Primary Structure of the Brain α-Spectrin

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Abstract. We have determined the nucleotide sequence coding for the chicken brain α-spectrin. It is derived both from the cDNA and genomic sequences, comprises the entire coding frame, 5′ and 3′ untranslated sequences, and terminates in the poly(A)-tail. The deduced amino acid sequence was used to map the domain structure of the protein. The α-chain of brain spectrin contains 22 segments of which 20 correspond to the repeat of the human erythrocyte spectrin (Speicher, D. W., and V. T. Marchesi. 1984. *Nature (Lond.).* 311:177–180.), typically made of 106 residues. These homologous segments probably account for the flexible, rod-like structure of spectrin. Secondary structure prediction suggests predominantly α-helical structure for the entire chain.

Parts of the primary structure are excluded from the repetitive pattern and they reside in the middle part of the sequence and in its COOH terminus. Search for homology in other proteins showed the presence of the following distinct structures in these nonrepetitive regions: (*a*) the COOH-terminal part of the molecule that shows homology with α-actinin, (*b*) two typical EF-hand (i.e., Ca^{2+}-binding) structures in this region, (*c*) a sequence close to the EF-hand that fulfills the criteria for a calmodulin-binding site, and (*d*) a domain in the middle of the sequence that is homologous to a NH_2-terminal segment of several *src*-tyrosine kinases and to a domain of phospholipase C. These regions are good candidates to carry some established as well as some yet unestablished functions of spectrin. Comparative analysis showed that α-spectrin is well conserved across the species boundaries from *Xenopus* to man, and that the human erythrocyte α-spectrin is divergent from the other spectrins.

SPECTRIN is the major constituent of the cytoskeletal network underlying the plasma membrane (for a review see Marchesi, 1985). It was considered to be specific for red blood cells until spectrin-like proteins were detected immunologically in many types of cells (Goodman et al., 1981; Levine and Willard, 1981; Bennett et al., 1982; Burridge et al., 1982; Glenney et al., 1982*a*,*b*; Repasky et al., 1982; Kakiuchi et al., 1982; Lehto and Virtanen, 1983). At present we know several proteins related to the red blood cell spectrin. Their kinship has been primarily investigated by peptide mapping and immunological techniques (Repasky et al., 1982; Glenney et al., 1983; Glenney and Glenney, 1984*a*, *b*; Harris et al., 1985). These studies have shown that spectrins in different tissues occur as heterodimers and possess a common (α, M_r from 230 to 260 kD) and a variant (β or γ, M_r from 220 to 260 kD) subunit (Lazarides and Nelson, 1985). The latter show a high degree of variation while the common subunits are much alike in different types of cells (Glenney et al., 1982*b*). The mammalian erythroid α-chain is, however, a deviant member of the family and diverges from the others by its immunological and structural properties (Glenney and Glenney, 1984*a*; Harris et al., 1985).

The first spectrin-like molecule to be detected outside the realm of the red blood cells was found in brain (Levine and Willard, 1981; Goodman et al., 1981) and is also called fodrin (Levine and Willard, 1981) or calspectin (Kakiuchi et al., 1982). Recently it has been shown that mammalian brain contains two isoforms of spectrin (Lazarides and Nelson, 1983; Lazarides et al., 1984; Riederer et al., 1986; Virtanen et al., 1986), one located primarily in the axons and the other in the cell bodies and dendrites. Another spectrin-like protein that is more thoroughly characterized, was detected in the avian intestinal tissue (Glenney et al., 1982*b*). This terminal web (TW) 260/240-protein differs from the others by its location in the terminal web of the enterocytes, distant from the plasma membrane.

First data on the primary structure of spectrin were presented by Speicher et al. (1983). They sequenced some tryptic peptides of the human erythrocyte spectrin α- and β-chains. The sequences revealed repetitive structure where each unit typically consists of 106 amino acids. However, the compiled fragmentary data cover only ~48% of the α- and 18% of the β-chain (Speicher and Marchesi, 1984). The first cDNA sequence of a nonerythroid spectrin was determined by us for a clone isolated from a chicken gizzard expression library (Wasenius et al., 1985). This 1.5-kb sequence showed that the basic architecture of the erythroid spectrin (i.e., the multiply repeated 106-residue motif) is also found in the nonerythroid spectrins. This finding was confirmed by Birkenmeier et al. (1985). Later, McMahon et al. (1987), Leto et al. (1988), and Giebelhaus et al. (1987) have provided

partial sequences of human spectrin, rat and human spectrins, and of *Xenopus* oocyte spectrin, respectively.

In this paper we present the primary structure of α-spectrin, based on the cDNA and genomic sequences, and map its domain structure. The primary structure reveals a multidomain molecule. It is mostly comprised of regular homologous repeats but also contains in its middle and COOH-terminal parts nonrepetitive sequences. The latter show sequence similarities to some functionally defined proteins and may thus carry specific functions of spectrin.

Materials and Methods

General Methods and Reagents

General procedures, such as isolation of DNA, restriction enzyme digestions, PAGE and agarose gel electrophoresis, purification of DNA fragments from the gels, and nick translation of the probes, were performed by standard methods (Maniatis et al., 1982). Restriction and modification enzymes and polymerases were purchased from Boehringer Mannheim Biochemica GmbH (Mannheim, FRG) unless indicated otherwise and radionucleotides from Amersham International (Amersham, UK).

Isolation of cDNA and Genomic Clones

A cDNA library was made in lambda gt10 phage as described (Huynh et al., 1985). Total RNAs were prepared from various tissues of 14-d chicken embryos by a modified guanidine isothiocyanate/cesium chloride method as described earlier (Chirgwin et al., 1979). Poly $(A)^+$-RNA was isolated by two cycles on an oligo-dT-cellulose column (Pharmacia Fine Chemicals, Piscataway, NJ). The RNA isolated from brain was used for cDNA synthesis. This was carried out by RNase H method (Gubler and Hoffman, 1983) using oligo-dT (Promega Biotec, Madison, WI) as a primer. cDNA was then treated with Eco RI-methylase, Eco RI linkered, digested with Eco RI, and size selected on a Sepharose CL-4B column (Pharmacia Fine Chemicals). cDNA fragments over 1 kb were ligated overnight at 14°C to the Eco RI-digested and phosphatase-treated lambda gt10 vector (Promega Biotec) and packaged into virus particles. *Escherichia coli* c600hfl host cells were transfected and plated. A chicken genomic library was constructed by using the DNA isolated from the brains of 14-d-old chicken embryos. The DNA was partially digested with Eco RI, ligated into lambda gt10-vector, packaged, and transfected into *E. coli* c600hfl cells following the procedures described for cDNA library construction (see above).

The cDNA library was spread on culture plates, replicated onto nitrocellulose filters which were then screened by hybridization with nick-translated 18-3a clone (Wasenius et al., 1985). Screening of $\sim 1.8 \times 10^4$ plaques under stringent conditions (final wash with 0.1× SSC, 0.1% SDS at 65°C for 1 h) yielded 23 positive clones. The positive clones were localized, picked up, and plaque purified. The isolated lambda DNAs were digested with Eco RI to release the inserts. These fragments were isolated from agarose gels by trapping to strips of DEAE-nitrocellulose filters and subcloned into M13 vectors for subsequent sequence analysis. A "sublibrary" was prepared using a specific oligonucleotide primer (5'CTCTTCCAGAAGATTCT3') (see Fig. 1) and screened as described in the text. The genomic library was screened using some clones from the 5' end of spectrin cDNA to obtain clones that cover further the 5' end of the coding sequence. Inserts were isolated and cloned for sequence analysis as above.

DNA Sequence Analysis

Sequencing of the DNAs subcloned into M13 mp18 and mp19 vectors was performed by Sanger's dideoxy chain termination method (Sanger et al., 1980; Biggin et al., 1983) with both the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD) and modified T7 DNA polymerase from United States Biochemical Corp. (Cleveland, OH). Exo III-nuclease technique was used to generate deleted inserts (Henikoff, 1984). M13 universal primer and several specific primers were used.

Computer Analysis

Nucleotide sequencing was aided by the Staden programs (Staden, 1987). Amino acid sequence homologies were studied with a computer program, DIAGON (Staden, 1982); the parameters in dot matrix analyses are specified in the figure legends. Secondary structure was predicted using the algorithm of Garnier et al. (1978). Search for homologous sequences was carried out using the protein identification resource, PIR (database 12.0, March 1987), as a reference.

Oligonucleotide Synthesis

Deoxyoligonucleotides were synthesized with synthesizer (model 381A; Applied Biosystems, Inc., Foster City, CA).

Results

Isolation of cDNA and Genomic Clones for Brain α-Spectrin

Brain was chosen for the construction of the cDNA library on the basis of its relatively high content of spectrin mRNA; this was found by Northern hybridization of mRNAs from various tissues with the α-spectrin probe 18-3a (not shown). The library was screened by hybridization with the same probe. The longest cDNA insert detected was ~4.7 kb. The nucleotide sequence analysis revealed that its 3' end corresponded to the poly$(A)^+$-tail of the mRNA. The clone contains an authentic Eco RI site that divides it into two halves which are designated CLONE IA and CLONE IB in Fig. 1. The 18-3a sequence is present in the middle of the CLONE I, confirming that the isolated clone represents α-spectrin; it is marked with a dashed overline in Fig. 2 (nucleotides 5,041–6,459). Sequencing errors in the 5' end of the published 18-3a sequence (Wasenius et al., 1985) and in the 12 carboxy-terminal residues in our previously published partial sequence (Wasenius et al., 1987) are corrected in Fig. 2.

