Purification, characterization and molecular cloning of muscle paranemin

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Purification, characterization and molecular cloning of muscle paranemin

by

Philip Mark Hemken

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# TABLE OF CONTENTS

## INTRODUCTION
- Dissertation Organization .................................................. 1
- General Overview .............................................................. 2
- Literature Review .............................................................. 3
- References ............................................................................ 38

## PARANEMIN IS A GLYCOPROTEIN LOCALIZED AT Z-LINES OF ADULT CARDIAC AND SKELETAL MUSCLE
- Summary .............................................................................. 51
- Introduction ......................................................................... 51
- Materials and Methods ......................................................... 52
- Results ................................................................................. 54
- Discussion ............................................................................. 56
- References ............................................................................ 58

## PURIFICATION OF THE INTERMEDIATE FILAMENT FAMILY PROTEIN, PARANEMIN, FROM EMBRYONIC AVIAN SKELETAL MUSCLE
- Summary .............................................................................. 70
- Introduction ......................................................................... 71
- Materials and Methods ......................................................... 73
- Results ................................................................................. 79
- Discussion ............................................................................. 82
- References ............................................................................ 85

## MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF PARANEMIN, A NOVEL TYPE VI INTERMEDIATE FILAMENT PROTEIN
- Summary .............................................................................. 105
- Introduction ......................................................................... 106
- Experimental Procedures .................................................... 108
- Results ................................................................................. 115
- Discussion ............................................................................. 120
- References ............................................................................ 125

## OVERALL SUMMARY .................................................................................. 152

## BIBLIOGRAPHY .................................................................................. 155

## ACKNOWLEDGMENTS ........................................................................... 180
INTRODUCTION

Dissertation Organization

The alternate format is used in my dissertation with the inclusion of three papers. The main body consists of: one short paper, which will be submitted for publication to Biochemical Biophysical Research Communications; a second long paper, which will be submitted for publication to Biochemical Journal; and a third long paper, which will be submitted for publication to The Journal of Biological Chemistry. I was responsible for planning and implementing essentially all of the experiments in these three papers. I received help with confocal microscopy, amino acid analysis, and cDNA sequencing from the Iowa State University Confocal Microscope Facility, Protein Facility and DNA Sequencing and Synthesis Facility, respectively. Dr. Bruno Becker, a former postdoctoral fellow in our laboratory, did the majority of the work in preparing a β-galactosidase-paranemin fusion protein, which is shown in the third paper. The protease calpain used in an experiment in the third paper was purified by Dr. Elisabeth Lonergan, a former graduate student in our laboratory. The references cited in the Introduction and in each of the three papers immediately follow each one in a Reference section. A comprehensive reference list, including titles of the articles, is found in the Bibliography section at the end of this dissertation.
General Overview

Intermediate filaments (IFs) comprise one of the three major filament networks in nearly all eukaryotic cells (for reviews see Robson, 1989, 1995; Fuchs and Weber, 1994), with the other two filament systems being microfilaments (actin filaments) and microtubules. The term “intermediate-sized filament” was first used by Ishikawa et al. (1968), when they observed filaments, which had an intermediate diameter (~10 nm) compared to actin filaments (~7 nm) and thick myosin filaments (~15 nm) between the myofibrils in developing muscle cells. Later the name has come to be focused on the fact that the IFs also have a diameter intermediate in size between the actin filaments and microtubules (23 nm) observed in nonmuscle cells. The IFs have been studied for over 30 years now, but they still represent one of the last functionally important protein assemblies in the cell that do not have a clear biological role (Traub and Shoeman, 1994). As molecular biology techniques became available and the IF proteins were sequenced, it became clear in the 1980’s that IFs from different cell types are composed of proteins that share common sequence and structural features. Today the IF protein superfamily contains over 50 proteins that are classified into as many as six classes or types based upon sequence, and there are probably others that have not been discovered. The major IF protein in most mature muscle cells is desmin (Huiatt et al., 1980; O’Shea et al., 1981). In mature striated muscle cells, most of the IFs are located in a collar-like arrangement around the periphery of the myofibrillar Z-lines (Robson, 1995). It is unknown, however, how the desmin-containing IFs are linked to the myofibrils. Paranemin is a minor protein
constituent in muscle cells, but because it copurifies from embryonic muscle with desmin and vimentin IF proteins and is colocalized with desmin at myofibrillar Z-lines (Breckler and Lazarides, 1982; Price and Lazarides, 1983), we propose it may have a role in attaching desmin IFs to the Z-lines and possibly to the muscle cell membrane. Because very little was known about paranemin's properties and function, the focus of the studies that follow is on the purification, characterization and molecular cloning of muscle paranemin. Paranemin will be shown to be a novel IF protein, rather than an IF-associated protein (IFAP) as has been believed since its discovery. The results that will be described are supportive of a role for paranemin in attachment of IFs to other structures such as myofibrillar Z-lines and/or the cell membrane at cell-matrix adhesion plaques.

