

# From the spectrin gene to the assembly of the membrane skeleton

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**ABSTRACT.** The complete nucleotide sequence coding for the chicken brain  $\alpha$ -spectrin was determined. It comprises the entire coding frame, 5'- and 3'-untranslated sequences terminating in a poly(A)-tail. The deduced amino acid sequence shows that the  $\alpha$ -chain contains 22 segments, 20 of which correspond to the typical 106 residue repeat of the human erythrocyte spectrin. Some segments non-homologous to the repeat structure reside in the middle and COOH-terminal regions. Sequence comparisons with other proteins show that these segments evidently harbour some structural and functional features such as: homology to  $\alpha$ -actinin and dystrophin, two typical EF-hand structures (calcium-binding) and a putative calmodulin-binding site in the COOH-terminus and a sequence homologous to various src-tyrosine kinases and to phospholipase C in the middle of the molecule. Comparison of our sequence with other partial  $\alpha$ -spectrin sequences shows that  $\alpha$ -spectrin is well conserved in different species and that the human erythrocyte  $\alpha$ -spectrin is divergent.

**KEY WORDS:** *spectrin, membrane skeleton, gene, assembly, sequence*

## Introduction

Spectrin is the major constituent of the so-called membrane skeleton which forms a tightly-woven filament network at the cytoplasmic face of the plasma membrane (for a review, see Marchesi, 1985). The best characterized membrane skeleton is that of red blood cells. It is composed for the most part of two non-identical spectrin polypeptides called  $\alpha$ - and  $\beta$ -spectrins. They form  $\alpha/\beta$ -heterodimers which show antiparallel side-by-side orientation. Heterodimers, on the other hand, form tetramers by head-to-head association. Tetramers seem to be the major organizational form of spectrin in erythrocytes although hexamers, octamers and higher oligomers exist as well.

For a long time spectrin was considered to be specific for red blood cells. During the past five years, however, spectrins and spectrin-like proteins have been found in virtually all types of cells (for a review see Lazarides and Nelson, 1985). From these studies it has become clear that non-erythroid spectrins also occur as heterodimers formed by a common ( $\alpha$ ; M, 230-260 kDa) and a variant ( $\beta$  or  $\gamma$ ; M, 220-260kDa) subunit. They also share other characteristics which can be regarded as criteria for the proteins to be included in the family of spectrin-like proteins (Marchesi, 1985). These are: (i) a capacity to self-associate at least to tetrameric forms; (ii) a capacity to interact with F-actin; (iii) a capacity to bind ankyrin, another membrane skeletal protein and, (iv) an elongated rod-like form with a high  $\alpha$ -helical content.

Some of the spectrin-like proteins found in cells other than erythrocytes are known by names not immediately referring to spectrins. Thus, for instance, spectrin-like protein in brain is also called fodrin (Levine and Willard, 1981) or calspetrin (Kakiuchi *et al.*, 1982). The spectrin-like protein in bovine lens fiber cells, which we discovered by using immunological techniques (Lehto and Virtanen, 1983), is called p230 according to its molecular weight. A special type of spectrin-like protein,

TW260/240 is present in the chicken intestinal epithelium (Glenney *et al.*, 1982). Unlike other members of the spectrin family, this protein is located in the terminal web of the enterocytes, at some distance from the plasma membrane. Besides the above-mentioned cells and tissues, spectrin-like proteins have been detected in a large variety of different cells including myoblasts, human fibroblasts, human sperm cells, mouse oocytes, *Acanthamoeba* Neff. cells, *Drosophila* S3 cells, and *Xenopus laevis* oocytes.

Ultrastructural characterization of different types of spectrins has revealed the morphology of a long, flexible, double-stranded rod in non-erythroid spectrins as well (Marchesi, 1985). They show a contour length of 100 nm. Members of the spectrin family are also thoroughly characterized by using immunological techniques. These studies have shown that antibodies raised against non-mammalian erythrocyte spectrin usually cross-react with spectrin in non-erythroid cells while the antibodies against mammalian erythrocyte spectrin do not show this cross-reactivity. Thus, it has been concluded that mammalian erythrocyte spectrin is divergent from the other members of the family (Glenney and Glenney, 1984). This is also supported by structural studies including tryptic peptide mapping of various spectrins.

