Drosophila Spectrin. II. Conserved Features of the Alpha-Subunit Are Revealed by Analysis of cDNA Clones and Fusion Proteins

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Abstract. *Drosophila* alpha-spectrin cDNA sequences were isolated from a lambda gtll expression library. These cDNA clones encode fusion proteins that include portions of the *Drosophila* alpha-spectrin polypeptide as shown by a number of structural and functional criteria. The fusion proteins elicited antibodies that reacted strongly with *Drosophila* and vertebrate alpha-spectrins and a comparison of cyanogen bromide peptide maps demonstrated a clear structural correspondence between one fusion protein and purified *Drosophila* alpha-spectrin. Alpha-spectrin fusion protein also displayed calcium-dependent calmodulin-binding activity in blot overlay experiments and one fusion protein bound specifically to both *Drosophila* and bovine brain beta-spectrin subunits on protein blots. A region of the *Drosophila* cDNA cross-hybridized at lowered stringency with an avian alpha-spectrin cDNA. Together these data show that the composition, structure, and binding properties of the spectrin family of proteins have been remarkably well conserved between arthropods and vertebrates. *Drosophila* cDNA hybridized to an mRNA of ≥9 kb on blots of total *Drosophila* poly A^+ RNA; and hybridized in situ to a single site in polytene region 62B, 1-7. This result and Southern blot analysis of genomic DNA indicate that the sequences are likely to be single copy in the *Drosophila* genome.

SPECTRIN isoforms from vertebrate species display a high degree of conservation with respect to many structural characteristics and binding properties. These characteristics include a dimeric subunit composition, antigenic crossreactivity, calmodulin-binding activity, and a periodic, segmented structure (Bennett, 1985; Marchesi, 1985; Speicher, 1986). Conserved interactions between the alpha- and beta-subunits have also been demonstrated by the formation of hybrid molecules composed of subunits from different species. For example, human erythrocyte beta-spectrin was shown to form hybrid molecules with the alpha-spectrin subunit of both avian erythrocytes and brush borders (Coleman et al., 1987).

The spectrin family of proteins is widely distributed, but little is known of spectrins in invertebrate species where molecular and genetic studies of function and role in development are facilitated. We have recently purified a spectrin from *Drosphila* that displays many of the characteristic properties of vertebrate spectrins (Dubreuil et al., 1987). Here we report the isolation and characterization of cDNA clones derived from the gene that encodes *Drosophila* alpha-spectrin. We also describe the surprising result that alpha-spectrin fusion proteins bind specifically to *Drosophila* and vertebrate beta-spectrin subunits. Our findings demonstrate that several aspects of the spectrin family that are conserved between mammals and birds show evolutionary conservation even to a species as distantly related as *Drosophila*.

A preliminary account of this work was presented at the 1986 meeting of the American Society for Cell Biology (1986. *J. Cell Biol.* 103[5, Pt. 2]:540*a*.[Abstr.]).

Materials and Methods

Isolation of Alpha-Spectrin Clones

A cDNA expression library in lambda gtll prepared from *Drosophila* head poly A^+ RNA (Itoh et al., 1986) was screened with antibodies using the system of Young and Davis (1984) as modified by Goldstein et al. (1986). 12 150-mm petri plates were screened at a density of ~80,000 plaques per plate. Rabbit antiserum 675 (Dubreuil et al., 1987), diluted 1:250 into a milk/protein-containing buffer (150 mM NaCl, 10 mM $NaPO_4$, pH 7.5, 5% nonfat dry milk, 0.01% thimerosal; Johnson and Elder, 1983), served as the primary antibody. A boiled lysate of *Escherichia coli* strain Y1089 (lambda gtll lysogen) was included to adsorb antibodies against *E. coli* antigens. Affinity-purified goat anti-rabbit antibody conjugated to horseradish peroxidase (Zymed Laboratories, South San Francisco, CA) was used to detect primary antibody binding. Positive plaques were replated at successively lower densities to obtain clonal phage populations.

Hybridization Analysis of cDNA Sequences

DNA was prepared from positive lambda gtll clones, digested with Eco RI, separated on an agarose gel, and transferred to nitrocellulose (Southern, 1975) for cross-hybridization analysis. The cDNA inserts of clones 9a and 10 were subcloned into the pUC18 vector (pUC18 is identical to pUC19

[Yanisch-Perron et al., 1985] except that the polylinker is in the reverse orientation). To prepare hybridization probes, the insert DNA bands of pUC18:9a and pUC18:10 were excised from agarose gels, electroeluted, and biotinylated with photoactivatable biotin (Clontech Laboratories, Palo Alto, CA). Biotinylation, hybridization, washes, and detection of biotinylated DNA probes were carried out as described by Forster et al. (1985).

For hybridization to Northern and genomic Southern blots, and for lowered stringency hybridization, insert DNA or restriction fragments from pUC18 subclones were purified from agarose gels and nick translated using ^{32}P-dATP. Northern blots were prepared and hybridization performed as in Goldstein et al. (1986). Genomic DNA was prepared from the *Drosophila* Schneider's 3 cell line (Schneider and Blumenthal, 1978), essentially as described by Maniatis et al. (1982), using SDS instead of *N*-lauroylsarcosine for the initial cell lysis. The DNA was digested to completion with either Eco RI or Bam HI. Digested DNA was separated on a 0.8% agarose gel and transferred to nitrocellulose (Southern, 1975). Hybridization and washes were carried out as described by Goldstein et al. (1986). Conditions for lowered stringency hybridization were the same, except that 30% formamide rather than 50% formamide, was used during hybridization, and the most stringent wash was in 2× SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0) plus 1% SDS for 2 h at 55°C instead of 1× SSC at 65°C.

Biotinylated cDNA inserts (prepared as above) from pUC18:9a and pUC18:10 were hybridized in situ to the polytene chromosomes of Oregon R larvae, essentially as in Engels et al. (1986). Labeled chromosomes were photographed under phase contrast optics without counterstaining.

