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Detection and characterization of a mouse α-spectrin cDNA clone by its expression in *Escherichia coli*

(expression library/erythrocyte membrane/immunological screening)

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ABSTRACT **A cloned segment of mouse α-spectrin mRNA has been identified by immunological techniques. Double-stranded cDNA derived from spleens of anemic mice was introduced into a bacterial expression vector, pUC, and transformed *Escherichia coli* colonies were screened by using an antiserum to erythrocyte membrane ghost proteins. Of 17 positive colonies, 2 bound antibody to mouse spectrin, and these 2 colonies contained 750-base-pair inserts that cross-hybridized. Transfer of the 750-base-pair insert to an expression vector containing the P_L promoter of phage λ produced larger amounts of peptides that were bound by antibody to mouse spectrin. The spectrin-like peptides made in *E. coli* elicited antibody that reacted only with the α-spectrin subunit of erythrocyte membranes. This clone will be useful for the study of the structure and expression of the spectrin gene, particularly in understanding the role of spectrin in human inherited hemolytic anemias.**

Spectrin is the major component of the plasma membrane of circulating erythrocytes (for a review, see refs. 1–3) and forms, in association with actin and bands 2.1, 3, and 4.1, a network believed to be responsible for maintaining the shape of mature erythrocytes and conferring structural integrity (4). The observations that several hemolytic anemias are associated with structural abnormalities, decreased levels of spectrin, or both (1, 5) strongly support the importance of this protein in maintaining the stability of the erythrocyte cytoskeleton. In some rare strains of mice whose erythrocytes contain only trace amounts of spectrin (6), the homozygous state is lethal and the heterozygous state confers a spherocytosis syndrome. Erythrocyte spectrin is composed of two nonidentical subunits now generally designated as α (M_r, 250,000) and β (M_r, 225,000) chains. The two subunits exist as rodlike structures each approximately 1000 Å long, the dimeric form of the molecule resulting in a side-by-side association of parallel monomers (1). Recent evidence has shown that spectrin, once thought to be present only in erythrocytes, is also expressed in many nonerythroid cells (for a review, see ref. 3). It is now commonly accepted that spectrins constitute a family of polypeptides thought to mediate linkage of actin filaments to the plasma membrane in a wide variety of cell types (4, 7–9). Most cells express a common subunit, the α chain, in association with a polymorphic cell-type-specific subunit, termed fodrin or TW260K. Models for the structure of human erythrocyte spectrin have been described during the years, but only recently have Speicher *et al.* (10, 11) reported the amino acid sequence of one of the nine domains that form the structure of the protein.

Expression vectors have been used to identify cloned sequences of several genes (12, 13). We have used this approach to clone erythrocyte membrane proteins so that we can study their coordinate expression during erythropoiesis. In this report, employing the pUC system (12), we describe the isolation from a mouse expression library of a cDNA clone that expresses a protein antigenically related to the α subunit of mouse spectrin.

MATERIALS AND METHODS

Bacterial Strains and Vectors. Plasmids pUC7, pUC8, and pUC9 were kindly supplied by J. Messing (University of Minnesota). Plasmids pTG908, pTG920, and pTG161 (each of which utilizes the P_L promoter of bacteriophage λ) were provided by R. Lathe and M. Courtney (Transgene, Strasbourg, France), who also provided the host *Escherichia coli* strain TG900 carrying the λ heat-sensitive repressor gene (14). *E. coli* strains DHI and TG900 were transformed according to the procedure of Hanahan (15).

Protein was prepared from *E. coli* by sonication in the presence of Triton X-100 and lysozyme and was estimated by the procedure of Schaffner and Weissmann (16).

Isolation and Electrophoresis of RNA. Poly(A)$^+$ RNA was prepared from spleens of anemic mice as previously described (17). Poly(A)$^+$ RNA was fractionated on a 1.2% agarose gel in the presence of 2.2 M formaldehyde (18). Transfer to nitrocellulose and prehybridization and hybridization procedures were essentially as previously described (17, 19). cDNA probes were prepared from cloned sequences by restriction enzyme digestion and gel electrophoresis purification and were labeled by using calf thymus primers and *E. coli* DNA polymerase I (20).