In view of the rigid structure of the spectrin network and the abundance of spectrin in most types of cells, it is natural to think that spectrin probably has a skeletal, structural role in the cell. In mammalian red blood cells spectrin is known to be responsible for the stability and maintenance of the biconcave shape of the cell and for the lipid asymmetry of the plasma membrane (Marchesi, 1985). Such membrane supporting functions have also been ascribed to non-erythroid spectrins. It is becoming clear, however, that both in erythrocytes and in non-erythroid cells spectrins also have more dynamic functions. These include regulation of the distribution and movement of the plasma membrane phospholipids (Cohen, 1983), functioning as a specific linker between membrane receptors and the filament

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cytoskeleton as shown by lymphocyte capping experiments (Levine and Willard, 1983; Nelson *et al.*, 1983), participation in the intracellular movement of proteins and organelles (Bennett, 1985), and in exocytosis (Perrin *et al.*, 1987).

One of the characteristic features of the spectrin-like proteins is their multiple interactions with various cytoskeletal, peripheral, and transmembrane proteins. Structural and immunological studies have shown that spectrins are multidomain proteins in which different parts of the molecule are responsible for specific interactions with other proteins. For instance, the site for dimer formation has been localized to the NH<sub>2</sub>-terminal end of the  $\alpha$ -chain and to the COOH-terminus of the  $\beta$ -chain. Tetramer formation is mediated and enhanced by the interaction with actin oligomers and protein band 4.1. This complex, on the other hand, is hinged to the cytoplasmic face of the plasma membrane via another protein, ankyrin, which binds to the COOH-terminal end of the  $\beta$ -chain. Ankyrin then connects the whole complex to the transmembrane protein called band 3, the anion transporter protein (Branton *et al.*, 1981). There are also additional interactions between the spectrin-based network and the plasma membrane, including the binding of protein band 4.1. to glycoporphin A, a transmembrane protein (Marchesi, 1985), and an ankyrin independent, as yet poorly characterized, direct binding of spectrin to the plasma membrane (Steiner and Bennett, 1988).

Immunoanalogues of erythroid ankyrin and protein band 4.1 have also been detected and isolated from non-erythroid sources. Similarly to their erythroid counterparts, they also interact with non-erythroid spectrins (Bennett and Davis, 1981). The interactions of e.g. brain spectrin with other proteins extend, however, beyond the typical erythroid membrane skeletal proteins. It has been shown for instance that brain spectrin is able to bundle microtubules and to modulate actin-activated myosin ATPase in a calcium-dependent manner (Wagner, 1984; Bennett, 1985). Our own studies have suggested that spectrin interacts in an as yet unknown manner with intermediate filaments (Lehto and Virtanen, 1983). It also shows affinity for p36 (calpactin 1), the major substrate for the *src*-oncogene encoded tyrosine-specific protein kinase (Lehto *et al.*, 1983; Gerke and Weber, 1984).

### The aim of the study

Spectrins are known to have multiple interactions and functions. The functional domains have not, however, been accurately defined due to the paucity of the primary structure data. The aim of this study was to determine and to analyze the primary structure of the non-erythroid spectrin  $\alpha$ -chain in order to lay the basis for further work to analyze the structure/function-relationships of various spectrins.

## Results and discussion

### The nucleotide and the deduced amino acid sequence of brain -spectrin.

The overlapping cDNA and DNA clones comprised 7774 kb. It covered the entire coding region and the 3'-untranslated region of the cDNA. In this sequence a single open reading frame of 7431 nucleotides coding for 2477 amino acid residues was observed. There was a putative initiation codon 124 basis from the 5'-end of the sequence. In the upstream sequence, typical promotor elements such as TATA-box and CAAT-box

were identified. After the TGA-stop codon, there was a 219 base pair untranslated sequence ending in a poly (A) tail (see Wasenius *et al.*, 1989).

### Repeat structure of $\alpha$ -spectrin

The amino acid sequence was subjected to the DIAGON-analysis (Staden, 1982) to look at the internal homologies of  $\alpha$ -spectrin. This analysis indicated that there is an overall homology between the NH<sub>2</sub>- and COOH-terminal halves of the molecule (Fig. 1). Furthermore, there were smaller repeats of ca. 106-amino acid residues long to be found throughout the molecule. There were, however, three regions, two in the middle and one in the COOH-terminal part of the protein that appeared to deviate from this general pattern of repeat structure. These domains are designated  $\alpha$ 10,  $\alpha$ 11 and  $\alpha$ 22.