Electrophoretic Techniques

SDS-PAGE was performed using the buffer system described by Laemmli (1971). For transfer and analytical purposes, 80 × 100 × 0.8 mm slab gels were run with 7% acrylamide, 0.08% *N,N'*-methylene bisacrylamide in the running gel, and 3% acrylamide, 0.08% bis in the stacking gel.

For preparative work, 3- or 4.5-mm-thick gels were cast of 5% polyacrylamide and heavily loaded with sample. Protein bands were transiently visualized with 4 M Na-acetate (Higgins and Dahmus, 1979) and gel bands were excised with a razor blade. Polypeptides were electroeluted from the gel slices in an electrophoretic concentrator (ISCO, Inc., Lincoln, NB) for 6 h at 150 V with 25 mM Tris, 192 mM glycine, 0.1% SDS in the outer reservoirs, and 8 mM Tris, 64 mM glycine, and 0.1% SDS in the sample chamber. To remove free SDS, electrophoresis was continued for 2 h at 150 V after replacing the buffers in the sample chamber and the outer reservoirs with fresh Tris-glycine buffers without SDS (the contents of the collection cup were left undisturbed). To further remove SDS, the samples containing the electroeluted polypeptides were dialyzed overnight against a buffer containing 0.1% Tween 20, 50 mM NaCl, 20 mM Na-phosphate (pH 7.5), and 1 mM EDTA. Relative protein concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. Final purity was checked by reelectrophoresis on analytical gels. Glycerol was added to 50% for storage at −20°C.

Protein blots (Burnette, 1981) were prepared from SDS-PAGE gels in which a uniform curtain of sample was separated by electrophoresis through the gel. 10–20-fold less protein was loaded on gels for transfer than on gels for staining with Coomassie Blue. To better resolve closely spaced doublets, electrophoresis was sometimes continued after the dye front had left the gel. The gels were electrophoretically transferred to nitrocellulose as described (Dubreuil et al., 1987). The nitrocellulose blots were reversibly stained with Ponceau Red S (Sigma Chemical Co., St. Louis, MO) and cut into ~3-mm-wide strips whose length ran from the top to the bottom of the corresponding gel.

Production and Purification of Fusion Proteins

The DNA inserts from pUC18:9a and pUC18:10 were subcloned into each of the pATH1, 10, and 11 vectors (see Dieckmann and Tzagoloff, 1985, for representative pATH vectors), which represent each of the three possible reading frames. HB101 host bacteria carrying recombinant plasmids were screened for the production of fusion proteins as follows. Small cultures were grown in M9CA medium (Maniatis et al., 1982) plus 20 μg/ml tryptophan and 50 μg/ml ampicillin at 37°C. Midlog cultures (grown in the absence of tryptophan) were induced to begin Trp E synthesis by the addition of 1 mg/ml indoleacrylic acid and were incubated further for 2–3 h at 37°C. Cells were pelleted and resuspended in SDS sample buffer and analyzed by SDS-PAGE. For purification, fusion proteins were produced in large scale cultures, separated on preparative polyacrylamide gels, electroeluted, and dialyzed as described above. Purified fusion proteins will be referred to as fusion protein 9a and fusion protein 10.

Antibody Production and Affinity Purification

50 μg of purified fusion protein 9a was emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.) and injected into the popliteal lymph nodes of a rabbit (Sigel et al., 1983). After 1 mo, the rabbit was boosted subdermally with 50 μg of fusion protein 9a emulsified with Freund's incomplete adjuvant. Serum reactivity with *Drosophila* spectrin was detected within 2 wk of the boost.

Protein samples to be used for the affinity purification of antibodies were separated by SDS-PAGE and transferred to nitrocellulose (as above). Proteins on the blot were visualized with Ponceau Red S (Sigma Chemical Co.). Bands of interest were excised and incubated with antiserum diluted 1:250 in Tween buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) plus 5% newborn calf serum. Elution of bound antibodies was carried out according to Olmsted (1981), as modified by Goldstein et al. (1986).

Peptide Mapping

Fusion protein 9a and *Drosophila* alpha-spectrin bands were cut out of SDS-polyacrylamide gels after staining with Coomassie Blue and the polypeptides were digested within the gel slices with cyanogen bromide (Sigma Chemical Co.). The digestion and washes were carried out according to the method of Pepinsky (1983). Digestion products were eluted from the gel slices into a 10–15% gradient polyacrylamide gel. The fragments separated on the gel transferred to nitrocellulose for the detection of peptide bands with antibody probes.

Protein Biotinylation

Purified fusion proteins were labeled with biotin by mixing 30 μg of protein (~0.5 mg/ml) with 1 μl of 150 mM biotin-epsilon-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem, San Diego, CA) and incubated at 4°C for 2 h. This mixture was then diluted to 100 μl with a buffer containing 50 mM NaCl, 10 mM Na-phosphate pH 7.5, 1 mM EDTA, and 0.1% Tween 20, and centrifuged through Sephadex G-50 medium resin (Pharmacia, Inc., Piscataway, NJ) in a 1.5-ml microfuge tube spin column equilibrated and rinsed with the same buffer. Calmodulin was prepared and labeled with biotin as in Dubreuil et al. (1987).

Blot Overlays

Strips of the protein blots were preincubated in 5% newborn calf serum (HyClone Laboratories, Logan, UT) in Tween buffer, then incubated in the same buffer containing either (*a*) diluted polyclonal antiserum or (*b*) biotinylated protein (with unlabeled competing polypeptides as noted) for 1–3 h. Serum was omitted from incubations with biotin-labeled calmodulin. The strips were washed in Tween buffer, then incubated in Tween buffer plus 5% serum with either (*a*) alkaline phosphatase–labeled goat anti–rabbit (1:2,000) (Zymed) or (*b*) streptavidin-alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, MD) (0.5 μg/ml) for 1 h. After washing with Tween buffer, the blots were rinsed in 0.15 M Tris-Cl, pH 8.8 and reacted with 60 μg/ml nitro blue tetrazolium and 60 μg/ml 5-bromo-4-chloro-3-indoyl phosphate in the same buffer to detect alkaline phosphatase on the blot.