Literature Review

In this literature review, I start by briefly describing the general structure and function of skeletal and cardiac muscle, and then describe the structure and arrangement of filaments in the striated muscle myofibril. Next is a discussion of the major myofibrillar proteins and of vertebrate striated muscle contraction. This will be followed by a brief discussion of smooth muscle and some of its differences in comparison to striated muscle. Sections describing specific aspects of striated muscle development, intermediate filaments (IFs) and their associated proteins (IFAPs), which is penultimate, and lastly a section discussing the similarities between transcription factors and intermediate filaments complete the literature review.
Skeletal muscle

Collagen is the primary structural component of connective tissue in muscle, which provides a network of fibers throughout each muscle body (Light and Champion, 1984). The epimysium surrounds the entire muscle like a sheath and is continuous with the tendon in skeletal muscle. The epimysium is, in turn, continuous with the perimysium, which surrounds bundles of muscle fibers and gives meat its texture. The perimysium is continuous with the endomysium, which surrounds each muscle fiber (cell) and can be seen with the aid of the light microscope. This collagen network is continuous from the tendon throughout the entire muscle to the endomysium and individual muscle cells. In this way the force of muscle contraction is transmitted through connective tissue, via the tendon, to the bone (Light and Champion, 1984).

The smallest units in muscle that can give a physiological response are the muscle fibers, each of which contains a complex contractile apparatus enclosed by an electrically polarized membrane (Huxley, 1972). Muscle fibers characteristically have diameters between 20-100 μm. Lengths of fibers depend on the muscle and its construction. Common laboratory models have included frog sartorius and rabbit psoas muscles because they are several cm in length. The term skeletal muscle fiber is synonymous with muscle cell; therefore, muscle fibers are the cellular units within skeletal muscle.

Each fiber of skeletal muscle is a multinucleated cell, formed by fusion of mononucleated myoblasts during embryonic development (reviewed in Obinata, 1994). In
early stages of developing muscle cells, the nuclei are centrally located and they move
during development to a position directly underneath the sarcolemma, a trilaminar
membrane that surrounds the muscle fiber. The mitochondria, ribosomes, storage granules,
glycogen, and Golgi complex of muscle fibers are considered structurally and functionally
similar to their counterparts in nonmuscle cells (Goll et al., 1984). A transverse tubule
system, also called the T-system or the T-tubules, which are open to the exterior of the
fiber, carry electrical impulses into the interior of the cell rapidly and efficiently (for
reviews see Huxley, 1972; Lytton and MacLennan, 1992). An elaborate intracellular
membrane system called the sarcoplasmic reticulum (SR) is the muscle analog of the
nonmuscle endoplasmic reticulum, and regulates the levels of free Ca\(^{+2}\) that in turn control
muscle contraction. The SR has two main regions, including (1) the junctional SR, which
is a specialized contact region between the SR and either T-tubules or the sarcolemma, and
(2) the free SR, which is found between the junctional SRs. In the middle of the free SR
the fenestrated collar surrounds the middle of the myofibrillar sarcomere (Huxley, 1972;
Lytton and MacLennan, 1992).