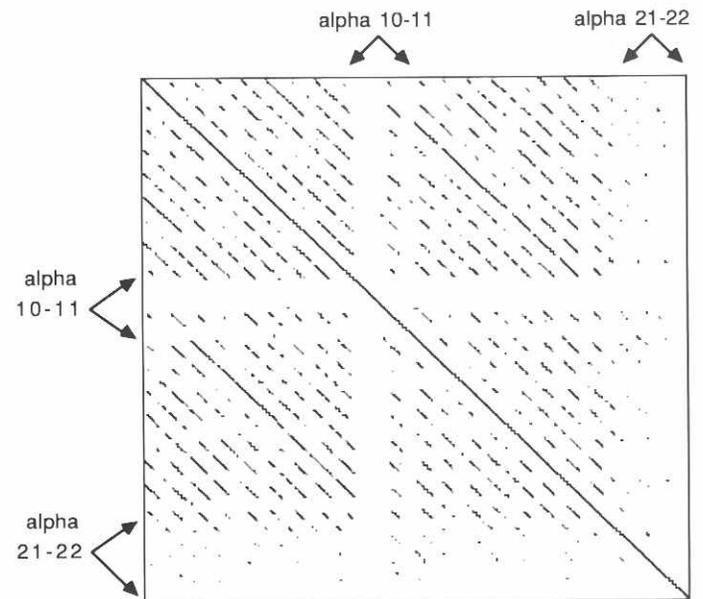


Fig. 1. DIAGON-analysis of the  $\alpha$ -spectrin amino acid sequence. The amino acid sequence was compared with itself. The lines running parallel to the diagonal represent repetitive sequences.

The odd span length was 21 and scoring level 250. The arrows at  $\alpha$ 10- $\alpha$ 11 and at  $\alpha$ 21- $\alpha$ 22 indicate the longest unique (non-repetitive) stretches.

After this preliminary analysis, the optimal alignment of the internal repeats was searched. This analysis revealed the preservation of the 106-residue repeat in 14 out of 20 homologous units, indicating that the exact length of the repeat is of importance for the structure of the molecule. Most of the repeats also possessed invariant residues in certain locations. The overall homology between the repeats was fairly high. This analysis also clearly showed the deviant nature of the repeats  $\alpha$ 10,  $\alpha$ 11 and  $\alpha$ 22.

The present sequence is the first complete nucleotide and amino acid sequence of the  $\alpha$ -chain of any spectrin-like molecule. Therefore it was compared with the published partial sequence of various other  $\alpha$ -spectrins (Table 1).

Some sequences, such as *Xenopus* oocyte  $\alpha$ -spectrin (Giebelhaus *et al.*, 1987) showed virtual identity with the chicken brain  $\alpha$ -spectrin sequence. Likewise, the human fibroblast

TABLE 1  
COMPARISON OF CHICKEN  $\alpha$ -SPECTRIN WITH OTHER SPECTRINS (PARTIAL SEQUENCES). %-SIMILARITY BASED ON THE NUMBER OF IDENTICAL AMINO ACIDS IN THE CORRESPONDING POSITIONS

	%-similarity
human erythrocyte $\alpha$ -spectrin	41
human fibroblast $\alpha$ -spectrin	96
rat brain $\alpha$ -spectrin	95
<i>Xenopus</i> oocyte $\alpha$ -spectrin	92

$\alpha$ -spectrin (McMahon *et al.*, 1987) appears to be nearly identical with the present sequence. These comparisons thus corroborate the observations from the earlier structural studies indicating that non-erythroid  $\alpha$ -spectrins from various species are closely related (ca. 90% of the residues are identical). On the other hand, comparison with the human erythroid  $\alpha$ -spectrin showed that mammalian erythroid  $\alpha$ -chain is clearly deviant from the chicken brain  $\alpha$ -spectrin (ca. 41% of the residues are identical).

On the basis of the previous biophysical studies, it is concluded that spectrin has, for the most part, an  $\alpha$ -helical conformation. This  $\alpha$ -helicity has been considered to underlie the observed rod-like structure of the molecule. Speicher and Mar-

chesi (1984) have presented evidence, based on the primary structure data, that each repeat unit contains three  $\alpha$ -helices connected by reverse turns and random coils. Secondary structure prediction of the current sequence revealed a high overall  $\alpha$ -helicity of the chicken brain  $\alpha$ -spectrin. It was not possible, however, to predict three  $\alpha$ -helical regions per repeat as suggested by Speicher and Marchesi (1984). Thus, from this data it is evident that more refined modelling is necessary to understand the secondary structure of spectrin.