Miscellaneous Techniques

Human RBC ghosts were prepared as in Dodge et al. (1963) and bovine brain membranes as in Davis and Bennett (1983). *Drosophila* head homogenates were prepared by freezing Oregon R adults in liquid N_2, removing the heads while frozen, then boiling and triturating in SDS sample buffer (Laemmli, 1971). Insoluble material was removed by centrifugation.

Results

Isolation of cDNA Sequences Coding for Drosophila Alpha-Spectrin

Rabbit antiserum 675 (Dubreuil et al., 1987) was used to screen a lambda gtll cDNA expression library produced from *Drosophila* head poly A$^+$ RNA (Itoh et al., 1986). The rabbit antiserum reacted strongly with the 234-kD *Drosophila* spectrin band on blots of *Drosophila* head ho-

mogenates (Fig. 1) and weakly with two higher molecular mass polypeptides that contaminated the immunogen; under the same conditions preimmune serum did not react significantly with any *Drosophila* polypeptides. A screen of ~1 × 10^6 plaques yielded 32 recombinant lambda gt11 clones that retained positive antibody reactivity after plaque purification.

Lysogens were produced as described by Goldstein et al. (1986) and used in the preliminary screening of six positive clones that reacted strongly with the antiserum. The lysogen data will be described for completeness, but are not shown in favor of data presented later concerning Trp E fusion proteins. Lysogen cultures were induced with isopropyl beta-D-thiogalactoside and lysates were prepared for SDS-PAGE. Each lysogen lysate contained one or more bands that were not present in control lysogens (lambda gt 11 clone without an insert, producing only beta-galactosidase), and these bands reacted with antiserum 675 after transfer to nitrocellulose. For five clones, antibodies purified by affinity to these bands on nitrocellulose specifically recognized only the 234-kD spectrin band on protein blots of Schneider's 3 (Schneider and Blumenthal, 1978) cell homogenates and in two cases, also bound to bovine brain alpha-spectrin (clones 9 and 10). Antibodies purified by affinity to polypeptides expressed by the sixth clone reacted only with one of the non-spectrin, high molecular mass bands that contaminated the immunogen preparation. This clone was not investigated further.

Characterization of Drosophila Alpha-Spectrin cDNAs

DNA was purified from all 32 positive lambda gt11 clones, digested with Eco RI, separated on an agarose gel, and transferred to nitrocellulose to examine cross-hybridization between the insert sequences. The clone 9 cDNA insert contained two Eco RI fragments, of 3.8 (9a) and 0.5 kb (9b), indicating an internal Eco RI site (Fig. 2). Clone 10 had a single insert of 3.9 kb without internal Eco RI sites. The cDNA inserts of 23 clones hybridized with the biotinylated cDNA insert of clone 9a. Of those, five hybridized exclusively to 9a; 13 hybridized strongly and five weakly to the biotinylated 10 sequence. Only one cDNA hybridized strongly to 10, but weakly to 9a. The remaining nine cDNA inserts did not hybridize to either the 9a or 10 sequences.

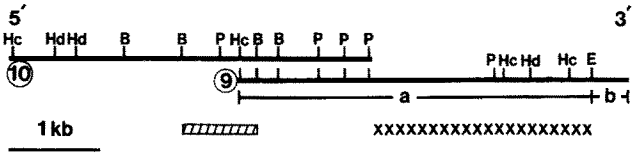

Figure 2. Partial restriction map of the lambda gt11 cDNA inserts 9 and 10 showing the area of overlap between the clones and the region of homology to a vertebrate alpha-spectrin. *B*, Bam HI; *E*, Eco RI; *Hc*, Hinc II; *Hd*, Hind III; *P*, Pst I. The two Eco RI fragments of clone 9 were subcloned independently into pUC18 and labeled *a* and *b*; 9b has not been restriction mapped. *X's* delineate the region that cross-hybridizes with avian alpha-spectrin cDNA pUC8-13a. (*Cross-hatched box*) The 0.76-kb Bam HI fragment of clone 10 used for genomic Southern analysis. The pattern of Pst I fragments in the region of overlap is clearly identical between 9A and 10, but the placement of these sites on the map is tentative.

Restriction mapping analysis clearly indicated that the pUC18:9a and pUC18:10 inserts overlapped (Fig. 2), although it is not known whether these two cDNAs are in fact derived from identical mRNA's. With that caveat, a linear total of ~6.8 kb of cDNA has so far been isolated.

Hybridization Analysis of the Drosophila Alpha-Spectrin Gene

Gel isolated pUC18:9a insert DNA was nick-translated with ^{32}P-dATP and used as a hybridization probe against a Northern blot of *Drosophila* S2 cell poly A$^+$ RNA. A single message of ≥9,000 nucleotides was detected (Fig. 3). This

Figure 1. Antiserum 675 reacts with *Drosophila* alpha-spectrin in head homogenates. Lane *1*, Coomassie Blue stain; lane *2*, polyclonal antiserum 675; lane *3*, preimmune serum. The polypeptides in a lysate of adult *Drosophila* heads were separated by SDS-PAGE and stained with Coomassie Blue, or transferred to nitrocellulose. The Coomassie Blue-stained lane is from a gel that was more heavily loaded than the gel for transfer to show the total profile of proteins in the sample. Neighboring strips of the nitrocellulose blot were incubated in a 1:2,000 dilution of immune or preimmune serum, and bound antibody was visualized as described in Materials and Methods. The heavy antibody reaction in lane 2 corresponds to the position of the 234-kD alpha-spectrin band (Dubreuil et al., 1987).