Construction of the Expression Library of Mouse Anemic Spleen. One hundred micrograms of a plasmid library containing cDNA sequences from mouse anemic spleen (21) was digested to completion with 200 units of *Pst* I. To isolate the cDNAs from the pBR322 vector, the digested material was electrophoresed on a 1% low-melting agarose gel, portions of the gel were excised, and the cDNAs were extracted and purified, omitting the linearized pBR322. The plasmid vectors pUC7, pUC8, and pUC9 (22) were digested to completion with *Pst* I and treated with calf intestinal alkaline phosphatase to reduce recircularization. One microgram of cDNA was ligated to each vector at a weight ratio of vector to cDNA of 4:1. *E. coli* strain DH1 was transformed according to Hanahan (15). One million ampicillin-resistant colonies were collected for each ligation with the three different vectors, and plasmids were extracted from the colonies (23). Under these conditions approximately one bacterial clone in six should have a cDNA insert in the right orientation and in the exact reading frame. The three different expression libraries were kept in separate pools. The pooled plasmids were used to transfect *E. coli* DH1. The cDNA libraries were

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Abbreviation: bp, base pair(s).

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immunologically screened by using the procedure described by Helfman *et al.* (12).

Preparation of Antibodies. Mouse erythrocyte membranes were prepared as described (24). One milligram of erythrocyte membrane proteins was boiled (100°C) for 5 min in 1% $NaDodSO_4$/5 mM sodium phosphate buffer, pH 8.0, and then emulsified with Freund's complete adjuvant for the immunization of two New Zealand White rabbits; booster injections were administered every 2 weeks. Blood was collected 45 days after the initial immunization and sera were tested against $NaDodSO_4$-denatured erythrocyte membrane proteins in a solid-phase radioimmunoassay (25) using an ^{125}I-labeled $F(ab')_2$ fragment of antiserum to rabbit IgG (Amersham, 15 μCi/μg; 1 Ci = 37 GBq).

In other experiments, rabbits were injected at 1-week intervals with slices of acrylamide gel containing the antigen under study (≈40 μg of protein) crushed in phosphate-buffered saline (26). Sera were collected 1 month after the initial injection. The sera were then extensively preabsorbed with boiled lysates from the parental bacterial strain, DH-1, containing pUC8 plasmid without an insert (12).

Antisera to mouse spectrin and IgG were kindly supplied by P. A. Marks (Sloan–Kettering Institute, New York), S. R. Goodman (Hershey Medical Center, Pennsylvania State University, Hershey, PA), and M. M. B. Kay (Texas A & M University, Temple, TX).

Immunoautoradiography. Proteins were separated by $NaDodSO_4$/6–12% polyacrylamide gel electrophoresis based on the systems of Laemmli (27) and Fairbanks *et al.* (24). Immunoblots and autoradiography were performed as described (28).

RESULTS

Specificity of the Rabbit Antiserum to Mouse Erythrocyte Membrane Protein. Mouse erythrocyte ghosts prepared by the methods of Fairbanks *et al.* (24) were electrophoresed in a $NaDodSO_4$/7% polyacrylamide gel. Proteins were transferred to nitrocellulose and allowed to react with a 1:1000 dilution of the rabbit antiserum to mouse erythrocyte ghosts (28). As shown in Fig. 1, the antiserum reacted predominantly with high molecular weight proteins, namely the α chain of spectrin and band 2.1. Lower reactivity was present against the β chain of spectrin, band 3, and bands 4.1a and b. It must be pointed out that the antibodies were raised against denatured proteins. Since the bacterial colonies carried only a fragment of a cDNA, they would be less likely to express native antigens.

Immunological Screening of the cDNA Library. Twenty thousand transformants for each pool of the expression library (60,000 in total) were plated directly onto agar plates containing ampicillin to give ≈1000 colonies per plate. Colonies were replica-plated onto nitrocellulose filters and immediately lysed (12). The filters were then incubated at room temperature with rabbit antiserum to mouse erythrocyte membrane protein diluted 1:1000, washed with saline at room temperature, and incubated with ^{125}I-$F(ab')_2$. After 2 hr of incubation the filters were washed in saline, dried, and autoradiographed overnight.