#### Identification of distinct domains in $\alpha$ -spectrin

It is probable that the major function of the spectrin repeats is to confer on the molecule its rod-like structure and to permit the alignment of the subunits. Therefore, we focused our attention on the deviant regions ( $\alpha 10$ ,  $\alpha 11$ ,  $\alpha 22$ ), which, according to our hypothesis, could carry some of the more specific functions of spectrin. As a first approach to unraveling the functions of these regions, we carried out a systematic homology search in protein banks. Such a search yielded the following types of homologies: (i) a region in  $\alpha 22$ -domain showing prototypical EF-hand sequences representing putative calcium binding sites; (ii) a region in  $\alpha 22$  showing homology with calmodulin binding proteins; (iii) a high degree of homology with  $\alpha$ -actinin in a stretch starting from  $\alpha 20$  and extending to the very COOH-terminus of  $\alpha$ -spectrin; (iv) a domain in  $\alpha 10$  showing a high degree of homology to the members of the non-receptor tyrosine kinase oncoproteins and phospholipase C.



Fig. 2. The optimized alignment of the chicken brain  $\alpha$ -spectrin. Asterisks indicate the identical residues between the neighboring repeats. Non-homologous regions are shown in boxes. The domain designations are given to the left and the lengths of the domains (amino acids) to the right.

No calcium-binding sites have been demonstrated in non-erythroid spectrins. The presence of two typical EF-hand structures, homologous to the calcium-binding site in other proteins (Tufty and Kretsinger, 1975), strongly suggests, however, that spectrin is able to bind calcium. In fact, spectrin is known to be involved in various calcium-regulated events, such as complex formation with calpactin (Gerke and Weber, 1984), degradation by a calcium regulated protease (Siman *et al.*, 1984) and modulation of the  $Mg^{2+}$ -ATPase activity of the smooth muscle actinomyosin (Wagner, 1984). Moreover, Fishkind *et al.*, (1987) have recently shown that  $\alpha$ -spectrin binds actin in a calcium-dependent manner. One of the functions of the putative calcium-binding sites in spectrin could be to modulate the actin-binding of spectrin, since  $\alpha$ -spectrin binds actin with its COOH-terminus to which the EF-hand structure maps.

One of the distinguishing features of the members of the non-erythroid spectrin family is their capacity to bind calmodulin by their  $\alpha$ -subunit (Glenney *et al.* 1982b; Kakiuchi *et al.*, 1982). On the basis of the homology studies we localized the putative calmodulin-binding site to the COOH-terminal end of the molecule. Tsukita *et al.* (1983) have, however, suggested that the calmodulin-binding site resides in the  $NH_2$ -terminus of  $\alpha$ -spectrin. On the other hand, Harris and Morrow (1988) have recently identified a region in the area corresponding to the  $\alpha 11$ -extension in our sequence that seems to have a capacity to bind calmodulin. Clearly, further biochemical studies are needed to establish the exact location of the calmodulin-binding sites in non-erythroid spectrin.

The homology search also revealed a sequence similarity between  $\alpha$ -actinin and  $\alpha$ -spectrin (see also Wasenius *et al.*, 1987). The homologous region in  $\alpha$ -actinin is known to be responsible for the  $\alpha$ -helical structure of the protein which is the prerequisite for the dimer formation between the two  $\alpha$ -actinin subunits (Baron *et al.*, 1987). The homologous region encompasses the entire COOH-terminal half of  $\alpha$ -actinin, whereas there is no sequence similarity between  $\alpha$ -spectrin and the  $NH_2$ -terminal half of the molecule that is known to be responsible for the actin-binding function. Thus,  $\alpha$ -actinin appears to be a hybrid molecule with a unique actin-binding  $NH_2$ -terminus and a spectrin-like COOH-terminal half.

The deviant domain  $\alpha 10$ , located in the middle of the  $\alpha$ -chain, showed homology to the proteins belonging to the non-receptor class of the tyrosine kinase proteins, also called *src*-oncogene family, and with phospholipase C (Lehto *et al.*, 1988). The homology with  $\alpha 10$  is localized to the  $NH_2$ -terminal part of the «*src*-proteins» which exerts a modulatory effect on the tyrosine kinase activity which, on the other hand, resides in the COOH-terminal parts of the proteins (Jove and Hanafusa, 1987). The functional significance of this homology is currently unknown.