We were unable to isolate from the original cDNA library clones that would extend the sequence further into the 5' direction. Thus a 17-mer primer (5'CTCTTCCAGAAGATTCT3')

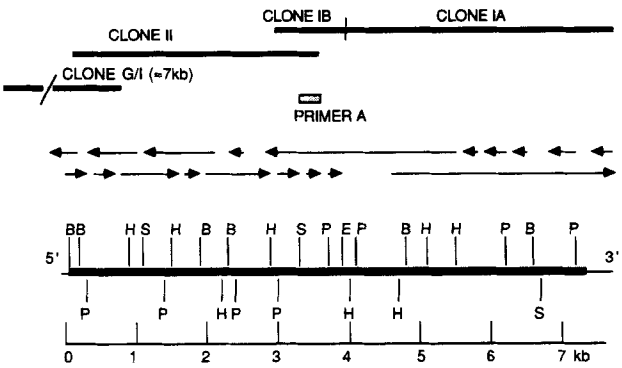


Figure 1. Restriction enzyme map and sequencing strategy for the α-spectrin cDNA and genomic clones. The mapped restriction sites are Bam HI (*B*), Eco RI (*E*), Hind III (*H*), Pst I (*P*), and Sph I (*S*). The dideoxy chain termination method (Sanger, 1980) was applied on CLONE I which was divided in two halves (*CLONE IA* and *IB*) by an authentic Eco RI site. A 17-mer oligonucleotide primer A was used to prime a sublibrary which was screened with the CLONE IB; thus, the CLONE II that spans most of the frame coding for the NH_2-terminal sequence was isolated. A genomic library was constructed from the DNA isolated from embryonal brain tissue and screened with the 5' end fragments of the CLONE II. The genomic clone CLONE G/I, that contains the 5' end of the coding frame was partially sequenced by specific priming. *Arrows*, the consensus sequence obtained for both strands.

was designed complementary to the 5′ end of the CLONE IB (Fig. 1) and used as a primer to produce a sublibrary using the RNase H method described in Materials and Methods. The sublibrary was screened with the CLONE IB as a probe. From it a new clone (CLONE II) that overlaps the original 4.7-kb clone was obtained.

To obtain clones covering the 5′ end of the coding sequence further, the genomic library was screened with some 5′ fragments of the CLONE II as probes. This yielded a ~7-kb genomic clone (CLONE G/I) that overlaps the CLONE II by ~650 bp.

The restriction map and the sequencing strategy of the CLONES I, II, and G/I are summarized in Fig. 1.

The Nucleotide and Deduced Amino Acid Sequence of the Brain α-Spectrin

The nucleotide sequence of the entire coding region was obtained from the two overlapping cDNA clones and from a partial sequence of the genomic clone. Also, the entire untranslated 3′ region was sequenced. Fig. 2 shows the consensus nucleotide and deduced amino acid sequences. The former comprises 7,774 bp and contains an open reading frame (7,431 nucleotides) that codes for 2,477 amino acid residues yielding a mol mass of 285,369 for the translation product. The putative initiation codon is 124 bases from the 5′ end of the shown sequence. It is flanked by a sequence that is in good agreement with the Kozak's rule for the functional initiation codon (Kozak, 1986). This ATG codon is preceded by a typical TATA box (between −45 and −40) and a CAAT box (−111 to −108). After the stop codon, there is a 219-bp untranslated 3′ sequence with a poly(A)-tail. The polyadenylation signal AATAAA occurs 19 bp from the poly(A)-tail (nucleotides 7,606–7,611). These signals are underlined in Fig. 2. The Eco RI site in CLONE I was not overlapped by sequencing. However, a previously determined homologous sequence has already covered this site (McMahon et al., 1987).

Repetitive Structure of the α-Spectrin

Internal repeats were systematically studied using a computer program, DIAGON (Staden, 1982). Fig. 3, depicting a diagonal plot, shows that the molecule is composed of homologous NH_2- and COOH-terminal halves indicated by a long contiguous diagonal line (Fig. 3, *arrow*), and of multiply repeated, homologous units indicated by the numerous parallel evenly spaced lines. These basic repeats are designated α1, α2, α3, etc. following the nomenclature introduced by Speicher and Marchesi (1984). They encompass ~80% of the molecule excluding two regions (in α10 and α11) in the middle and one (in α21 and α22) at the carboxy-terminal part of the protein (Fig. 4). The latter are revealed as "white strips" in the dot matrix, which indicates that they are different from the other segments.

The optimal alignment of the homologous repeats is shown in Fig. 4. The particular alignment (α1 begins with the amino acid residue 15) has been chosen to make it match with the published pattern of Speicher and Marchesi (1984) and of Wasenius et al. (1985). This leaves 14 NH_2-terminal residues as an overhang, designated α′1. Most of the repeats (α2–5, α7–9, α12–14, and α16–18) are 106 amino acids long, thus conforming to the common repetitive pattern of the spectrin structure (Speicher and Marchesi, 1984; Wasenius et al. 1985). One gap is required for the optimal alignment in α1 and α6. α15 is longer than the other repeats due to the unique insertion in position 70 (Fig. 4).

The alignment shows a faithful occurrence of certain amino acids in the fixed positions in most of the repeats (Fig. 4). These include isoleucine (in positions 1 and 46), tryptophan (12 and 45), leucine (15 and 26), arginine (22), aspartic acid (38), glutamic acid (48), lysine (71), and histidine (72 and 101). The amino-terminal ends of the repeats seem to be more strongly conserved than the carboxy-terminal ends.

The segments α20 and α21 are longer and show a lower degree of homology to the other repeats. In addition, the segments called α10 and α22 are qualitatively different from the rest of α-spectrin chain. Another "nonhomologous" region can be found as an extension of the α11 unit.

In Table I all the pairwise comparisons of the units α1–α22 are shown. The highest degree of homology is seen between the repeats in the corresponding positions of the amino- and carboxy-terminal halves of the molecule; the α2–α8 stretch is closely related to a sequence covering α11–α17 (Fig. 3, *arrow*). This may be a track left by the latest duplication event in the evolution of spectrin (see Discussion). This comparison also shows that the divergent regions α10 and α22 are totally unrelated to the homologous repeat units and to each other.

Secondary structure prediction was carried out using the algorithm of Garnier et al. (1978; and data not shown). It revealed predominantly α-helical structure. Some of the homologous repeats (α2, α4, α5, α11, α14, α16, α18, α20, and α21) show an α-helical structure without any or with only a slight tendency to breaks. In α1, α3, α6–α9, α12, α13, and α15, on the other hand, several helix-breaking turns and coil structures are predicted. In many repeats they tend to cluster around the positions 58–68 and 80–85.

Comparison of Various α-Spectrins

Alignment of the present sequence with the known partial sequences of various α-spectrins is shown in Fig. 5. The published *Xenopus* oocyte α-spectrin sequence (Giebelhaus et al., 1987) corresponds to the residues 568–1,021, the human fibroblast α-spectrin sequence (McMahon et al., 1987) to the residues 676–1,599, and the rat brain α-spectrin sequence (Leto et al., 1988) to the residues 1,776–2,250. The fragments of the human erythroid α-spectrin sequence (Speicher and Marchesi, 1984) cover scattered parts along the chicken sequence except the COOH terminus.

Comparison between our sequence and the human fibroblast α-spectrin reveals that these sequences are practically identical. It also confirms that the 60-bp insert found in one of the clones of McMahon et al. (1987) represents the predominant transcript and is not a cloning artifact since the same sequence is present here (amino acid residues 1,053–1,073). The observation of McMahon et al. (1987) that there is a 36 amino acid extension in α11 (residues 1,168–1,204 in our sequence) is also corroborated by our sequence.

Xenopus sequence covers only ~17% of the current sequence in the middle part of the molecule. It also shows virtual identity with the chicken brain α-spectrin sequence.

Human erythroid α-spectrin has a significantly lower similarity to the chicken brain sequence than the human