Seventeen positive colonies were obtained from such a screening; the reactivities of the colonies varied from very strong to very weak (Fig. 2*a*). To select colonies that synthesized a protein related to spectrin, the panel of 17 colonies was allowed to react (12) with a 1:1000 dilution of IgG preparation purified from a monovalent antiserum to mouse spectrin (provided by P. A. Marks). Fig. 2b shows that, among the 17 selected colonies, 2 of them bound the anti-spectrin antibody. The two positive colonies contained pUC7 plasmid with cDNA inserts of ≈750 base pairs (bp), which cross-hybridized. The panel of 17 colonies was subsequently

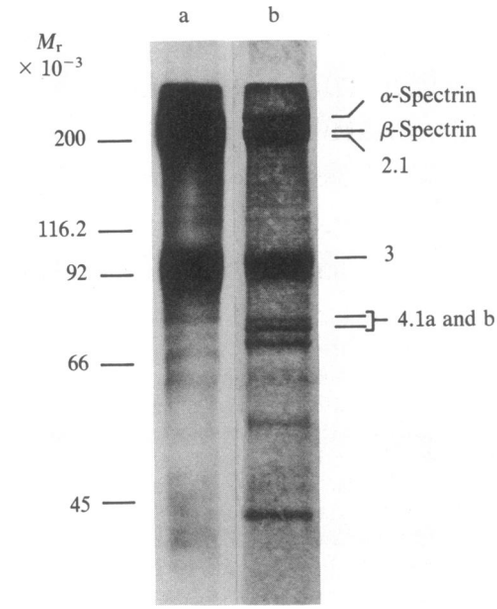

FIG. 1. Reactivity of the rabbit antiserum to mouse erythrocyte membrane proteins. About 30 μg of membrane proteins was electrophoresed in a discontinuous $NaDodSO_4$/10% polyacrylamide slab gel. One lane of the gel was stained with Coomassie blue (lane b), and one lane was transferred electrophoretically to nitrocellulose and allowed to react with a 1:1000 dilution of the rabbit antiserum to mouse proteins (lane a). Immunoautoradiography was performed according to Towbin (28). Molecular weight markers were run in parallel. The relative positions of the mouse erythrocyte membrane proteins were deduced from ref. 29.

screened with two additional antibodies to mouse spectrin (provided by S. R. Goodman and M. M. B. Kay), and the result was the same as shown in Fig. 2b—i.e., the same two colonies reacted with all three antibodies to mouse spectrin. Further analyses have been carried out with one of the two colonies containing pB129. This clone made quite readily detectable amounts of a peptide (M_r, 36,000) that reacted specifically with the antibody to spectrin after electrophoretic transfer to nitrocellulose (Fig. 3, lane d).

Antigenicity of the *E. coli*-Produced Spectrin-like Peptides. To obtain better expression of these spectrin-like proteins, the 750-bp insert was purified from pB129 and was ligated to *Pst* I-linearized, alkaline-phosphatase-treated pTG908 and pTG161. pTG908 has the *Pst* I site in the same reading frame as pUC7, whereas the *Pst* I site in pTG161 is in a different reading frame. The ligated material was then used to transform the TG900 strain of *E. coli*. Transformants were plated onto ampicillin/agar plates, grown at 28°C overnight, and then shifted to 37°C for 5 hr. Colonies reacting with anti-spectrin antiserum and some negative colonies were selected. Bacterial lysates were prepared from several transformants, grown at 28°C, and shifted to 37°C for 5 hr. Fig. 3 shows the immunoautoradiography using anti-spectrin antiserum on lysates from selected cultures. Two spectrin-like peptides (M_r ≈ 31,000 and 36,000, respectively) were produced by pTG908-B129, in an amount greater than that produced by the original pUC7-B129 in the host DH-1 cells (Fig. 3, lanes a and d). No detectable reactivity with anti-spectrin antiserum was present in the TG900 lysate (data not shown). However, two proteins antigenically related to spectrin were produced by pTG161-B129, one of which migrated with the M_r 31,000 protein produced by pTG908-B129 (Fig. 3, lane c). In this vector the B129 insert is not located in the correct reading frame, and one would have expected no expression of spectrin-related proteins. One possible explanation is the presence in the 750-bp insert of an additional strong internal AUG initiation signal that was utilized in the pTG161 vector