#### **A family of proteins with spectrin-like structural motifs**

The homology between  $\alpha$ -actinin and  $\alpha$ -spectrin suggests that there may be a larger family of proteins displaying spectrin-like structural motifs. This is further strengthened by another observation yielded by the homology search, viz. a homology between Duchenne muscular dystrophy (DMD)-protein, also called dystrophin (Koenig *et al.*, 1988), and spectrin. Dystrophin, like spectrin, shows an internal repeat structure with each repeat having 109 amino acid residues. Furthermore, by DIAGON analysis we were able to show that repeats homologous to spectrin repeats occur regularly and several times in DMD

protein. Thus, it is apparent that  $\alpha$ -spectrin,  $\alpha$ -actinin and dystrophin belong to the same family of cytoskeletal proteins which contain homologous but distinct repeat units and possess a common architectural design (Table 2). These regions may also represent common functional features, such as are needed for instance for antiparallel dimer formation (Davison and Critchley, 1988).

TABLE 2  
PROTEINS WITH SPECTRIN-LIKE REPEAT STRUCTURE

Protein	Length of repeat	Number of homologous repeats	Proportion of protein encompassed by the homologous repeats
$\alpha$ -spectrin	106	18	80%
$\alpha$ -actinin	120	4	53%
dystrophin	109	26	73%

#### **A model for the evolution of spectrin**

The revelation of the primary structure of spectrin and its comparison with the other spectrins and spectrin-like molecules also permits us to ponder the evolution of the spectrin molecule. On the basis of the repetitive structure, it can be suggested that present-day spectrins have evolved by several contiguous duplications from an ancestral gene coding for 106-residue repeat unit (Speicher and Marchesi, 1984). It seems probable that the ancestral repeat unit first underwent three duplication steps to reach an eight-repeat stage. In some as yet unknown manner, one additional repeat was inserted making a nine-repeat structure, after which the final duplication took place. Possibly by exon shuffling the gene has also acquired new domains  $\alpha 10$ , and  $\alpha 22$ , and the extension of  $\alpha 11$ . The derivation of these segments is currently unknown.

#### **Prospects**

The unraveling of the primary structure of spectrin gives a clear prediction of the secondary structure of the molecule. This is important in order to understand the functions of spectrin. Thus, primary structure analysis has allowed us to identify structurally distinct domains which are hypothesized to be the sites carrying some of the distinct functions of spectrin. Thus, the view of the gene of  $\alpha$ -spectrin has furnished us with important clues for further, more biochemically and cell-biologically oriented work on the structure and function of spectrin. The availability of the sequence information and clones of spectrin brings the analysis of the spectrin-based cytoskeleton and its interaction to a new level which was not possible with the classical techniques. Moreover, it is clear from the results obtained from the preliminary sequence comparisons that new concepts of the evolution and structuring of the cytoskeletal network will probably emerge. Alpha-spectrin is one of the longest structural proteins currently known by its primary structure, and this may give important insight into the structure and regulation of large structural proteins in general.

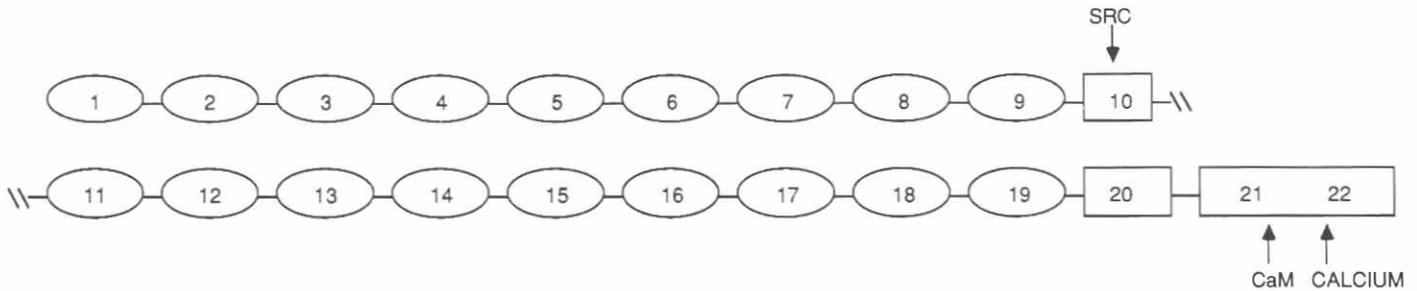


Fig. 3. A schematic representation of the  $\alpha$ -spectrin domain structure.