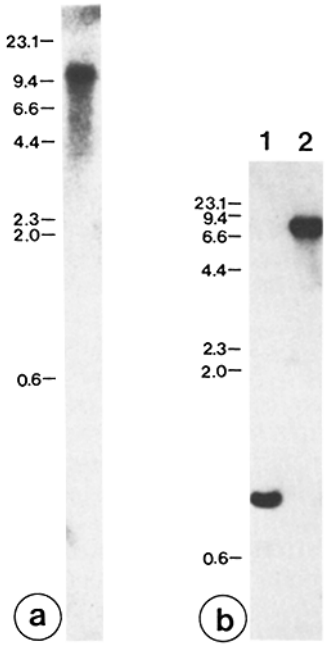

Figure 3. Northern and Southern blot analysis reveals that the *Drosophila* alpha-spectrin mRNA is ≥9 kb and suggests that the gene is single copy. (*a*) Autoradiogram showing the hybridization of ^{32}P-labeled pUC18:9a insert DNA to a Northern blot of 5 μg poly A$^+$ RNA from Schneider's 3 cells. (*b*) Genomic Southern analysis of (lane *1*) Eco RI-digested and (lane 2) Bam HI-digested genomic DNA from cultured *Drosophila* cells (~5 μg per lane). The ^{32}P-labeled 0.76-kb Bam HI fragment (see Fig. 2) was used to probe the digested genomic DNA. The positions of Hind III-digested lambda DNA fragments (*a*, denatured; *b*, not denatured) are indicated in kilobases.

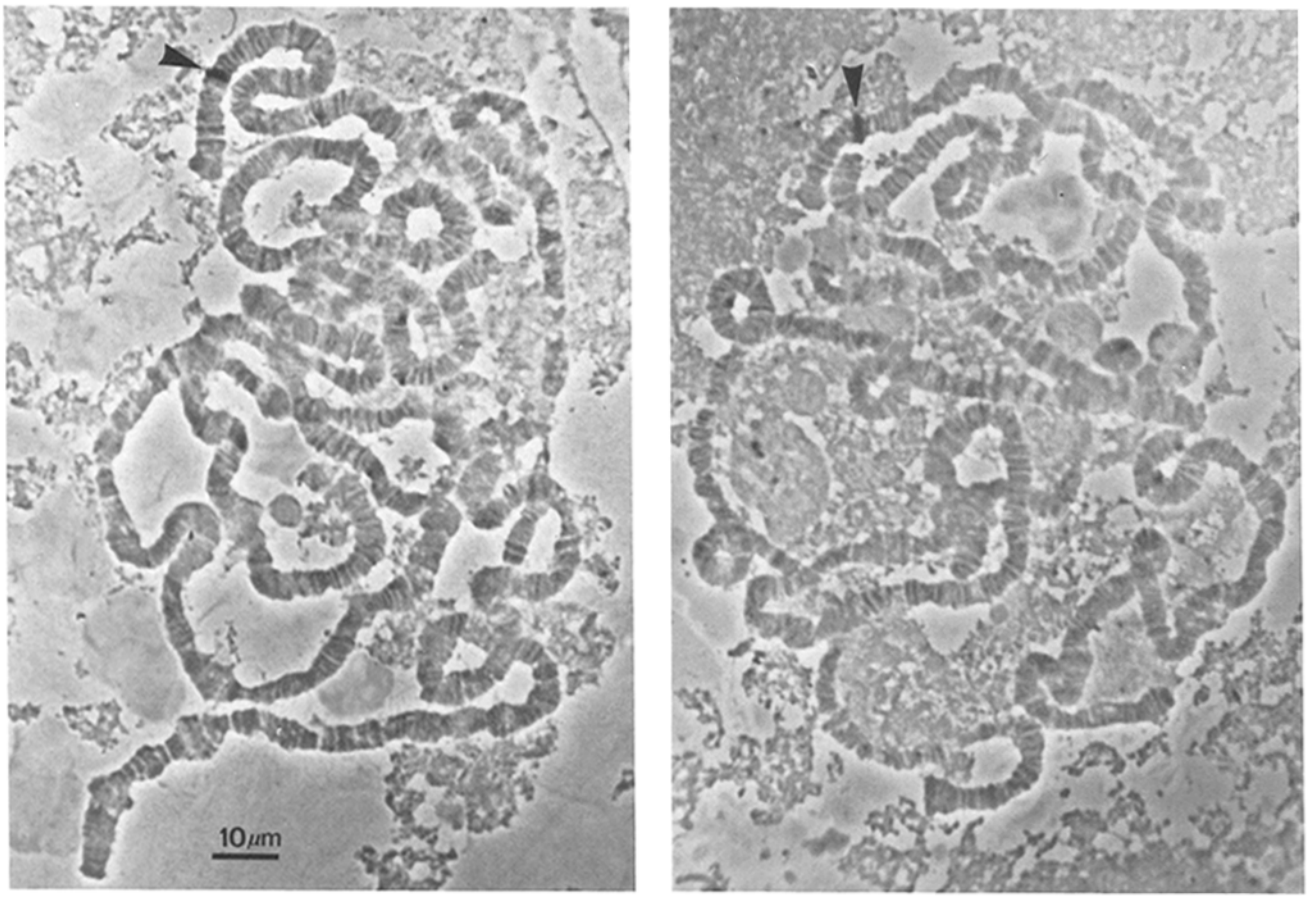

Figure 4. Biotinylated 9a cDNA insert hybridizes in situ to region 62B 1-7 on polytene chromosomes of Oregon R larvae. Only one site of hybridization (*arrowheads*) was seen in each of many chromosome preparations, two of which are shown here.

mRNA is of sufficient size to code for the large *Drosophila* alpha-spectrin protein.

A cDNA sequence coding for avian alpha-spectrin (pUC8-13a; Birkenmeier et al., 1985; kindly provided by J. Barker and C. Birkenmeier) hybridized specifically to the 9a sequence at lowered stringency (data not shown). The region which cross-hybridized is marked by the *X's* in Fig. 2. This demonstrates partial DNA sequence similarity between *Drosophila* and vertebrate alpha-spectrin genes, and further confirms the alpha-spectrin nature of the 234-kD *Drosophila* spectrin band.

The 0.76-kb Bam HI fragment from clone 10 (*diagonal bars*, Fig. 2) was isolated from an agarose gel, labeled by nick translation, and used to probe two restriction digests (Eco RI and Bam HI) of genomic DNA that had been transferred to nitrocellulose paper (Fig. 3). The 0.76-kb Bam HI fragment does not appear to be bisected by introns in genomic DNA (Coyne, R., and L. Goldstein, unpublished observations). A single band was detected for each restriction digest, indicating that at least this portion of the gene is represented as a single copy sequence in the genome. The same results were found with genomic DNA from adult and embryonic sources (data not shown).