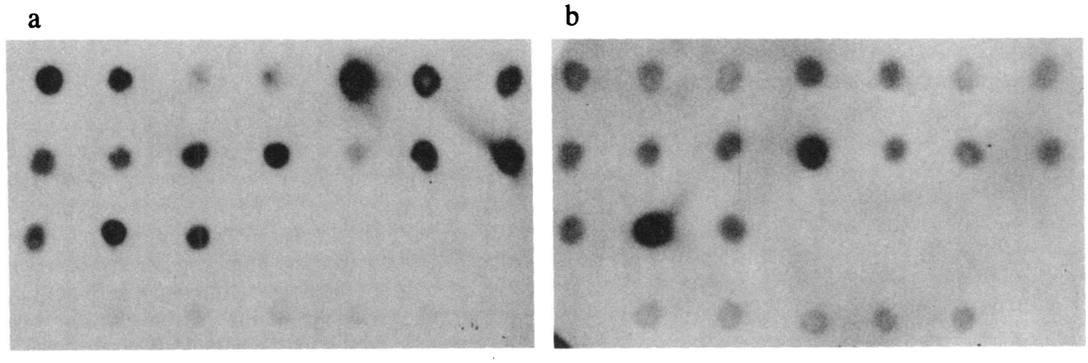

FIG. 2. Immunological detection of clones containing spectrin cDNA. The panel of 17 colonies reacting with the polyvalent antiserum to mouse erythrocyte membrane protein was replica plated. One filter was allowed to react with a 1:1000 dilution of the polyvalent antiserum to total mouse erythrocyte membrane proteins (*a*), the second with 1:1000 dilution of IgG purified from antiserum to mouse spectrin (provided by P. A. Marks) (*b*). Along with the 17 selected colonies, 5 negative colonies—i.e., DH1 cells containing pUC8 without an insert—were present on the filters (bottom row of colonies).

and presumably was responsible for the second band obtained with pTG908-B129 (Fig. 3, lane a). On the other hand, no spectrin-like proteins are produced by *E. coli* TG900 cells carrying the same plasmid with the B129 insert in the opposite orientation (Fig. 3, lane b).

Isolation of Antisera to the Bacterial B129 Peptides. *E. coli* TG900 containing pTG908-B129 were grown at 28°C overnight in a 50-ml culture and shifted to 37°C for 5 hr to induce expression. A bacterial lysate was prepared and electrophoresed on a $NaDodSO_4$/12% polyacrylamide preparative gel 1.2 mm thick. Gel slices containing spectrin-like proteins produced by the bacteria were excised, homogenized, and injected into rabbits. Sera from rabbits immunized with proteins of M_r 31,000–36,000 derived from pTG908-B129 were tested for their ability to react with spectrin. The immunoautoradiography shows the erythrocyte ghost proteins that reacted with a 1:10 dilution of preimmune serum (Fig. 4, lane a), with a 1:50 dilution of antiserum to M_r 31,000–36,000 B129 peptides (lane b), and with a 1:5000 dilution of anti-spectrin IgG (lane c). The serum from the rabbits immunized with the M_r 31,000–36,000 B129 peptides reacted specifically with the α subunit of mouse spectrin, which was clearly resolved from the β subunit in this electrophoretic system. The result indicates that the M_r 31,000–36,000 B129 protein contains an antigenic determinant of the mouse α-spectrin molecule.