The contractile units in muscle fibers are the myofibrils, each of which is usually 1-3
\(\mu m\) in diameter and normally extends the entire length of the muscle cell (Goldspink, 1970;
Craig, 1994). Each myofibril is surrounded in part by the SR and has a banded cross-
striated appearance along its length. Because all the myofibrils in a cell are aligned in
register, and all of the fibers also are arranged in register, the entire muscle appears cross-
striated.
Cardiac muscle

Cardiac and skeletal muscle differ mainly in their metabolism, because cardiac muscle, which depends entirely upon aerobic metabolism, contracts continuously throughout its lifetime. Cardiac cells are arranged in overlapping bundles, and the bundles in layers. Cell borders jut in and out in somewhat of a staggered or stairstep arrangement, thereby locking together adjoining ends of two cells (Sommer and Jennings, 1992). At the base of the ventricle lies the largest mass of cells where the greatest forces of contraction are generated. Cardiac cells are usually mononucleated and the nucleus is centrally located in the cell. Fibers are not synonymous with cells in cardiac muscle. Structurally, individual short cells exist along fibers, but physiologically the myocardium behaves like a syncytium, which means the heart has electrical continuity among cells throughout. Electrical coupling between cells exists in specialized cell membrane structures called intercalated disks that provide electrical and mechanical connections in the heart. Components of the intercalated disk, which exist at the ends of the cell, include the (1) nexus (gap junction, zonulae occludens), which lies parallel to the long axis of the cell, (2) the desmosome (maculae adherens), which functions as a mechanical connection between cells, and the intermediate junction (fasciae adherens), which takes the place of the terminal Z-line (Page and Fozzard, 1973; Goll et al., 1984; Sommer and Jennings, 1992).
Structure of the myofibril in striated muscle

The myofibril is the contractile organelle that separates muscle from nonmuscle cells and comprises over one-half of the muscle volume. In the light microscope a system of repeating transverse bands are seen, which reveal clues to the structure of the myofibril. The repeating transverse bands arise because a variation in density is present along the length of the myofibrils. Bands in adjacent myofibrils within a cell, and even those in adjacent cells, are precisely aligned with each other from one end to the other. Thus, the myofibrils are responsible for the crossbanded structure in skeletal and cardiac muscle, and give rise to the term striated muscle. The myofibril is not completely enclosed by a membrane. The complex of proteins (>20) within the myofibril (Huxley, 1972; Robson, 1995) is insoluble and can exist without a membrane completely surrounding it.

Each longitudinal repeat of the pattern along the long axis of a myofibril is known as a sarcomere. In vertebrate muscles, sarcomere lengths usually are about 2.3-2.8 μm at normal resting states. The ends of the sarcomere are defined by the Z-line structure, which appears as a dense narrow line or disk running perpendicular to the long axis of the myofibril and cell (Huxley, 1972). Within each sarcomere a more dense, bright birefringent zone, is seen under the polarized light microscope, and is known as the A (anisotropic) band. The A-band is located symmetrically in the middle of the sarcomere and is separated from the Z-lines by zones of lower density and weaker birefringence known as I (isotropic) bands. In a muscle at rest length and a sarcomere length of about 2.5 μm, the A-band is about 1.5 μm long and the two halves of the I-band flanking the Z-
line are each about 0.5 μm long, giving an overall I-band length of about 1 μm. The central region of the A-band in rest-length muscle is somewhat less dense than its lateral regions and is known as the H-zone, which is approximately 0.5 μm in length. When a muscle contracts, birefringence decreases 40-45%, indicating that a fundamental change occurs in the A-band while the muscle is contracting (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954; Huxley, 1957). During muscle contraction, sarcomere lengths decrease to ~2-2.25 μm, depending on the extent of the contraction.