Biotinylated pUC18:9a and pUC18:10 DNA inserts were used as probes for in situ hybridization to polytene chromosomes (Fig. 4). Both probes hybridized predominantly to a single locus in the region 62B 1-7 (labeling with insert 10 DNA not shown). This provides further evidence that the *Drosophila* alpha-spectrin gene is single copy.

Production of Alpha-Spectrin Fusion Proteins

The cDNA sequences 9a and 10 were subcloned into pATH vectors for large scale expression of fusion proteins. Hybrid proteins expressed by these vectors contain the amino-terminal portion of the bacterial Trp E enzyme fused to a polypeptide encoded by the recombinant cDNA insert. Upon induction with indoleacrylic acid, Trp E fusion proteins of the expected molecular mass were produced by cDNA clones in only one orientation and one of the three possible frames. These were the pATH10 vector containing the 9a sequence (fusion protein 9a), and pATH1 vector containing the 10 sequence (fusion protein 10). Using the fact that only one orientation was productive, the 3′ and 5′ ends of the cDNA inserts could be inferred and are indicated on Fig. 2. Fusion protein 9a migrated as a ~185-kD polypeptide; fusion protein 10 as a ~190-kD polypeptide (Fig. 5). Because of their large size, these Trp E fusion proteins could be separated from most bacterial proteins by SDS-PAGE and were purified by electroelution (Fig. 5).

Alpha-Spectrin Fusion Proteins Are Antigenically Related to Drosophila and Vertebrate Alpha-Spectrins

Purified fusion protein 9a was used to elicit an immune response in a rabbit. The resulting antiserum (designated 905),

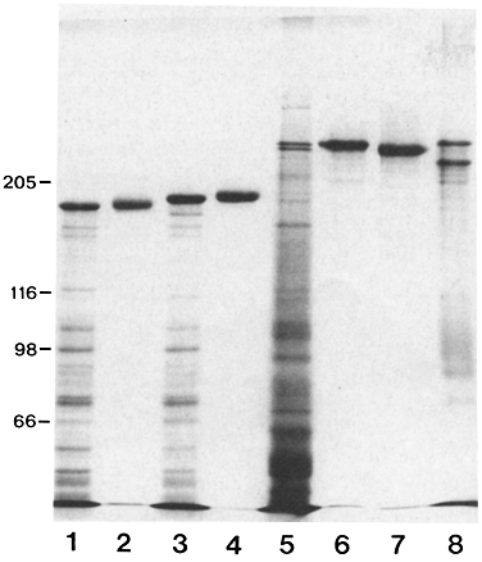

Figure 5. SDS-PAGE analysis of the purity and composition of materials. Lane *1*, lysate of bacteria expressing fusion protein 9a. Lane *2*, fusion protein 9a purified from a gel; lane *3*, lysate of bacteria expressing fusion protein 10; lane *4*, fusion protein 10 purified from a gel; lane *5*, bovine brain membranes (spectrin enriched preparation); lanes *6* and *7*, brain alpha- and beta-spectrin, respectively, purified from the fraction shown in lane *5*; lane *8*, human erythrocyte ghost membrane preparation. All lanes are stained with Coomassie Blue. The position of molecular mass standards are indicated on the left ($\times 10^{-3}$).

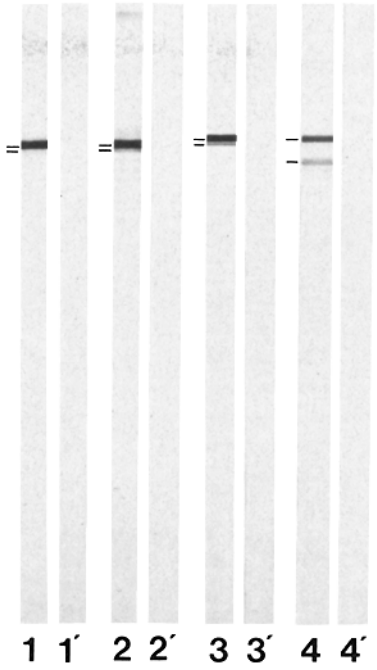

Figure 6. Anti-fusion protein 9a (antiserum 905) demonstrates shared epitopes between *Drosophila* and vertebrate spectrins. Lanes *1* and *1′*, adult *Drosophila* head lysate; lanes *2* and *2′*, phosphocellulose purified *Drosophila* spectrin (Dubreuil et al., 1987); lanes *3* and *3′*, bovine brain membranes; lanes *4* and *4′*, erythrocyte ghosts. (Fig. 5 includes representative protein profiles of the brain and erythrocyte preparations, gels for transfer were loaded with 10–20-fold less material). In each case, the samples were transferred to nitrocellulose after SDS-PAGE and probed with either (lanes *1–4*) a 1:5,000 dilution of antiserum 905 or (lanes *1′–4′*) a 1:5,000 dilution of preimmune serum from the same rabbit. In each case, dashes mark the positions of the corresponding spectrin alpha- and beta-chains.

reacted with a 234-kD band on protein blots of adult *Drosophila* head lysates and with the 234-kD alpha-spectrin band of purified *Drosophila* spectrin (Fig. 6). Preimmune serum from this rabbit did not react with these bands. Antiserum 905 also reacted primarily with the alpha-spectrin band of bovine brain spectrin and human RBC ghosts (Fig. 6). Monoclonal antibodies with several different epitope specificities have been produced against fusion protein 10 (Dubreuil et al., 1987). The fact that several different monoclonal antibodies react with both the fusion proteins and with purified *Drosophila* spectrin argues that the cross-reactivity is not due to one strong determinant shared between these polypeptides. Thus, the 9a and 10 *Drosophila* cDNA sequences code for polypeptides that probably represent portions of the *Drosophila* alpha-spectrin protein.