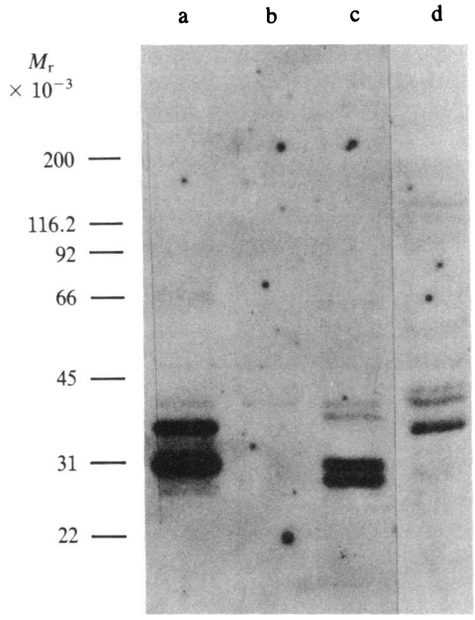

FIG. 3. Spectrin-like peptides synthesized by the expression vectors. *E. coli* strain DH1 carrying pB129 was grown at 37°C. *E. coli* strain TG900 carrying either pTG908-B129 or pTG161-B129 was grown at 28°C overnight and then shifted at 37°C for 5 hr. Cells were lysed by sonication in the presence of Triton X-100, DNase, and lysozyme (15), and the lysates were electrophoresed in a $NaDodSO_4$/12% acrylamide gel. The proteins were then transferred to nitrocellulose and allowed to react with a 1:1000 dilution of a rabbit IgG antibody to mouse spectrin followed by ^{125}I-labeled $F(ab')_2$ fragment of antibody to rabbit IgG, according to Towbin (28). The blot was washed several times, dried, and autoradiographed with a DuPont Cronex intensifying screen. The proteins specified by the TG900 cells carrying pTG908-B129 (lane a), pTG161-B129 (with the 750-bp insert in the wrong orientation) (lane b), or pTG161-B129 (lane c) and by DH1 cells carrying pB129 (lane d) are illustrated. Each lane contained approximately 10 μg of total bacterial proteins. The faint band in lane d with M_r about 45,000 may represent trace antibodies to *E. coli*.

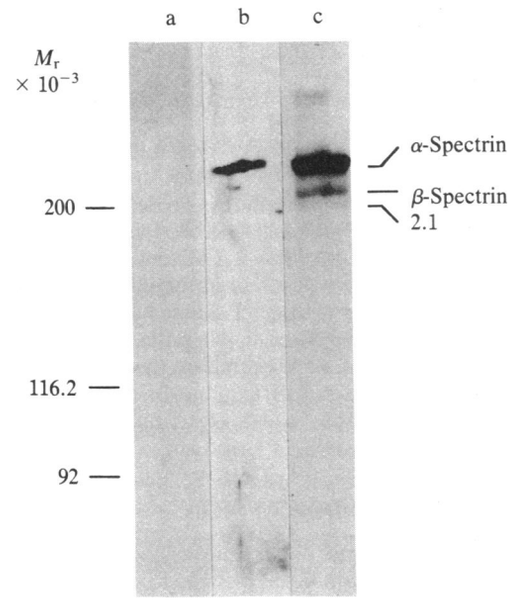

FIG. 4. Reactivity of the antiserum against the bacterial B129 peptides. Samples ($\approx$10 μg) of mouse erythrocyte membrane proteins were electrophoresed in a $NaDodSO_4$/6% polyacrylamide gel. After electrophoretic transfer to nitrocellulose, the blots were allowed to react with 1:10 dilution of preimmune serum (lane a), a 1:50 dilution of the rabbit serum raised against the B129 proteins (lane b), or with a 1:5000 dilution of rabbit anti-mouse spectrin IgG (lane c, provided by P. A. Marks). After 1-hr incubation, filters were washed and the attached proteins were allowed to react with ^{125}I-$F(ab')_2$ (1×10^6 cpm/ml) specific for rabbit IgG as previously described (12). The dried filters were exposed for 1 hr, using a DuPont Cronex intensifying screen.

