{125}I-labeled rabbit anti-mouse IgG (28). Total protein from normal erythrocytes (lane E) and purified erythrocyte membranes (lane F) were electrophoresed in parallel as standards. The protein 4.1 variants detected here are slightly different in size than originally reported (6), probably because of differences in the types of gels used in the two different analyses. Thus, the 77-kDa variant appears on our gel system to be of slightly reduced mobility (≈82 kDa). For consistency, we have used the original approximations for the molecular masses of these proteins.

By 100 hr, the majority of the cells have become reticulocyte- and erythrocyte-like (7); they do not, however, express the 100- and 115-kDa variants that are predominant in normal adult erythrocytes (lane E).

Indirect immunofluorescence was used to follow the expression of protein 4.1 in individual cells before and after the shift to 42°C. Fig. 5*A* shows that in unshifted HD3 cells only weak fluorescence is observed, corresponding to the relatively low amounts of protein 4.1 that have accumulated in these cells. After the cells are induced to differentiate, a brightly localized region is seen in some of the cells; most have the diffuse delocalized staining pattern characteristic of reticulocytes and erythrocytes. This staining pattern persists throughout the time course of erythroid cellular maturation. By comparison, mature erythrocytes are invariably uniformly labeled (Fig. 5*E*) in the diffuse cytoplasmic pattern.

DISCUSSION

We report in this paper the isolation of a monoclonal antibody for protein 4.1 and, subsequently, a cDNA clone corresponding to the gene encoding erythroid protein 4.1, a major central component in defining the architecture of the erythrocyte cytoskeleton. The predicted amino acid sequence of a small segment of the cDNA clone closely matches the amino acid sequence of a fragment of the human 4.1 protein, confirming the identity of this clone. Genomic blot analysis suggests that the gene is present as a single copy in the chicken genome. If the sum of the hybridizing genomic fragments (Fig. 2) corresponding to the exons contained within the 1.9-kbp 4.1A cDNA clone is representative of one complete gene, the entire 7-kb mRNA is likely to be encoded by a cellular 4.1 gene in excess of 100 kbp.

There appears to be a single 4.1 message of ≈7 kb in erythroid cells. Thus, the multiple protein variants do not appear to arise from multiple mRNAs but are probably generated by translational or posttranslational events. However, the protein variants could also arise from slight alterations (by differential processing) in the same primary tran-

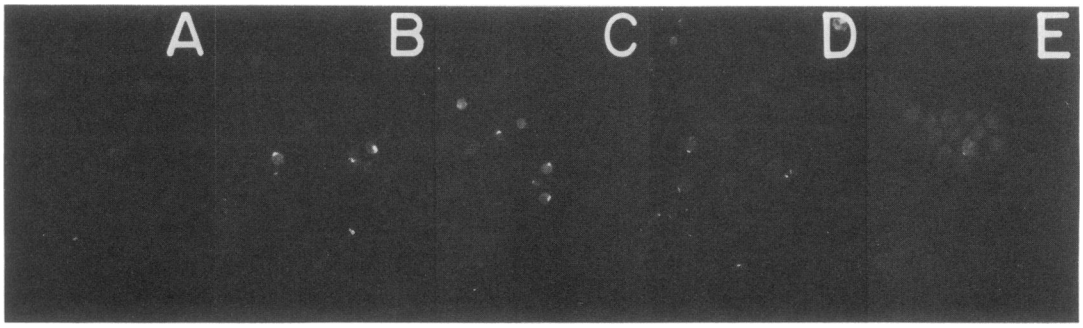

FIG. 5. Immunofluorescence analysis of protein 4.1 during erythroid cellular maturation. HD3 cells, after being induced to differentiate at 42°C, were examined by indirect immunofluorescence for the expression of protein 4.1. Cells were cytocentrifuged onto microscope slides at 0 (*A*), 36 (*B*), 60 (*C*), and 84 (*D*) hr after induction. Adult (definitive) erythrocytes were also examined (*E*). After fixation, cells were incubated with the anti-protein 4.1 monoclonal antibody and then incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Cooper Biomedical, Malvern, PA).

scription unit, or by transcription initiation at close multiple sites. Thus, one might visualize only a "single" mRNA of 7 kb on RNA blots, since the resolution of individual mRNAs of this size on this type of gel is relatively poor. Fine structure analysis of full-length cDNAs will resolve these two possibilities.

The size of the protein 4.1 message appears to be the same in both AEV-transformed erythroblasts and more mature reticulocytes, indicating that the changing pattern of variants during erythrocyte differentiation does not arise from any temporal change in the transcriptional unit. We have no evidence to distinguish between the possibilities that the very low level of mRNA detected in MSB1 (splenic lymphoma) cells is due to low-level transcription of the erythroid band 4.1 gene in those cells, or might rather be due to the expression of a distantly related (and therefore poorly hybridizing) 4.1-like gene in nonerythroid cells.

If, as stated previously, erythroleukemic cells transformed by AEV most accurately represent the erythroid colony-forming unit stage of erythrocyte maturation (7), then protein 4.1 is one of the earliest components expressed in the erythroid cytoskeleton—e.g., band 3 cannot be detected in HD6 cells by immunological methods (J.L.G. and N.S.Y., unpublished observations). In Friend-transformed murine erythroleukemia (MEL) cells, protein 4.1 has also been found to be expressed before induction, although MEL cells are thought to be blocked at a later stage of differentiation than the avian erythroid colony-forming unit (29, 30). The smaller 77 and 87 kDa variants of chicken protein 4.1 are notably less abundant than the 100- and 115-kDa variants in the total protein of mature erythrocytes but are equally or more abundant in the purified membrane fraction. The 100- and 115-kDa variants are synthesized in excess over what is effectively incorporated into the membrane, whereas nearly all of the 77- and 87-kDa variant polypeptides appear to be assembled (31). The developmentally early and relatively abundant expression of the 77- and 87-kDa variants suggest that they may be among the first components involved in the assembly of the cytoskeleton, perhaps by providing the anchoring sites upon which the spectrin–actin network can be formed. The brightly localized regions of protein 4.1 detected in intermediate staged cells in the immunofluorescence experiment suggest that such sites may be compartmentalized. Although we cannot exclude the possibility that such a staining pattern is due to an artifact induced by fixation, the same stages of cells stained with an anti-band 3 monoclonal antibody exhibit a diffuse immunofluorescence pattern throughout the time course of erythroid cell maturation (J.L.G., unpublished observations). However, if the brightly staining punctate pattern visualized at intermediate stages of maturation does represent a cytoskeleton nucleation event, we would expect that the diffuse staining pattern of protein 4.1 detected in mature erythrocytes is a result of the subsequent assembly of cytoskeletal components from such nucleation sites.

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