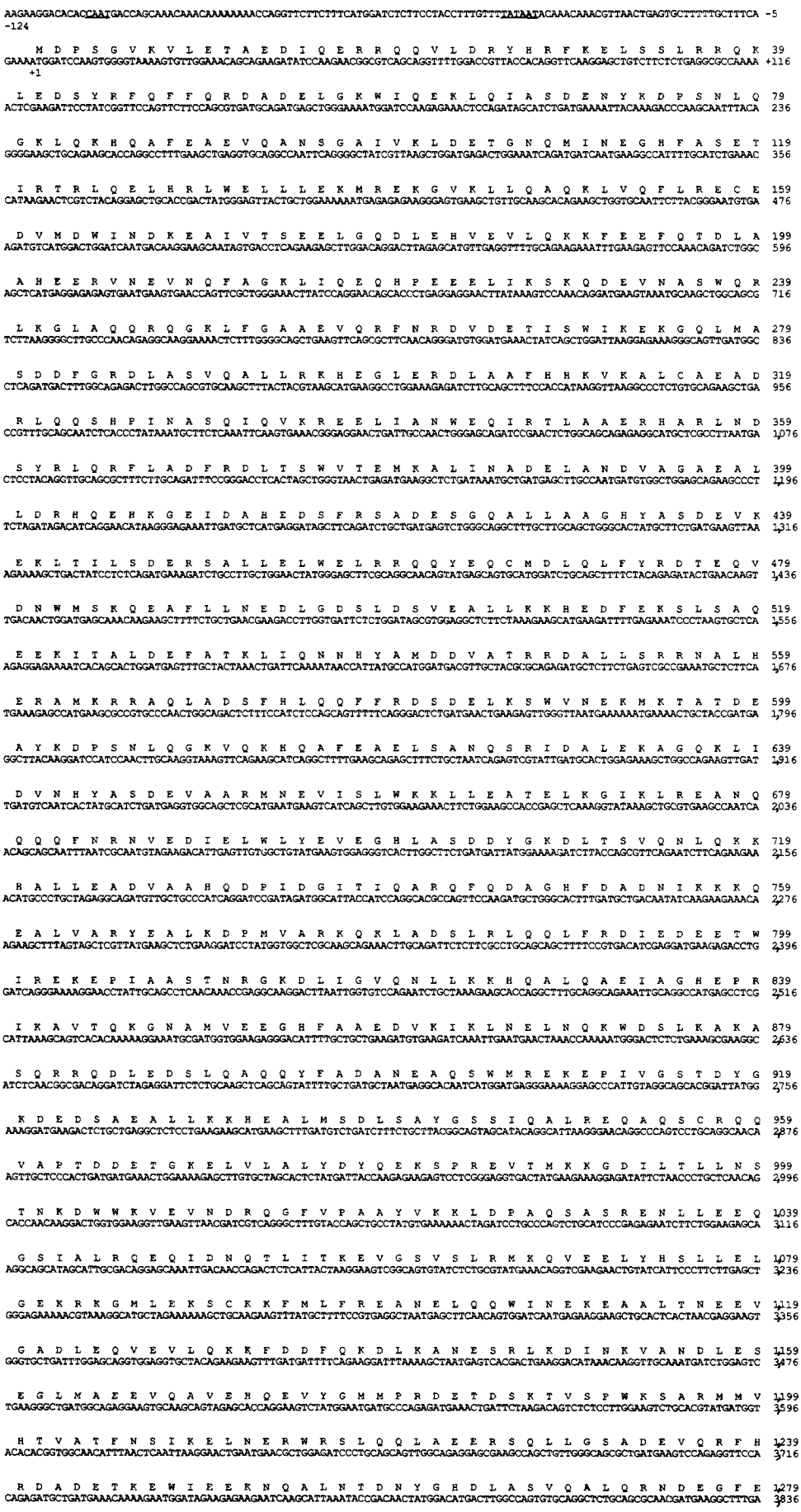
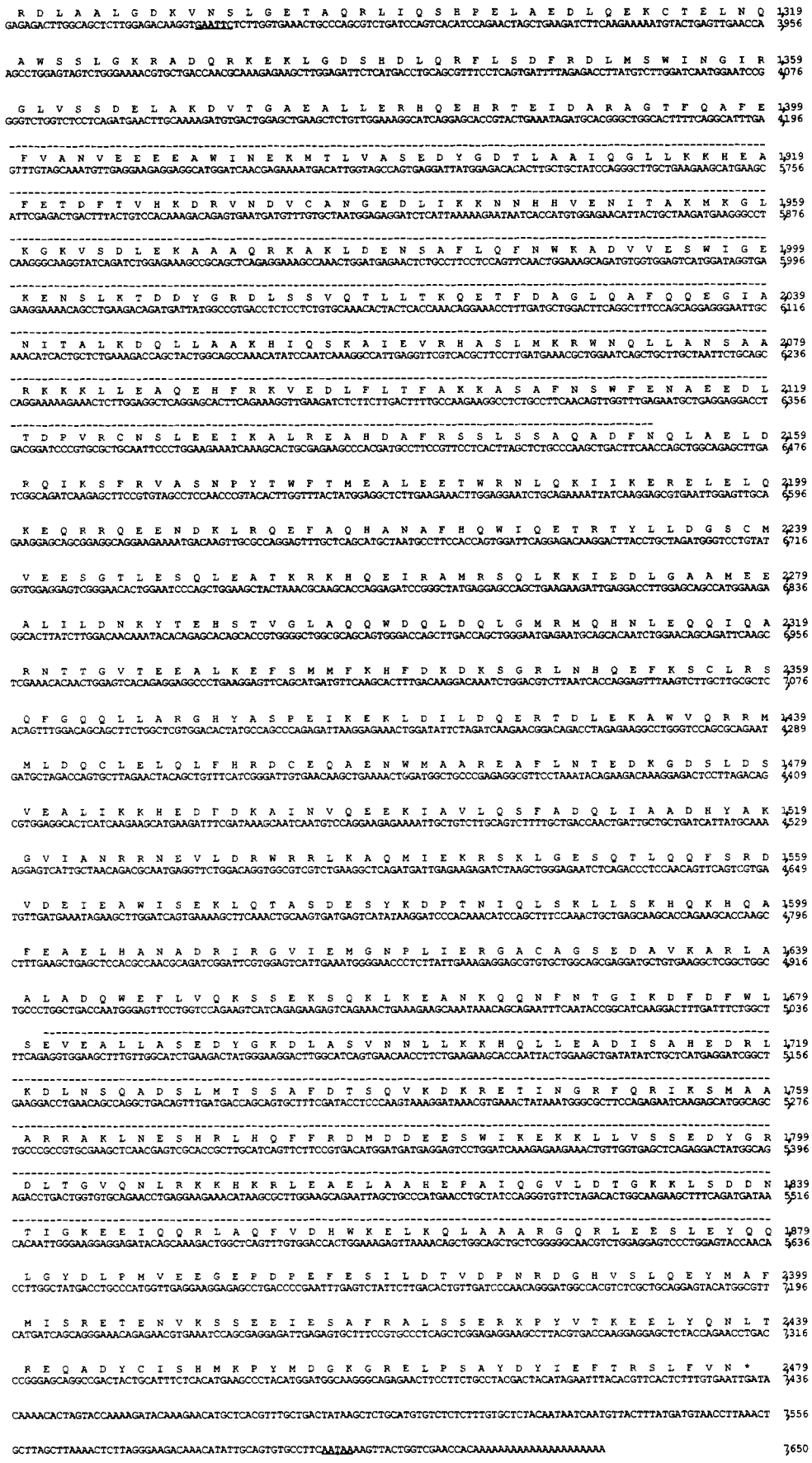


Figure 2. Complete nucleotide and amino acid sequences derived from the α-spectrin cDNA CLONES I and II and the genomic CLONE G/I. The amino acid sequence predicted for the long open reading frame is shown with the single letter code. Numbering of the amino acid sequence begins at the postulated initiator methionine. For the nucleotides, the positions upstream of the initiator codon have negative numbers. Numbering is shown on the right. The nucleotide sequence from −124 to +82 was obtained from the genomic clone with a specific primer. The CAAT and TATAAT sequences at the 5′ end and the polyadenylation signal AATAAA at the 3′ end are underlined. The sequence that is identical with the partial chicken gizzard α-spectrin sequence (18-3a; Wasenius et al., 1985) is overlined. The authentic Eco RI site at position 3,867 is underlined.



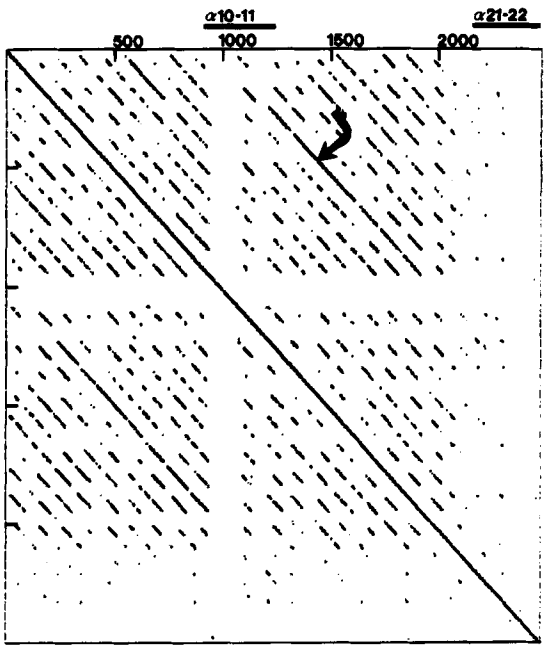


Figure 3. Dot plot of the α-spectrin amino acid sequence. The predicted amino acid sequence was compared with itself using the computer program DIAGON (Staden, 1982). The odd span length was 21 and the scoring level 250, corresponding to the double matching probability of $<5 \times 10^{-5}$. The lines parallel to the diagonal represent repetitive sequences. The residue numbers are indicated on the axes. Bars at α10–α11 and at α21–α22 indicate the longest continuous stretches that fall outside the repetitive pattern. *Arrow*, the "duplication" line. (see also Table I).

fibroblast and the frog oocyte spectrins. An unambiguous alignment between its sequenced fragments and the complete brain protein could, however, be made. On that basis a new positioning of the α-V domain was found. α-V domain represents the tryptic 41-kD COOH-terminal peptide of erythroid α-spectrin (T41), and its amino-terminal end had been tentatively placed in the α18 segment (Speicher and Marchesi, 1984). The present comparison, however, unequivocally places the terminal sequence of this peptide in the α19 segment as already suggested by Speicher (1986) (the H-RBC peptide placed in α19; Fig. 5, *black arrows*). The erythroid spectrin peptide, which was found to be unrelated to the typical repeat by Speicher and Marchesi (1984), finds a match to the boundary of α10 and α11. However, a gap has to be introduced in it to allow optimal alignment (α10–α11, Fig. 5, *asterisks*). This gap corresponds to the 60-bp insert of McMahon et al. (1987) (see above).