### Epilogue/VPL

In 1975 I joined the active and merry group of scientists working at the Third Department of Pathology, University of Helsinki. My experience was the same as that of many other doctors entering the third floor of the Meilahti complex; I became enchanted with the scientifically rigorous and socially relaxed atmosphere of the Department. Such is the logic of the cumulative effect of the build-up of knowledge in science that I was able to benefit from the collective knowledge and skills of the group working in the Department without actually knowing where the roots of this remarkable activity were to be found. Only slowly—and often through hindsight—did it become clear to me that I was benefiting from a long tradition of consistent perseverance in scientific endeavor the foundations of which were laid by Professors Ekman, Toivonen and Saxén and the school of developmental biologists around them. In my view, it was the genial recognition by this group of the value of model system provided by the induction phenomenon, their concentrated focus on this phenomenon, and the application of the most recent and modern techniques that gave the newcomer the intellectual framework and the practical tools for research and made it easy for her or him to tackle almost any problem in developmental biology or in the emerging fields of cell biology and molecular biology.

Together with Ismo Virtanen I started my experimental research work by first focusing on the problem of cytoskeleton-plasma membrane interactions. I was especially intrigued by the results from the studies on plasma membrane structure which indicated that there are probably transmembrane proteins to be found that mediate the linkage between extracellular matrix and the cell interior. As a further extension of that link, we thought, there must also be a linkage from the plasma membrane to the nucleus where the ultimate events leading to the switching on and off of e.g. differentiation genes in response to extracellular stimuli are thought to take place. This led Ismo and myself to recognize the intermediate filaments as one putative link between the plasma membrane and the nucleus. Now, 10 years later, the molecular mechanisms of such linkages between the plasma membrane and the intermediate filament, on the one hand, and the intermediate filaments and the nucleus, on the other, have been established. Another effort to explore the chain of events leading from the extracellular matrix to the nucleus was to isolate transmembrane proteins which would link the intermediate filaments to the matrix proteins outside the cell. As a by-product of these studies we identified a new high molecular-weight protein that turned out to be non-erythroid spectrin. This was a novel protein since at that time spectrin was considered to be present only in erythroid cells.

In this paper we have reviewed our studies on spectrin. I have chosen spectrin as a topic since it very nicely illustrates the value of modern techniques in dissecting the structure and functions of proteins. On the other hand we feel that in our studies we have come to a point where the biological relevance of the observations based on the gene approach must ultimately be tested in the relevant biological context, i.e. in a living cell or organism. This will bring us back to the model systems, approximations of biologically important phenomena which in the future will provide the final tests to the ideas from more reductionist approaches.

### Materials and methods

Cloning of the chicken brain  $\alpha$ -spectrin cDNA was carried out according to the following protocol: First, an expression cDNA-library was constructed by starting from a chicken gizzard mRNA (Helfman *et al.*, 1983). It was then screened with antibodies against bovine spectrin (anti-p230 antibodies; Lehto and Virtanen, 1983). Second, by using the clone pUC8-13a (obtained from the immunoscreening of the gizzard library; Wasenius *et al.*, 1985), as a probe, further clones from cDNA libraries were constructed starting from the chicken brain mRNA and chicken genomic DNA. Standard techniques were used for the isolation of the total cellular RNA (Chirgwin *et al.*, 1983) and poly (A)-mRNA, and for the cDNA synthesis and the transformation of the host cells (Maniatis, 1982). The cDNA libraries were constructed in bacteriophage lambda gt10-vector (Promega Biotec) as previously described (Huyng *et al.*, 1985).

Screening of the expression library was carried out by Dr. David Helfman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY USA) by using anti-p230 antibodies.

#### DNA-sequencing

Sequencing was carried out according to Sanger by using the dideoxy chain termination method (Sanger, 1980; Biggin *et al.*, 1983).

#### Computer analysis

Nucleotide sequence analysis was aided by the Staden program (Staden, 1987). For the amino acid sequence homology studies, DIAGON-program (Staden, 1982) was used. Search for the homologous sequences was carried out by using the protein identification resource PIR (database 12.0, March 1987) as a reference. Secondary structure prediction was based on the Chou-Fasman procedure (Chou and Fasman, 1978) and the algorithm of Garnier *et al.* (1978).

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