Fusion Protein 9a and Drosophila Alpha-Spectrin Display Common Peptide Fragments

Fusion protein 9a and *Drosophila* alpha-spectrin were digested with cyanogen bromide and the digestion products were separated by SDS-PAGE. The peptide bands were transferred from these gels to nitrocellulose and were probed with antiserum 905 that had been affinity purified against human erythrocyte alpha-spectrin (Fig. 7). The affinity-purification step was used to remove antibodies reactive to Trp E peptide fragments because the immunogen for the production of antiserum 905 (fusion protein 9a) contained a portion of the bacterial Trp E polypeptide. The structural correspondence between *Drosophila* alpha-spectrin and fusion protein 9a is evident: the majority of peptide bands recognized by

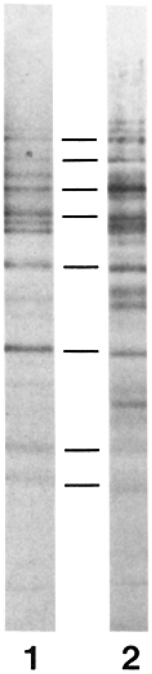

Figure 7. Peptide mapping shows primary sequence homology between *Drosophila* alpha-spectrin and fusion protein 9a. Lane *1*, *Drosophila* alpha-spectrin; lane *2*, fusion protein 9a. Cyanogen bromide digests of the indicated polypeptides were separated on 10–15% gradient polyacrylamide gels, blotted to nitrocellulose, and probed with antiserum 905 that had been affinity purified against human erythrocyte alpha-spectrin. Lines between the lanes illustrate prominent peptide bands that appear in both digests.

the antibody in the *Drosophila* alpha-spectrin lane are also prominent bands in the digest of fusion protein 9a.

Fusion Proteins Bind Calmodulin

Bacterial lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed for binding activity with biotin-labeled calmodulin (Fig. 8). The labeled calmodulin bound to both fusion protein 9a and fusion protein 10 in a calcium-dependent manner. This binding was inhibited by a 10-fold molar excess of unlabeled calmodulin (not shown). Other experiments with polypeptides expressed in pEV vectors, which express the product of the inserted cDNA clones with only a few additional amino acids (Crowl et al., 1985), also showed calcium-dependent calmodulin binding. Thus, the calmodulin binding illustrated in Fig. 8 represented binding to the expressed *Drosphila* spectrin fragments, not primarily to the Trp E portion of the protein.

Binding of Fusion Proteins to Drosophila and Vertebrate Spectrin Subunits

To further investigate the binding properties of the fusion proteins, fusion protein 9a was conjugated to biotin for use in blot overlay experiments. The biotin-labeled protein reacted primarily with the faster migrating, 226-kD band of purified *Drosophila* spectrin on protein blots, and with the beta-spectrin band of bovine brain membranes (Fig. 9). In competition experiments, the binding of labeled fusion protein 9a to *Drosophila* spectrin or to bovine spectrin was inhibited by either a 20-fold excess of unlabeled fusion protein 9a or a 20-fold molar excess of purified bovine brain alpha-spectrin. These results show that fusion protein and authentic alpha-spectrin are competing for the same interchain-binding sites on the two vertebrate beta-spectrins examined. A slight reaction of fusion protein 9a with *Drosophila* alpha-spectrin in Fig. 9 *a* (lanes *2–4*) helps to show the position of the alpha-subunits on the blot. The reaction of fusion protein 9a with alpha-spectrin was not appreciably inhibited in competition experiments (Fig. 9 *a*, lanes *3* and *4*).

The high degree of specificity and the fact that unlabeled alpha-chain competes with the immobilized protein for labeled fusion protein 9a binding argues that the assay is a valid measure of spectrin subunit–subunit binding. These data therefore confirm the alpha-spectrin nature of fusion protein 9a and provide further evidence that the faster migrating 226-kD band of the purified *Drosophila* spectrin doublet (Dubreuil et al., 1987) is a genuine beta-spectrin subunit.

When labeled fusion protein 9a was premixed with a 20-fold molar excess of purified bovine brain beta-spectrin before reaction with the bovine brain or *Drosophila* spectrin blots, labeling of the beta-spectrin band was reduced and shifted to predominant labeling of the alpha-spectrin band (Fig. 9, lanes *5*). This is presumably due to the association

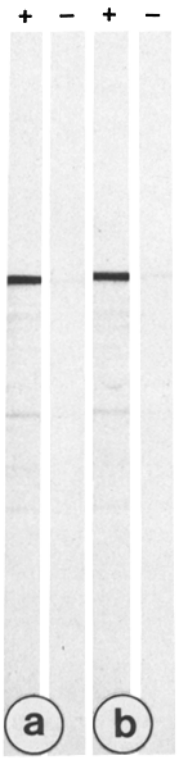

Figure 8. Calmodulin binds to the alpha-spectrin fusion proteins. (*a*) Lysate of *E. coli* expressing fusion protein 9a; (*b*) lysate of *E. coli* expressing fusion protein 10. In each case, the bacterial lysates were transferred to nitrocellulose after SDS-PAGE and probed with biotin-labeled calmodulin in either the presence of 1 mM $CaCl_2$ (+) or 1 mM EGTA (−) during incubation and subsequent rinses. The heavily stained bands in lanes *a* + and *b* + correspond to the positions of the fusion proteins.