The partial rat brain α-spectrin sequence and the present one are again for the most part virtually identical. The rat sequence seems to stop, however, ~227 residues short of the end of the chicken brain spectrin. There appears to be a stop codon which corresponds to the nucleotides 6,750–6,752 in our sequence and which causes a termination of the reading frame 681 nucleotides before the stop codon in the chicken sequence. There is a difference in the sequences that brings about this termination: the rat sequence has a T-insert corresponding to the position 6,717 of our sequence. It causes a shift to a frame which contains a TGA stop codon. We think that this may represent a sequencing error, since further downstream in the rat sequence there is an open reading frame coding for protein that would extend the homology to the COOH-terminal part of the chicken brain spectrin (see Discussion).

Distinct Domains in α-Spectrin

Systematic search in protein databases for sequences that are homologous to spectrin indicated four functionally interesting sites in α-spectrin. Firstly, the COOH terminus of the

Table I. Quantitation of the Conserved Residues Between the Repeats in Brain α-Spectrin

α	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	X	19	23	18	18	50	17	26	22	5	28	26	17	14	54	16	23	26	16	18	13	5
2		X	28	24	36	19	34	35	27	3	42	31	28	31	27	35	29	34	22	20	12	8
3			X	26	30	16	35	33	37	4	24	54	28	29	28	37	38	37	34	18	10	5
4				X	26	17	20	32	28	4	14	26	56	24	25	24	32	24	19	18	16	3
5					X	20	28	28	37	6	29	31	27	61	24	33	27	27	23	21	9	7
6						X	18	23	21	6	22	21	16	18	48	13	24	21	22	19	12	4
7							X	35	30	4	27	29	21	25	30	50	34	31	30	21	10	7
8								X	36	12	26	24	31	29	33	36	46	35	25	17	15	3
9									X	5	27	33	33	31	26	36	33	31	27	20	7	3
10										X	5	4	6	5	7	7	3	7	6	5	8	1
11											X	28	15	22	23	27	22	24	23	18	17	4
12												X	23	27	29	30	27	32	28	22	20	7
13													X	26	25	20	30	22	23	19	12	1
14														X	21	28	24	26	29	17	9	4
15															X	19	26	26	22	18	19	4
16																X	30	35	35	21	13	2
17																	X	37	32	15	10	4
18																		X	26	21	10	4
19																			X	24	15	5
20																				X	17	4
21																					X	3
22																						X

The numbers are identical residues between each repeat. The "duplication" line shown in Fig. 3 (*arrow*) is underlined. The segments α1–α22 are shown by the numbers on the top and on the left.

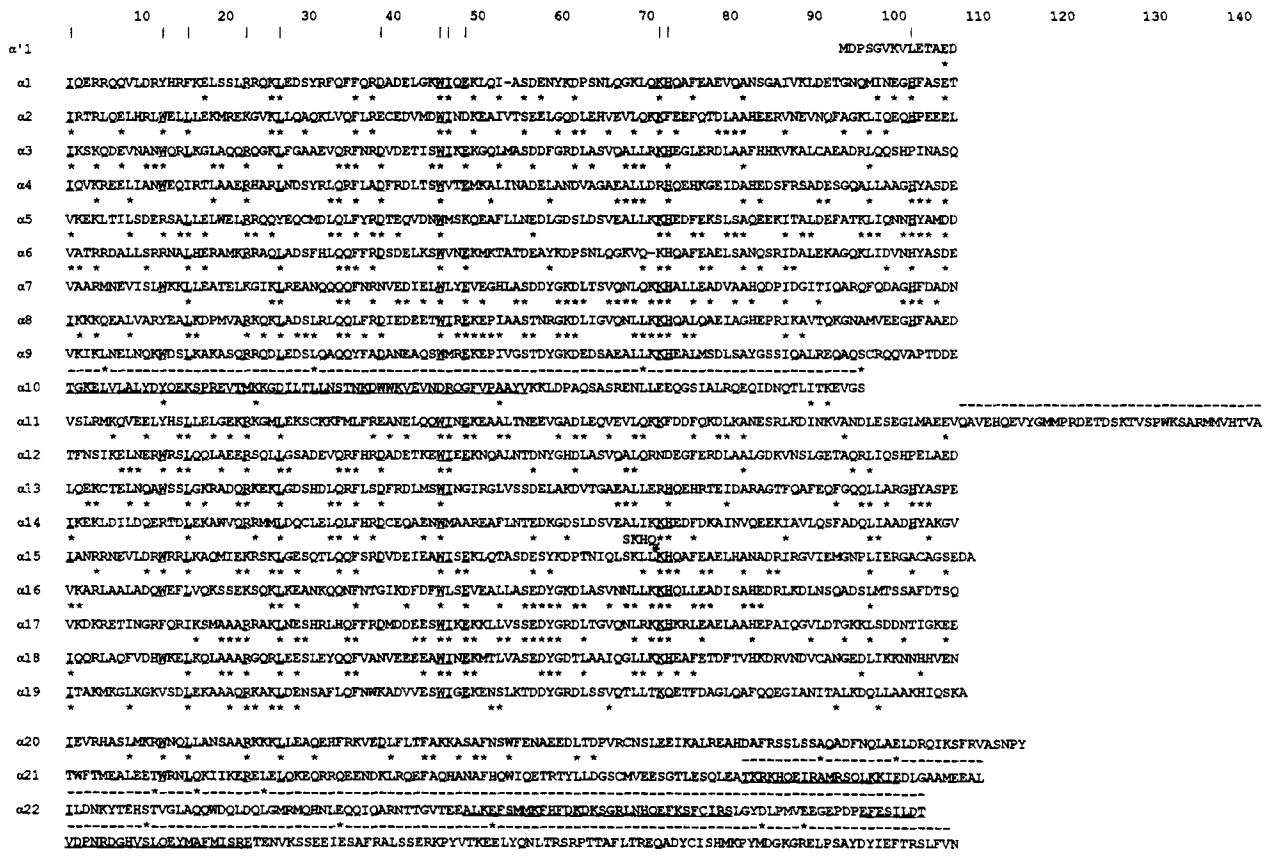


Figure 4. The alignment of the chicken brain α-spectrin segments α'1–α22. The optimal alignment of the repeats and the intervening nonhomologous regions located by the dot plot in Fig. 3 is shown. The beginning of the first repeat α1 is chosen to fit to the corresponding α1 unit of the human erythroid α-spectrin (Speicher and Marchesi, 1984). *Asterisks*, identical residues between neighboring repeats. The four-residue insert (SKHQ) of α15 is excluded from it and placed between the α14 and α15 lines. *Vertical lines*, the positions of some residues which tend to be conserved in most repeats. These residues are underlined. Nonhomologous regions are overlined. *Src*-like sequence (residues 967–1,021), the putative calmodulin-binding site (residues 2,253–2,371), and the EF-hand sequences (residues 2,332–2,404) are underlined. The domain designation is given to the left. *Dashes*, gaps created to optimize the alignment.

chain is clearly related to α-actinin. The latter contains three spectrin repeats as a part of its structure (Wasenius et al., 1987) and shows in its COOH terminus further homology to the carboxy terminus of α-spectrin (Fig. 6). Secondly, within that domain there are two so-called EF-hands (i.e., Ca^{2+}-binding loops) both in α-actinin and α-spectrin (Fig. 7). Before this sequence, there is, thirdly, a segment that could be a calmodulin-binding site. Fourthly, in the middle of the sequence, the distinct unit α10 shows a clear homology to the *src*-family of protein kinases and to phospholipase C (PLC).[1]

Fig. 6 *A* shows a DIAGON plot that compares the carboxy-termini of the chicken brain spectrin and the chicken α-actinin. In Fig. 6 *B* these sequences are aligned to further demonstrate their match; in the alignment 38% of the residues are identical. This clear match strengthens our interpretation of the DNA sequence: it is very likely that the stop codon for the reading frame of α-spectrin has been positioned correctly.

In Fig. 7 the putative calcium-binding sequences found in the COOH terminus of spectrin chain are aligned with several canonical EF-hand structures. There appears to be two calcium-binding loops in α-spectrin, as there are in α-actinin. The sequences shown in Fig. 7 correspond to a long loop that connects two helical segments in the binding sites found in the reference proteins such as parvalbumin and calmodulin (see Vyas et al., 1987).

By visual inspection we located in the end of the α21 domain a unique site (residues 2,253–2,371) that contains clusters of basic and hydrophobic residues. By these criteria it might represent a calmodulin-binding site (Kemp et al., 1987). Helical wheel analysis (Fig. 8) reveals its amphipathic nature and net positive charge, which is the consensus drawn for the calmodulin-binding sites in proteins (Erickson-Viitanen and De Grado, 1987).

The α10 domain is homologous to proteins belonging to the family of nonreceptor cytoplasmic tyrosine kinases (*src*, *syn*, *fgr*, *lyn*, *crk*, *yes*, *hck*, and *lsk*) as well as to PLC. Fig. 9 shows the optimal alignment of α10 with these sequences. The homology resides in a stretch of ~60 residues which occurs in the amino-terminal half of the *src*-proteins and in the middle portion of PLC. The degree of similarity ranges from

1. *Abbreviations used in this paper*: PLC, phospholipase C.