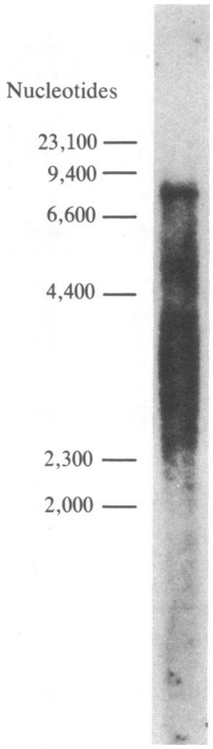

FIG. 5. Size of pB129-encoded mRNA. Mouse anemic spleen poly(A)$^+$ RNA (10 μg) was electrophoresed in a 1% agarose gel in the presence of 2.2 M formaldehyde (18) and transferred to nitrocellulose (19). B129 insert (200 ng) was labeled with ^{32}P by using calf thymus primers and *E. coli* DNA polymerase I (20). Hybridization was performed in 50% (vol/vol) formamide, using ^{32}P-labeled insert (1×10^6 cpm/ml) for 20 hr at 42°C. Blots were washed in 300 mM NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ three times at 37°C, then in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ for 30 min at 50°C. The filter was then dried and exposed to x-ray film overnight with a DuPont intensifying screen. Fragments of *Hin*dIII-digested λ DNA were used as size markers.

Size of the Encoded mRNA. The molecular weight of 220,000–250,000 for the two spectrin subunits requires a mRNA of at least 6000 nucleotides. To determine the size of the spectrin mRNA, poly(A)$^+$ RNA from mouse anemic spleen was electrophoresed on 2.2 M formaldehyde containing agarose gel and transferred to nitrocellulose (18, 19). The blots were hybridized with a ^{32}P-labeled 750-bp insert from pB129. The 750 bp hybridized with a poly(A)$^+$ RNA of about 8000 nucleotides (Fig. 5). Presumably the smaller RNA detected in the blot represents fragments of α-spectrin mRNA, which may be extremely sensitive to nucleases due to its large size.

DISCUSSION

This report describes the direct immunological detection of a plasmid, pB129, containing sequences for mouse α-spectrin mRNA. The cloning strategy was based on the use of a polyvalent antiserum raised in rabbits against denatured mouse erythrocyte membrane proteins, the major components of which are the two spectrin subunits, band 2.1, and band 3. This serum was used for immunological screening of a cDNA expression library employing the pUC vectors (12) derived from a mouse anemic spleen cDNA library in pBR322. Seventeen colonies were selected that showed different degrees of reactivity to the polyvalent antiserum. Among these colonies, two reacted with antibody to mouse spectrin (Fig. 2). Four reacted with an antibody to mouse band 3 (provided by M. M. B. Kay), and two reacted with an antibody to mouse band 2.1 (provided by S. R. Goodman) (data not shown).

Three criteria were used to establish that the colonies carry a plasmid encoding a part of the α-spectrin gene: (*i*) The specificity of the immunological reaction. Among the colonies selected during the first screening the same two colonies showed reactivity with the three different antispectrin antibodies. (*ii*) Antigenicity of the peptides expressed in *E. coli*. We transferred the insert of pB129 into an expression vector that is able to produce much higher levels of protein and is readily induced. Reactivity of *E. coli* with antibody to mouse spectrin depended upon the presence of the insert and upon its correct position in the plasmid. Extracts of *E. coli* with no plasmid did not contain any proteins sharing antigenic determinants with spectrin. In addition, immunization of rabbits with the peptides that bound spectrin antibody produced serum that reacted specifically with the α subunit of mouse spectrin (Fig. 4). (*iii*) The molecular weight of the encoded mRNA. The molecular weight of 250,000 for the α subunit of spectrin requires at least 6000 nucleotides. The 750-bp insert of pB129 hybridized with a poly(A)$^+$ RNA of about 8000 nucleotides (Fig. 5).

The availability of a mouse spectrin cDNA clone will facilitate the study of its gene structure and expression during erythroid differentiation. Furthermore, it will help elucidate the role, if any, of spectrin in the inherited anemias that involve the integrity of the membrane skeleton structure.

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