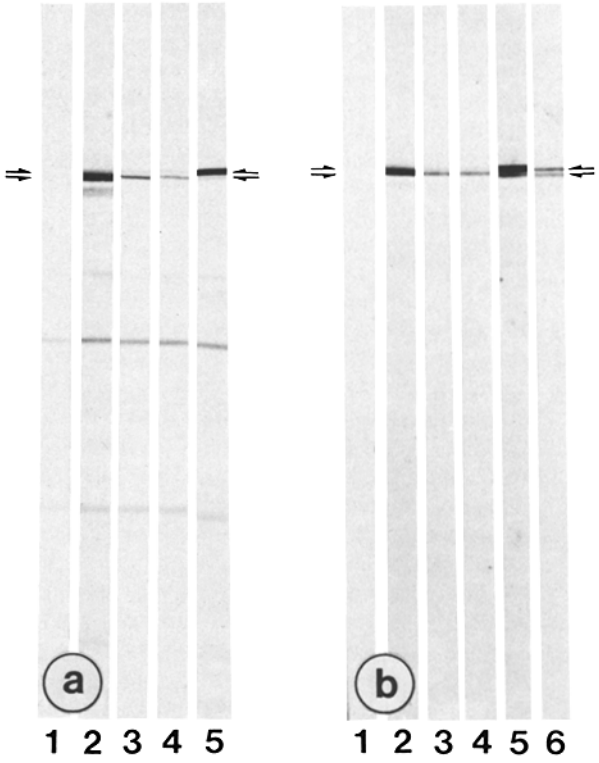

Figure 9. Alpha-spectrin fusion proteins bind to the beta-subunits of *Drosophila* and vertebrate spectrins. (*a*) Bovine brain membranes; (*b*) purified *Drosophila* spectrin. Spectrin enriched bovine brain membranes or purified *Drosophila* spectrin was transferred to nitrocellulose after SDS-PAGE. In each case, the nitrocellulose blots were probed with: lanes *1*, 0.5 μg/ml streptavidin-alkaline phosphatase alone; lanes *2*, 0.5 μg/ml biotin-labeled fusion protein 9a; lanes *3*, 0.5 μg/ml labeled fusion protein 9a plus 10 μg/ml unlabeled fusion protein 9a; lanes *4*, 0.5 μg/ml labeled fusion protein 9a plus ~14 μg/ml unlabeled brain alpha-spectrin; lanes *5*, 0.5 μg/ml labeled fusion protein 9a that had been premixed for 1 h with ~14 μg/ml unlabeled brain beta-spectrin; lane *6*, unlabeled brain alpha-spectrin (21 μg/ml) + the premixed components of lanes *5*. All incubations were carried out in a total volume of 200 μl. Arrows on either side of each set of blot strips mark the positions of the spectrin alpha- and beta-chains.

of labeled fusion protein 9a with the brain beta-subunit in solution, followed by an association of the beta-subunit (now complexed with labeled fusion protein) with the alpha-spectrin on the blot. Alpha-spectrin in solution competed with alpha-spectrin on the blot for binding of the putative-labeled fusion protein–beta–spectrin complex (Fig. 9*b*, lane *6*). It appears from these experiments that brain beta-spectrin has at least one binding site for brain or *Drosophila* alpha-spectrin that was not occupied by fusion protein 9a.

The binding of fusion protein 9a to beta-spectrins was probably not due to the bacterial TrpE moiety or to the attached biotin because no binding was detected when labeled fusion protein 10 was used in otherwise identical blot overlay experiments. Steric hindrance by the attachment of biotin within a binding site could explain the lack of fusion protein 10–binding activity, but we have independent evidence that this was not the case: antiserum 905 primarily labels brain alpha-spectrin (Fig. 6). Inclusion of an excess of unlabeled fusion protein 9a caused a shift in this reaction to the beta-spectrin band (data not shown). This was presumably due to 905 antibodies binding to the fusion protein 9a, and fusion protein 9a binding to beta-spectrin on the blot. Fusion protein 10 did not exhibit this behavior.

Discussion

Five lines of evidence indicate that the cDNA sequences we have isolated originate from the *Drosophila* alpha–spectrin gene: (*a*) fusion proteins encoded by the *Drosophila* sequences and expressed in *E. coli* shared multiple antigenic determinants with *Drosophila* and vertebrate alpha-spectrins; (*b*) peptide maps demonstrated several peptides in common between a fusion protein and *Drosophila* alpha-spectrin; (*c*) one of the fusion proteins had conserved binding sites for *Drosophila* and vertebrate beta–spectrin subunits; (*d*) the fusion proteins exhibited calcium-dependent calmodulin-binding activity as does purified *Drosophila* alpha-spectrin; and (*e*) a region of the *Drosophila* cDNA sequence contained sequences similar to a vertebrate alpha–spectrin cDNA. Consistent with this evidence is the finding that one of the *Drosophila* cDNA sequences hybridized to a *Drosophila* mRNA of adequate size to code for the large spectrin polypeptide. In situ hybridization and genomic Southern analysis suggest that this alpha-spectrin gene is a single copy sequence in the *Drosophila* genome.

The association of spectrin subunits is probably a cooperative, high affinity interaction (Marchesi et al., 1985). To date, the subunits have been separated from each other only after treatment with denaturants such as urea (Calvert et al., 1980, Davis and Bennett, 1983, Yoshino and Marchesi, 1984) or SDS (Speicher et al., 1980, Litman et al., 1980). Despite such harsh treatment, it has been shown that the isolated chains can renature sufficiently for reassembly of dimeric and tetrameric complexes (Calvert et al., 1980, Davis and Bennett, 1983, Yoshino and Marchesi, 1984, Coleman et al., 1987). In addition, it has been shown that ^{125}I-alpha-spectrin can associate with both brain and erythrocyte beta–spectrin subunits that have been subjected to SDS-PAGE and transferred to nitrocellulose (Davis and Bennett, 1983). Because the binding of biotinylated fusion protein 9a was highly specific and was inhibited by an excess of unlabeled fusion protein, we conclude that our blot overlays with labeled fusion proteins demonstrate binding sites for beta-spectrins that are conserved between a vertebrate alpha-spectrin and *Drosophila* alpha-spectrin. The fact that the 226-kD subunit of *Drosophila* spectrin shows the same binding specificities as vertebrate beta-spectrin affirms its identity as an authentic beta subunit.

Because fusion protein 10 did not bind to spectrin subunits on nitrocellulose under identical conditions as did fusion protein 9a, we infer that it does not have the same binding sites as fusion protein 9a. Thus, the region coding for the interchain binding site or sites must be located at least partially in the 3′ portion of the 9a cDNA sequence that is not shared by the 10 sequence. Similar reasoning will allow us to locate the calmodulin-binding site or sites of the *Drosophila* alpha–spectrin chain. If there is only one site, its coding sequence must be in the region of overlap between the 9a and 10 cDNA inserts, since both fusion protein 9a and fusion protein 10 bind calmodulin. Examination of fusion proteins expressed by vectors with systematic deletions in the regions of interest should allow precise localization of the sequences coding for these binding sites. Ultimately, mutagenesis of these sequences will allow an in depth study of the structural characteristics and functional implications of calmodulin and interchain binding both in vitro and in vivo.