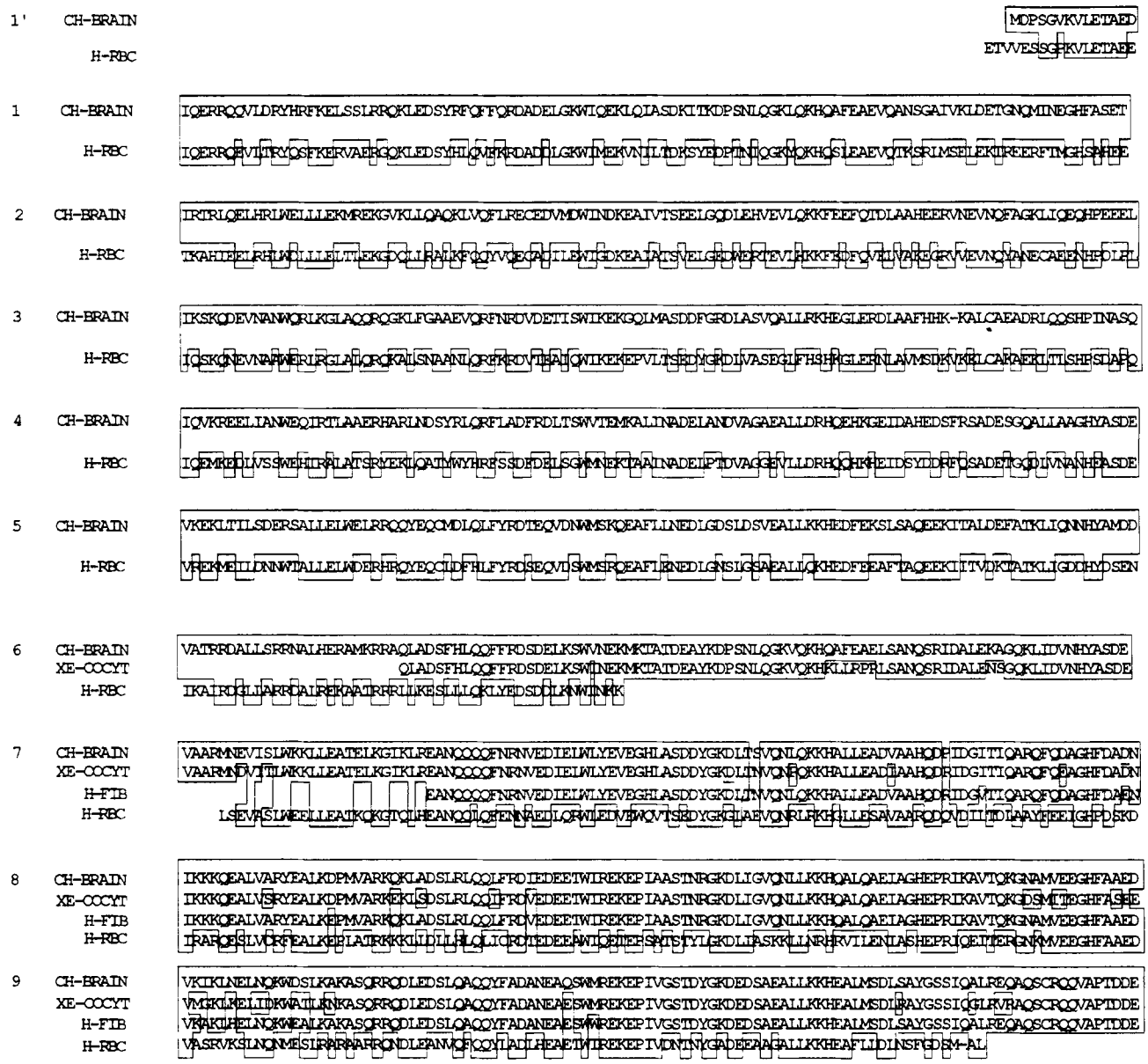
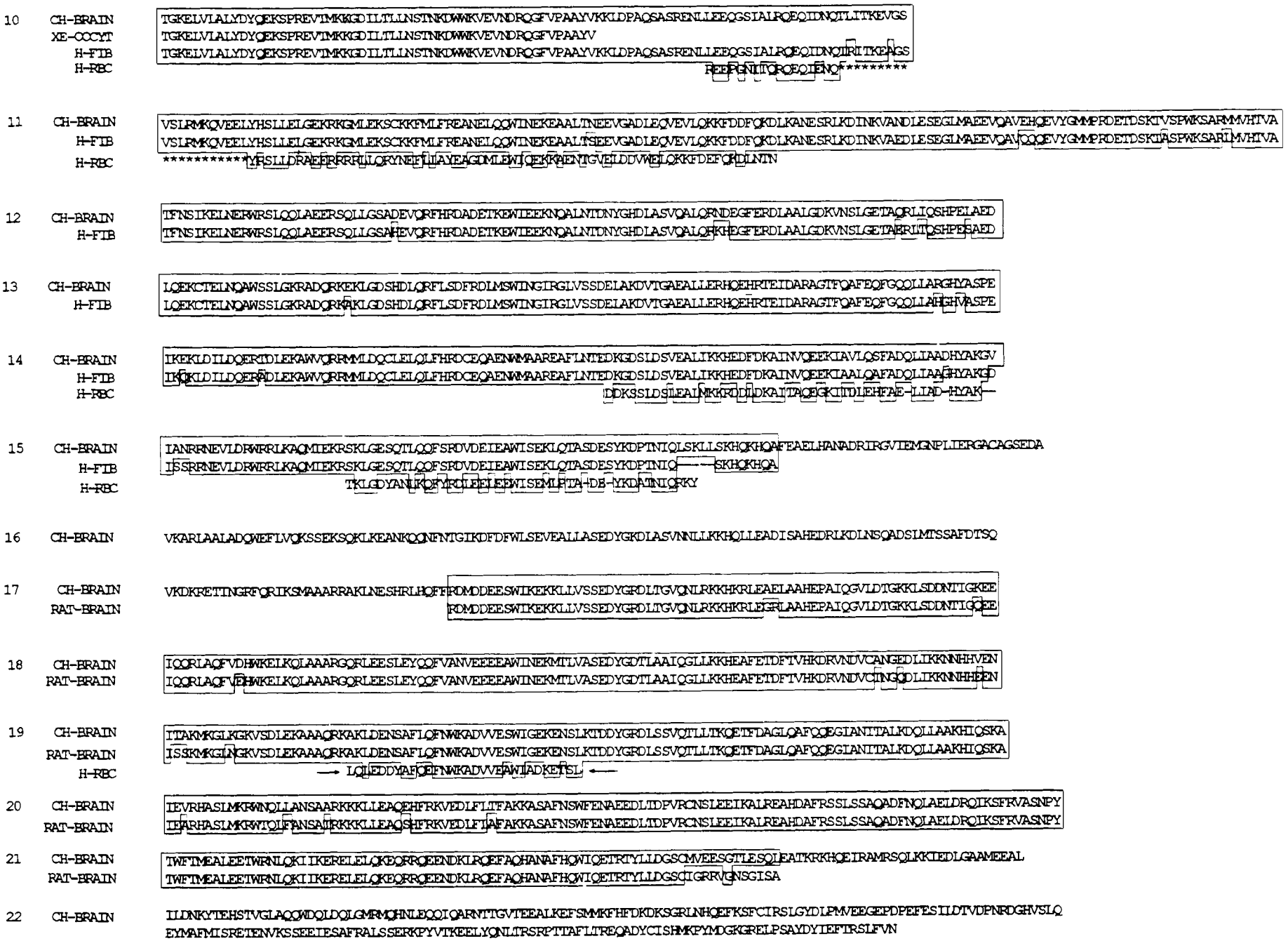


Figure 5. Comparison of the chicken brain α-spectrin (*CH-BRAIN*; current sequence), *Xenopus* oocyte α-spectrin (*XE-OOCYT*; Giebelhaus et al., 1987), human fibroblast α-spectrin (*H-FIB*; McMahon et al., 1987), rat brain α-spectrin (*RAT-BRAIN*; Leto et al., 1988), and human red blood cell α-spectrin (*H-RBC*; Speicher and Marchesi, 1984) sequences. Numbers on the left refer to the repeats of Fig. 4. Asterisks in the boundary of α10–α11 in H-RBC indicate the specific 20-residue insert in the nonerythroid proteins. *Black arrows*, the H-RBC peptide placed in α19.

24 to 32% (identical amino acids) and from 30 to 50% if conservative substitutions are counted. The residues with the consensus sequence ALYDY, KG, and WW in the positions corresponding to 89–92, 104 and 105, and 118 and 119 in *src*, respectively, are especially well conserved.

Discussion

We present here the nucleotide sequence covering the entire coding frame and the 3′ untranslated region plus a part of the 5′ flanking sequence of the mRNA for the spectrin α-subunit. The mRNA which was used as a template in the cDNA synthesis was isolated from the embryonic chicken brain. That the derived sequence represents the α-chain of brain spectrin can be deduced from the following: (*a*) the longest cDNA clone (4.7 kb) isolated from the primary library hybridizes to a probe which in our previous study has been shown to encode a protein that is immunoprecipitated by antibodies specific to α-spectrin, and the sequence of this probe is embedded in the present cDNA sequence; (*b*) the obtained sequence is more similar to the known α-spectrin than to the β-spectrin sequences (not shown); and (*c*) it shows a higher degree of homology to the nonerythroid α-spectrin than to the erythroid spectrin sequences.