Arrangement of thin and thick filaments within striated muscle myofibrils

The banding pattern in myofibrils is seen by light microscopy because they are constructed with overlapping arrays of longitudinally-running thick and thin filaments, which were first seen when thin sections of striated muscle were examined in the transmission electron microscope (Huxley, 1953). Only thin filaments (~7 nm in diameter) are seen in parts of the sarcomere corresponding to the I-bands, whereas both thin and thick (~15 nm in diameter) filaments are present in the A-band. The length of the A-band corresponds to the length of the thick filament, whereas the thin filament extends from the Z-line to the edge of the H-zone. The repeating banding patterns of thick and thin filaments observed by electron microscopy account for the sharp variation in density seen by light microscopy.

The thick filament often appears thicker at the very center of the A-band, and then tapers at either end for about 150 nm. Cross-bridges also can be observed, in well
preserved material, between thin and thick filaments, which occur at longitudinal intervals of about 43 nm between a given thick and thin filament (Pepe, 1967a, 1967b; Craig, 1994). In cross-section, six thin filaments surround each thick filament. The cross bridges are ~5-6 nm in diameter and ~18-19 nm long. Another structural feature or region observed, called the pseudo H-zone or bare zone, is that area in the center of the A-band where no cross-bridges extend from the surface of the thick filament. Another structural feature of the myofibril that is observed by electron microscopy is the M-line (disk). The M-line runs transverse to the long axis of the myofibril and is present at the middle of the A-band (Huxley, 1972; Goll et al., 1984). The M-line is composed of three proteins (Robson, 1995) and is believed to link each thick filament to the nearest six thick filaments in a hexagonal pattern. There are two thin filaments per one thick filament in cross-sections of vertebrate skeletal muscle, so there is always sharing of thin filaments between thick filaments. During contraction, muscles maintain virtually constant volume by changing the space between the thick and thin filaments (Goll et al., 1984). The Z-lines also have the ability to expand, because electron micrographs of cross-sections taken directly adjacent to the Z-line in shortened muscle show increased distances between neighboring thin filaments entering the Z-line. In relaxed muscle, these thin filaments are ~22-25 nm apart, and in contracted muscle they are ~28-30 nm apart (Goll et al., 1984; Vigoreaux, 1994).
Myofibrillar proteins

Myofibrillar proteins constitute 52-56% of total protein in skeletal muscle and 45-50% of the protein in cardiac muscle (Goll et al., 1984; Craig, 1994; Robson, 1995). Thick filaments contain all the myosin of the myofibril. Myosin makes up ~45% of total myofibrillar protein by weight, and constitutes approximately 94-96% of the protein in the thick filaments of vertebrate striated muscle. Most of the remaining protein in the thick filament is composed of C-protein and H-protein. The M-line is composed of three proteins, M-protein, myomesin, and creatine kinase. Actin, the principal component of the thin filament, makes up the second largest percentage (15-20%) of the myofibril by weight (Goll et al., 1984). Other major thin filament proteins, and their percentages of the myofibril by weight, include tropomyosin (4-6%), troponin (4-6%), and nebulin (~3%). Each thin filament is anchored at the Z-line, but is not continuous across the Z-line (Yamaguchi et al., 1982, 1985). The integral Z-line structure consists of actin, α-actinin, CapZ, and the ends of titin and nebulin (Labeit and Kolmerer, 1995a, 1995b; Robson, 1995). Peripheral Z-line proteins include the intermediate filaments, which are composed primarily of desmin (Stromer, 1990, 1995; Robson, 1995). The protein titin was discovered by Wang et al. (1979) and is 2.5-3.0 nm in diameter and >1 μm in length, with a single molecule spanning the distance from the Z-line all the way to the M-line (for a review see Small et al., 1992).