Our findings extend knowledge of the spectrin family of proteins into the higher invertebrates. Vertebrate spectrin sequences (reviewed by Speicher, 1986) exhibit a fascinating repeat structure that probably accounts for spectrin's flexibility and gel properties (Elgsaeter et al., 1987). Our results show antigenic determinants, interchain binding interactions, and nucleotide homology that are conserved between vertebrate and *Drosophila* spectrins. Sequence analysis of *Drosophila* alpha–spectrin cDNAs will determine whether *Drosophila* spectrin has a repeating segmental structure. Further description of the *Drosophila* spectrin gene will allow us to exploit this organism's potential for molecular and classical genetic analysis in order to understand the in vivo function of this conserved polypeptide.

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References

Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273–304.

Birkenmeier, C. S., D. M. Bodine, E. A. Repasky, D. M. Helfman, S. H. Hughes, and J. E. Barker. 1985. Remarkable homology among the internal repeats of erythroid and nonerythroid spectrin. *Proc. Natl. Acad. Sci. USA.* 82:5671–5675.

Burnette, W. N. 1981. Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

Calvert, R., P. Bennett, and W. Gratzer. 1980. Properties and structural role of the subunits of human spectrin. *Eur. J. Biochem.* 107:355–361.

Coleman, T. R., A. S. Harris, S. M. Mische, M. S. Mooseker and J. S. Morrow. 1987. Beta spectrin bestows protein 4.1 sensitivity on spectrin-actin interactions. *J. Cell Biol.* 104:519–526.

Crowl, R., C. Seamans, P. Lomedico, and S. McAndrew. 1985. Versatile expression vectors for high level synthesis of cloned gene products in *Escherichia coli*. *Gene.* 38:31–38.

Davis, J., and V. Bennett. 1983. Brain spectrin. Isolation of subunits and formation of hybrids with erythrocyte spectrin subunits. *J. Biol. Chem.* 258:7757–7766.

Dieckmann, C. L., and A. Tzagoloff. 1985. Assembly of the mitochondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. *J. Biol. Chem.* 260:1513–1520.

Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. Preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119–130.

Dubreuil, R., T. J. Byers, D. Branton, L. S. B. Goldstein, and D. P. Kiehart. 1987. *Drosophila* spectrin. I. Characterization of the purified protein. *J. Cell Biol.* 105:2095–2102.

Elgsaeter, A., B. T. Stokke, A. Mikkelsen, and D. Branton. 1987. The molecular basis of erythrocyte shape. *Science (Wash. DC).* 234:1217–1223.

Engels, W. R., C. R. Preston, P. Thompson, and W. B. Eggleston. 1986. In situ hybridization to Drosophila salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. *Focus (Idaho).* 8:6–8.

Forster, A. C., J. L. McInnes, D. C. Skingle, and R. H. Symons. 1985. Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Res.* 13:745–761.

Goldstein, L. S. B., R. A. Laymon, and J. R. McIntosh. 1986. A microtubulin-associated protein in *Drosophila melanogaster*: identification, characterization, and isolation of coding sequences. *J. Cell Biol.* 102:2076–2087.

Higgins, R. C., and M. E. Dahmus. 1979. Rapid visualization of protein bands in preparative SDS-polyacrylamide gels. *Anal. Biochem.* 93:257–260.

Itoh, N., J. R. Slemmon, D. H. Hawke, R. Williamson, E. Morita, K. Itakura, E. Roberts, J. E. Shively, G. D. Crawford, and P. Salvaterra. 1986. Cloning of *Drosophila* choline acetyltransferase cDNA. *Proc. Natl. Acad. Sci. USA.* 83:4081–4085.

Johnson, D. A., and J. H. Elder. 1983. Antibody directed to determinants of a Maloney virus derived MCF GP70 recognizes a thymic differentiation antigen. *J. Exp. Med.* 158:1751–1756.

Laemmli, U. K. 1971. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Litman, D., C. J. Hsu, and V. T. Marchesi. 1980. Evidence that spectrin binds to macromolecular complexes on the inner surface of the red cell membrane. *J. Cell Sci.* 42:1–22.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 280–281.

Marchesi, V. T. 1985. Stabilizing infrastructure of cell membranes. *Annu. Rev. Cell Biol.* 1:531–561.

Olmsted, J. B. 1981. Affinity-purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.

Pepinsky, R. B. 1983. Localization of lipid-protein and protein-protein interactions within the murine retrovirus *gag* precursor by a novel peptide-mapping technique. *J. Biol. Chem.* 258:11229–11235.

Schneider, I., and A. B. Blumenthal. 1978. Drosophila cell and tissue culture. *In* The Genetics and Biology of Drosophila. Vol. 2a. M. Ashburner and T. R. F. Wright, editors. Academic Press, Inc. New York. 266–315.

Sigel, M. B., Y. N. Sinha, and W. P. Vanderlaan. 1983. Production of antibodies by inoculation into lymph nodes. *Methods Enzymol.* 93:3–12.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.

Speicher, D. W. 1986. The present status of erythrocyte spectrin structure: the 106 residue repetitive structure is a basic feature of an entire class of proteins. *J. Cell. Biochem.* 30:245–258.

Speicher, D. W., J. S. Morrow, W. J. Knowles, and V. T. Marchesi. 1980. Identification of proteolytically resistant domains of human erythrocyte spectrin. *Proc. Natl. Acad. Sci. USA.* 77:5673–5677.

Yanisch-Perron, C., J. Viera, and J. Messing. 1985. Improved M13 cloning vectors and host strains. Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene (Amst.)* 33:103–119.

Yoshino, H., and V. T. Marchesi. 1984. Isolation of spectrin subunits and reassociation in vitro. Analysis by fluorescence polarization. *J. Biol. Chem.* 259: 4496–4500.

Young, R. A., and R. W. Davis. 1984. Yeast RNA polymerase II genes: isolation with antibody probes. *Science (Wash. DC).* 222:778–782.