10 CH-BRAIN TGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLDPAQSASRENLLEEQGSIALRQEQIDNQIRLITKEVGS
XE-COCYT TGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVNDRQGFVPAAYV
H-FIB TGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLDPAQSASRENLLEEQGSIALRQEQIDNQIRLITKEAGS
H-RBC REEPGNITTQRQEQIENQ*********
11 CH-BRAIN VSLRMKQVEELYHSLLELGEKRKGMLEKSCKKFMLFREANELQQWINEKEAALTNEEVGADLEQVEVLQKKFDDFQKDLKANESRLKDINKVANDLESEGLMAEEVQAVEHQEVYGMMPRDETDSKTVSPWKSARMMVHTVA
H-FIB VSLRMKQVEELYHSLLELGEKRKGMLEKSCKKFMLFREANELQQWINEKEAALTSEEVGADLEQVEVLQKKFDDFQKDLKANESRLKDINKVAEDLESEGLMAEEVQAVQQQEVYGMMPRDETDSKTASPWKSARLMVHTVA
H-RBC **********YRSLLDRAEERRRRLLQRYNEFLLAYEAGDMLEWIQEKRAENTGVELDDVWEQKKFDEFQRDLNTN
12 CH-BRAIN TFNSIKELNERWRSLQQLAEERSQLLGSADEVQRFHRDADETKEWIEEKNQALNTDNYGHDLASVQALQRNDEGFERDLAALGDKVNSLGETAQRLIQSHPELAED
H-FIB TFNSIKELNERWRSLQQLAEERSQLLGSAHEVQRFHRDADETKEWIEEKNQALNTDNYGHDLASVQALQKKHEGFERDLAALGDKVNSLGETAERLITQSHPESAED
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H-FIB LQEKCTELNQAWSSLGKRADQRKAKLGDSHDLQRFLSDFRDLMSWINGIRGLVSSDELAKDVTGAEALLERHQEHRTEIDARAGTFQAFEQFGQQLIAHGHVASPE
14 CH-BRAIN IKEKLDILDQERTDLEKAWVQRRMMLDQCLELQLFHRDCEQAENWMAAREAFLNTEDKGDSLDSVEALIKKHEDFDKAINVQEEKIAVLQSFADQLIAADHYAKGV
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H-RBC DDKSSLDSLEALMKKRQDLDKAITAQEGKITDLEHFAE-LIAD-HYAK—
15 CH-BRAIN IANRRNEVLDRWRRLKAQMIEKRSKLGESQTLQQFSRDVDEIEAWISEKLQTASDESYKDPTNIQLSKLLSKHQKHQAFEAELHANADRIRGVIEMGNPLIERGACAGSEDA
H-FIB ISSRRNEVLDRWRRLKAQMIEKRSKLGESQTLQQFSRDVDEIEAWISEKLQTASDESYKDPTNIQ---SKHQKHQA
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17 CH-BRAIN VKDKRETTNGRFQRIKSMAAARRAKLNESHRLHQFTRDMDDEESWIKEKKLLVSSEDYGRDLTGVQNLRKKHKRLEAELAAHEPAIQGVLDTGKKLSDDNTIGKEE
RAT-BRAIN RDMDDEESWIKEKKLLVSSEDYGRDLTGVQNLRKKHKRLEGRLAAHEPAIQGVLDTGKKLSDDNTIGQEE
18 CH-BRAIN IQQRLAQFVDHWKELKQLAAARGQRLEESLEYQQFVANVEEEEAWINEKMTLVASEDYGDTLAAIQGLLKKHEAFETDFTVHKDRVNDVCANGEDLIKKNNHHVEN
RAT-BRAIN IQQRLAQFVDHWKELKQLAAARGQRLEESLEYQQFVANVEEEEAWINEKMTLVASEDYGDTLAAIQGLLKKHEAFETDFTVHKDRVNDVCINGQDLIKKNNHHEEN
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RAT-BRAIN ISSKMKGLNGKVSDLEKAAAQRKAKLDENSAFLQFNWKADVVESWIGEKENSLKTDDYGRDLSSVQTLLTKQETFDAGLQAFQQEGIANITALKDQLLAAKHIQSKA
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RAT-BRAIN IEARHASLMKRWTQIFANSAIRKKKLLEAQSHFRKVEDLFTAFAKKASAFNSWFENAEEDLTDPVRCNSLEEIKALREAHDAFRSSLSSAQADFNQLAELDRQIKSFRVASNPY
21 CH-BRAIN TWFTMEALEETWRNLQKIIKERELELQKEQRRQEENDKLRQEFAQHANAFHQWIQETRTYLLDGSQMVEESGTLESQLEATKRKHQEIRAMRSQLKKIEDLGAAMEEAL
RAT-BRAIN TWFTMEALEETWRNLQKIIKERELELQKEQRRQEENDKLRQEFAQHANAFHQWIQETRTYLLDGSQIGRRVGNSGISA
22 CH-BRAIN ILDNKYTEHSTVGLAQQWDQLDQLGMRMQHNLEQQIQARNTTGVTEEALKEFSMMKFHFDKDKSGRLNHQEFKSFCIRSLGYDLPMVEEGEPDPEFESILDTVDPNRDGHVSLQ
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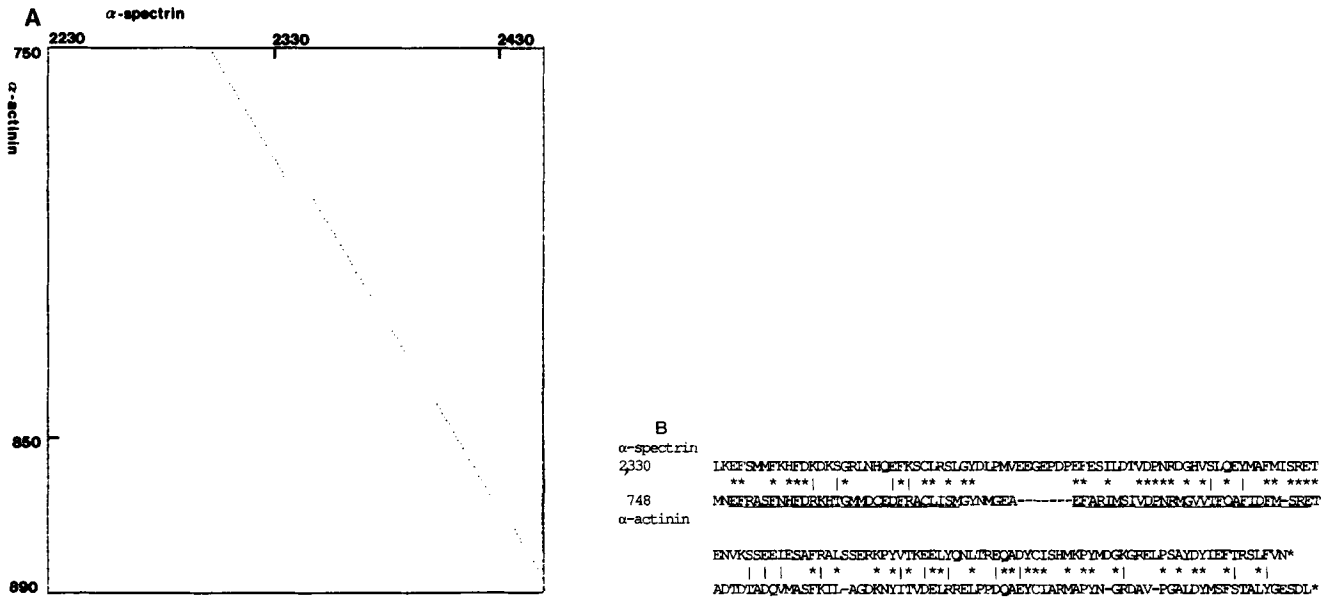

Figure 6. (*A*) Dot matrix comparison of the chicken α-actinin and α-spectrin. The COOH-terminal end of α-spectrin (residues 2,230–2,477, horizontal axis) and the α-actinin COOH-terminal end (residues 748–887) were compared for the sequence similarities using the computer program DIAGON (Staden, 1982). The odd span length was 21 and the scoring level 250 corresponding to the double matching probability of $<5 \times 10^{-5}$. The axes are labeled with residue numbers. (*B*) Alignment of the COOH termini of α-spectrin and α-actinin. The alignment follows the diagonal lines in *A*. Gaps were introduced to optimize the alignment. *Asterisks*, identical residues; *vertical lines*, conservative substitutions. The first residues taken to the alignment are numbered.

Comparison of Various Spectrins

The analysis of the current sequence establishes some of the structural principles that have been proposed for the spectrins on the basis of partial and fragmentary sequence information. First of all, the characteristic 106 amino acid repeat forms the basic structural motif of the brain α-spectrin. The remarkably precise conservation of its length in 14 out of 20 homologous units indicates that this preservation is of critical importance for the structure of spectrin. We surmise that the repeats account for the rod-like shape of the molecule and that the evolutionary constraint to preserve the 106 amino acid length is imposed by the formation of multiple contacts

			x	y	z	−x	−z				x	y	z	−x	−z	
Consensus			D	D/N	G	I/V/L	E				D	D/N	G	I/V/L	E	
α-SPECTRIN	I	2341	D	K	D	K	S	G	R	L	N	H	Q	E	2352	II
α-ACTININ chicken	I	761	D	R	K	H	T	G	M	M	D	C	E	D	772	II
α-ACTININ, dict.	I	743	D	K	D	N	D	N	K	L	N	R	L	E	754	II
CALMO	III	93	D	K	D	G	N	G	Y	I	S	A	A	E	104	IV
ICaBP	III	54	D	K	N	G	D	G	E	V	S	F	E	E	65	
PALB	CD	51	D	Q	D	K	S	G	F	I	E	E	D	E	62	EF
TROPO	III	106	D	K	N	A	D	G	F	I	D	I	E	E	117	IV

Consensus			D	D/N	G	I/V/L	E				D	D/N	G	I/V/L	E	
α-SPECTRIN	I	2383	D	P	N	R	D	G	H	V	S	L	Q	E	2394	
α-ACTININ chicken	I	797	D	P	N	R	M	G	V	V	T	F	Q	A	808	
α-ACTININ, dict.	I	779	D	T	D	G	N	G	T	I	S	F	E	E	790	
CALMO	III	129	D	I	D	G	D	G	E	V	N	Y	E	E	140	
ICaBP	III	54	D	K	N	G	D	G	E	V	S	F	E	E	65	
PALB	CD	90	D	S	D	G	D	G	K	I	G	V	D	E	101	
TROPO	III	142	D	K	N	N	D	G	R	I	D	F	D	E	153	

Figure 7. Potential calcium-binding sites in α-spectrin. EF-hand sequences of α-spectrin (residues 2,341–2,352 and 2,383–2,394) are separately aligned and compared with the EF-hand sequences of known calcium-binding proteins parvalbumin (*PALB*), troponin C (*TROPO*), intestinal calcium-binding protein (*ICaBP*), and calmodulin (*CALMO*) (Vyas et al., 1987), and of the putative calcium-binding sites of the *Dictyostelium* (Noegel et al., 1987) and chicken α-actinins (Baron et al., 1987). Only two loops (*III*, *IV*) of troponin C (*TROPO*) and calmodulin (*CALMO*) showing the best matches are shown. Asterisks above each sequence indicate its identities with the α-spectrin sequence. The EF-hand consensus sequence (Tufty and Kretsinger, 1975) is shown in the first line. Calcium-chelating side chains are marked with x, y, z, −x, and −z. The numbers on the left and right refer to the sequence positions.

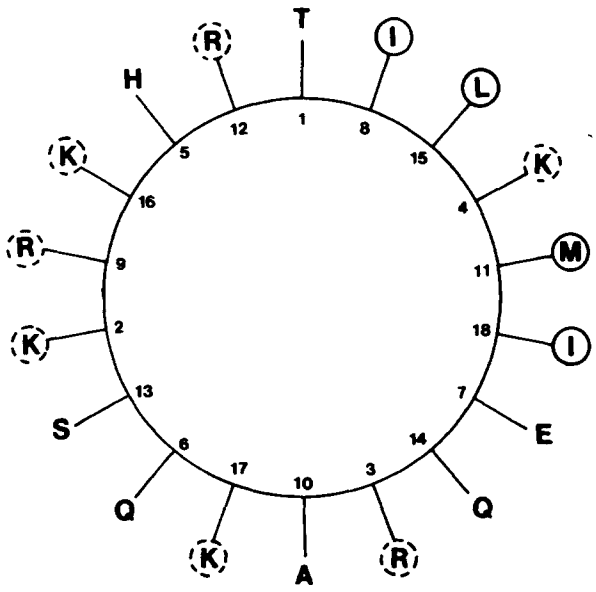

Figure 8. Helical wheel presentation of the putative binding site for calmodulin in α-spectrin. The numbering corresponds to the internal residues of a wheel. Hydrophobic residues are circled with continuous lines and basic residues with dashed lines.

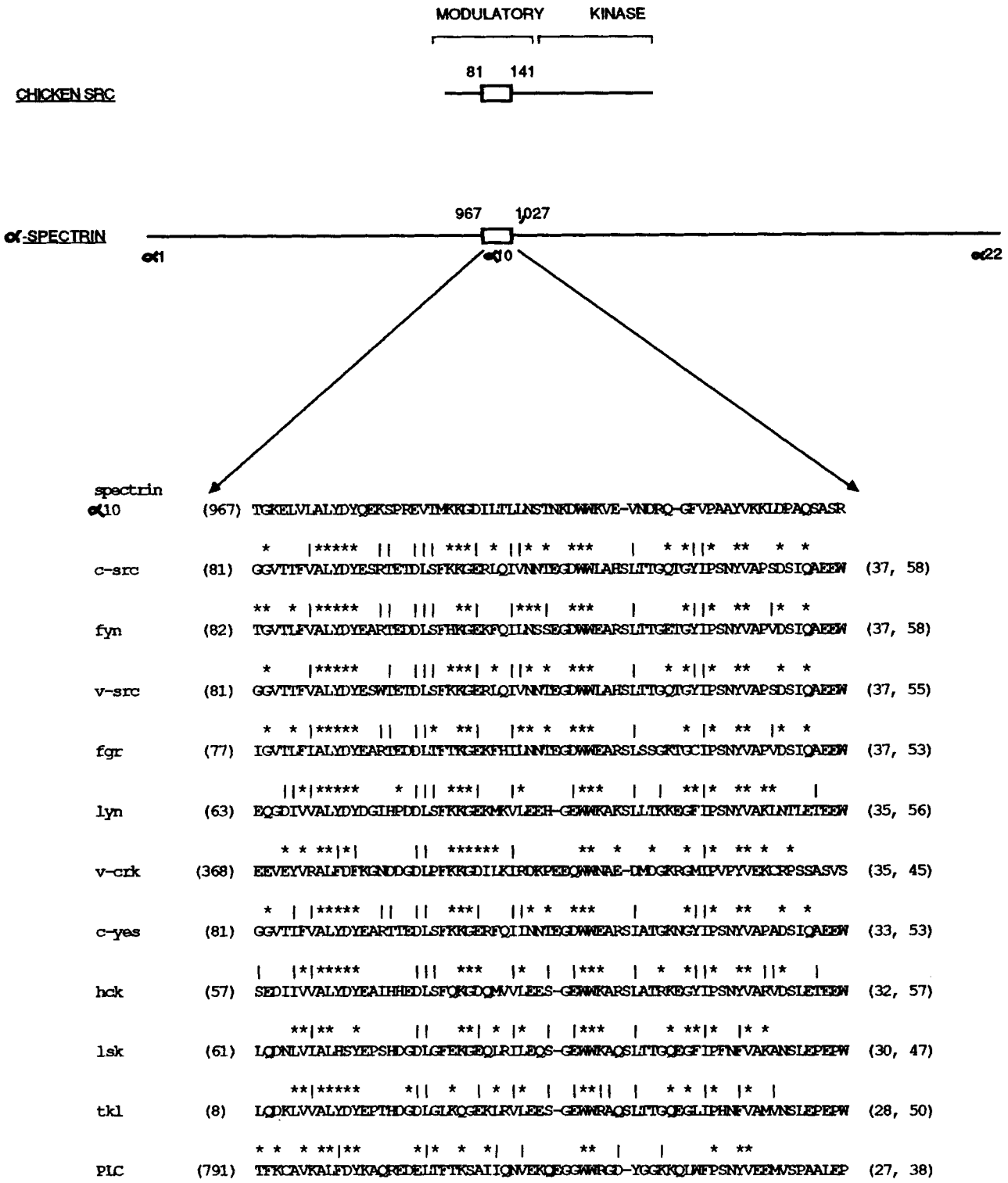

Figure 9. Homology of the α10 domain to the *src*-proteins and *src*-like proteins and to PLC. On the top, the chicken *src* tyrosine kinase and α-spectrin are schematically aligned to illustrate the location of this homologous region (□) in their linear maps. Modulatory and kinase refer to the two domains in the *src*-proteins. The homologous sequences are shown on the bottom. The numbers on the left refer to the first residues taken to the alignment. Asterisks and vertical lines indicate the identical and conservative substitutions, respectively, between α10 and each of the sequences. The two numbers on the right are taken from these pairwise comparisons and, again, refer to the number of identical (asterisks) and total conserved (asterisks and vertical lines) residues. The sequences are taken from the following references: c-*src*, Takeya and Hanafusa, 1982; *fyn*, Kawakami et al., 1986 and Semba et al., 1986; v-*src*, Takeya and Hanafusa, 1982 and Taylor and Hanafusa, 1983; *fgr*, Katamine et al., 1988; *lyn*, Yamanashi et al., 1987; c-*yes*, Sukegawa et al., 1987; *hck*, Quintrell et al., 1987; *lsk*, Marth et al., 1985; *tkl*, Strebhardt et al., 1985; v-*crk*, Mayer et al., 1988; PLC, Stahl et al., 1988.

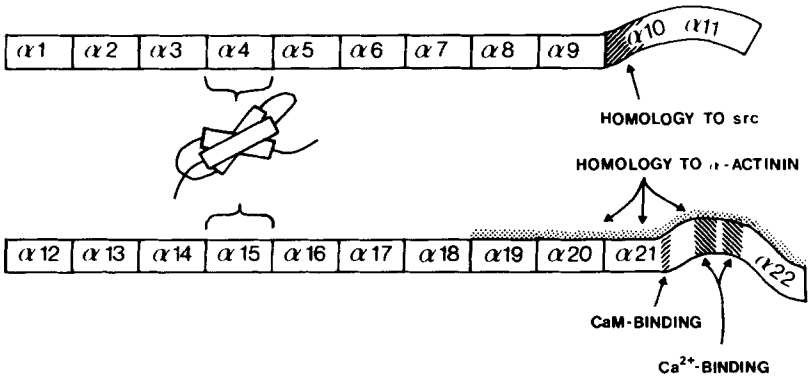

Figure 10. A schematic map for the α-spectrin domain structure.

with β-spectrin, which possesses the basically similar structural principle (Speicher and Marchesi, 1984).

In the chicken brain α-spectrin we can discern 18 repeats (α1–α9, α11–α19) with a relatively high degree of homology, two repeats (α20, α21) with a lower degree of homology, and the α10 and α22 segments as well as the 36 residue extension of the α11 that are unrelated to the homologous repeats. This divides the chain into 22 segments (Fig. 10).

Comparison of the present sequence with the published spectrin sequences corroborated the early observation (see McMahon et al., 1987 for detailed analysis) that the nonerythroid α-spectrins from different species, ranging in this study from *Xenopus* to man, show a high degree of mutual homology while the mammalian erythroid α-spectrin is a more distant protein. This indicates a rapid divergent evolution of the latter.

In accordance with McMahon et al. (1987) we found that the α10 and α11 units contain sequences unrelated to the homologous repeats. Interestingly, the homology of these segments between chicken and man is not different from that of the other domains of spectrin. This suggests that the distinct characteristics of these domains were established before the divergence of the human and avian species. Comparison of these with the corresponding regions of the erythroid spectrin is hampered by the paucity of sequence data. Interestingly, however, it seems that the amino acids encoded by the 60 nucleotide insert in the nonerythroid spectrins are lacking in the erythroid spectrin (McMahon et al., 1987) indicating a specific function for the α10–α11 domain in nonerythroid cells.

The mol mass calculated for our sequence is 285,369. This is ~10% higher than the highest estimates based on SDS-PAGE (Bennett et al., 1982). The 22 segments of the brain α-spectrin are two more than predicted for the erythroid α-spectrin by Speicher and Marchesi (1984). This discordance may be due to a mispositioning of the NH_2 terminus of tryptic fragment T41, belonging to the domain αV, in α18 instead of α19 (see above; Speicher, 1986). If this is taken into account in the modeling of the molecule, the number of the repeats in the human erythroid spectrin amounts to 21. We suggest that there is another (nonhomologous) segment in the COOH terminus of erythroid α-spectrin which corresponds to our α22. This is based on the following calculation: positioning of the amino terminus of the T41 in the beginning of the α19 would give erythroid α-spectrin a molecular mass of ~260 kD (the calculated molecular mass of the first 18 segments is 215 kD and that of T41 is 41 kD). The tryptic peptide, on the other hand, could accommodate four 106-residue units (4× ~11 kD ≈44 kD) raising the total number of segments to 22.

Secondary Structure

The model for the secondary structure of spectrin by Speicher and Marchesi (1984) predicts that each repeat may contain three helices; reverse turns would connect these long helices, and random coils the adjacent repeats. In our analysis only some of the repeats conform with such a model. Although we can conclude that the present primary structure is consistent with the predictions of the Speicher–Marchesi model, it is difficult to predict just three helices within the repeats. Overall, the sequence data supports the conclusion from spectroscopic studies (Burns et al., 1983) of the high α-helicity of spectrin.

The Nonhomologous Segments as the "Carriers" of the Distinct Functions of Spectrin

In previous studies (Wasenius et al., 1987; Närvänen et al., 1987) we have shown that there is an extensive homology between α-actinin and nonerythroid spectrin (see also Baron et al., 1987). In the present study, and with more sequence data available, we can extend this further to the COOH terminus in both molecules. The comparison shows that the COOH terminus of α-actinin is colinear with the COOH-terminal part of α-spectrin comprising the entire α22 domain. This lends further credence to our proposition that α-actinin can be viewed as a hybrid molecule composed of an actin-binding NH_2-terminal half and a spectrin-like COOH terminus (Wasenius et al., 1987).

Another specific feature of the α22 is the presence of two EF-hand structures which are also found in the α-actinin domain (Baron et al., 1987; Noegel et al., 1987). In α-actinin these sites have been thought to exert a calcium-dependent control on the actin cross-linking. In the antiparallel orientation of the subunits in α-actinin dimer, the actin-binding NH_2 terminus comes near the COOH terminus of the adjacent subunit which may then affect the binding (Noegel et al., 1987). In spectrin, actin binding occurs at the ends of the tetramers and involves the COOH terminus of the α-subunit and NH_2 terminus of the β-subunit (Morrow et al., 1980; Tsukita et al., 1983). Analogously to α-actinin, it can be surmised that the principal actin binding would occur at

the NH_2 terminus of the β-chain with the COOH terminus of the α-chain exerting control on the interaction. That spectrin can, indeed, bind actin in a calcium-dependent manner, is demonstrated by the recent finding of Fishkind et al. (1987) showing modulation of the spectrin–actin interaction by calcium in sea urchin egg.

Spectrin is also involved in other calcium-regulated events such as the complex formation with calpactin (Gerke and Weber, 1984) and the degradation by a Ca^{2+}-regulated protease (Siman et al., 1984). Furthermore, brain spectrin can also modulate the Mg^{2+}-ATPase activity of the smooth muscle actomyosin in a calcium-dependent manner (Wagner, 1984; Wang et al., 1987). This calcium sensitivity may at least partially be conferred by Ca^{2+} binding to the EF-hand structures in α22.

Nonerythroid spectrins are marked by their capacity to bind calmodulin to their α-subunit (Glenney et al., 1982*a*, *b*; Kakiuchi et al., 1982; Palfrey et al., 1982). The exact location of the binding site is, however, not resolved. In the present sequence there is a domain in α21 that fulfills the structural criteria proposed for calmodulin-binding sites. Such a positioning is supported by Carlin et al. (1983) who found that the major proteolytic breakdown product of fodrin (spectrin), which represents the COOH-terminal part of the chain is able to bind calmodulin. Tsukita et al. (1983) have, however, suggested that the calmodulin-binding site is close to the amino terminus of the α-chain. Subcloning and expression of the cDNA containing the putative calmodulin-binding site will enable us to test our proposal.

An intriguing finding from the homology search was the similarity between a defined region in α10, in PLC, and in the amino-terminal half of the *src*-proteins. The members of the "*src*-subfamily" are oncoproteins and all, except for *crk*, have kinase activity (Hanks et al., 1988; Mayer et al., 1988), a feature that has not been associated with spectrin. On the other hand, many of these proteins (Hunter and Cooper, 1985) are closely associated with the cytoplasmic side of the plasma membrane. In this regard these proteins are similar to spectrin.

The function of the α10 domain in spectrin is currently unknown. Similarly, the specific functions of the homologous domains in the *src*-proteins and PLC have not been elucidated. It is clear, however, from the studies with deletion mutants, that the NH_2-terminal portion of the *src*-proteins is not needed for the tyrosine kinase activity. On the other hand, the NH_2-terminal half has a modifying effect on the intrinsic kinase activity that resides in the COOH-terminal half (Jove and Hanafusa, 1987). The NH_2-terminal half has been suggested to recognize or bind the substrates which are then phosphorylated by the COOH-terminal catalytic domain. Hence, the term modulatory (Calothy et al., 1987) or recognition (Parsons et al., 1984) domain has been coined to the NH_2 terminus of the *src*.

Using this analogy, we may suggest that the α10 domain in spectrin could serve as a recognition site for some substrates of *src* tyrosine kinases. One candidate could be calpactin I, also known as p36, which interacts with α-spectrin (Lehto et al., 1983; Gerke and Weber, 1984) and which is also a major substrate of *src* tyrosine kinases (Hunter and Cooper, 1985). Alternatively, the homologous regions in spectrin, PLC, and *src*-proteins could anchor these proteins to some common cytoskeletal component. This would explain the reduced association of *src*-protein with cytoskeleton when they are deleted for the NH_2-terminal modulatory domain (Hamaguchi and Hanafusa, 1987).

Evolution of Spectrin

It is evident that spectrin has evolved from an ancestral gene coding for the basic 106-residue repeat by several contiguous duplications (Speicher and Marchesi, 1984). In our view, the ancestral repeat unit has first undergone three duplication steps to reach an 8-repeat stage. Possibly at this stage there has also been insertion of one more repeat to make a 9-repeat structure which has then undergone another, final duplication. Concurrently, the gene has also acquired the nonhomologous domains possibly by exon shuffling. The elucidation at what stage during evolution these divergent regions have been assimilated by spectrin—whether spectrin has acquired new functions during its recent evolutionary history—has to await primary structures of spectrins from lower organisms. The spectrin repeat has also been found in two other proteins, α-actinin (Wasenius et al., 1987) and dystrophin (Koenig et al., 1988). Hence, these proteins may have evolved from a common ancestor and possess a similar architectural design.

The present cDNA sequence of α-spectrin establishes that the same basic structural principle is found in various types of spectrin. It also clearly indicates regions which may carry some important functions of spectrin. Further studies to test the functional properties of these domains are now greatly facilitated by the availability of cDNA clones; fragments of the structure can now be expressed separately, and the produced polypeptides studied experimentally for the postulated functions.

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Note added in proof: Recently Harris et al. (Harris, A. S., D. E. Croall and J. S. Morrow. 1988. *J. Biol. Chem.* 263:15754–15761) have shown that one calmodulin-binding site in human fodrin resides in the terminal portion of the 11th repeat.

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