Myosin and actin are both necessary and completely sufficient for an in vitro contractile response (Sheetz and Spudich, 1983; Goll et al., 1984). Myosin consists of a diverse
family of proteins that are present in all eukaryotic cells (Goodson and Spudich, 1993). From gene cloning and DNA sequencing studies, more than 10 classes of the myosin gene have been found (for a review see Darnell et al., 1995). Myosin II, the form discovered in muscle, powers muscle contraction. Myosin II is a very large protein consisting of a long α-helical coiled-coil rod domain that is ~155 nm long and ~1.5 nm in diameter, with two pear-shaped heads at one end. The myosin II molecule contains three pairs of polypeptide chains, including two chains that comprise all of the long myosin rod domain and most of the mass of the two heads, and two pairs of light chains, with a member of each pair associated with each of the myosin heads (Craig, 1994). Myosin II has three physiologically important properties: (1) myosin spontaneously aggregates to form thick filaments; (2) myosin has the enzymatic ability to split ATP and release energy; in the absence of actin, myosin adenosine triphosphatase (ATPase) activity is inhibited by Mg\(^{2+}\), but when myosin is combined with actin, its ATPase activity is activated by Mg\(^{2+}\); and (3) myosin binds strongly to actin; the actin-myosin complex is specifically dissociated by ATP when Mg\(^{2+}\) is present (for review see Goll et al., 1984). The three-dimensional structure of myosin 'subfragment-1' has been determined at a resolution of 2.8 Å (Rayment et al., 1993a). A model of the actomyosin complex was reported at the same time (Rayment et al., 1993b). The conformational changes that occur in the actomyosin complex during contraction can now be studied and discussed in terms of molecular structure (Rayment and Holden, 1993, 1994).
Actin (42 kDa, ~375 residues), the most abundant intracellular protein in many nonmuscle eukaryotic cells, is encoded by a large, highly conserved gene family. Six actin isoforms are present in vertebrate cells (Herman, 1993). Actin is a much smaller molecule than myosin and contains only one polypeptide chain. In skeletal muscle, actin comprises about 10% by weight of the total protein (Darnell et al., 1995). Actin can exist as a globular monomer called G-actin and as a filamentous polymer called F-actin, which represents two long strings of G-actins arranged in a helical fashion (Goll et al., 1984). F-actin forms the core of the thin filament in muscle cells. Each actin monomer is capable of binding a single myosin S1 head. The atomic structure of G-actin (Kabsch et al., 1990) and an atomic model of the actin filament have been reported (Holmes et al., 1990).

Other proteins that are associated with the thin filaments in muscle that are particularly important are tropomyosin, troponin, CapZ and tropomodulin. Tropomyosin is a homodimer (66 kDa) with two 284-residue subunits forming an α-helical coiled-coil (Hitchcock-DeGregori and Varnell, 1990). Only the residues at the extreme ends of the molecules are not in the α-helices. A tropomyosin molecule is ~40 nm long and joins head to tail with successive molecules to form long cables wound in the grooves of the F-actin helix, so that each molecule of tropomyosin interacts with seven actin monomers (for review see Zot and Potter, 1987). Troponin (69 kDa) consists of three subunits: TnC, a Ca\(^{2+}\) binding protein that is 70% homologous to calmodulin; TnI, that binds to actin and inhibits actin-myosin interaction; and TnT, an elongated subunit that binds to the other two troponin subunits and also to tropomyosin at its head to tail junctions (Zot and Potter,
1987). Troponin is located on actin filaments of vertebrate striated muscle with a 38 nm periodicity (Ohtsuki et al., 1988). The troponin-tropomyosin complex regulates actin-myosin interactions in response to Ca\(^{2+}\) concentrations in the cell (Ebashi and Endo, 1968; Ohtsuki et al., 1986; Zot and Potter, 1987). CapZ is an integral Z-line protein that caps the barbed ends of actin filaments (Casella et al., 1986, 1987). It is a heterodimer (66 kDa), consisting of 36 kDa and 32 kDa subunits. CapZ has no actin severing activity and its actin binding ability is not affected by Ca\(^{2+}\). It was concluded that β-actinin, a protein first described in the mid 1960's, is equivalent to CapZ (Maruyama et al., 1990).

Tropomodulin caps the pointed end of actin filaments and is a 41 kDa tropomyosin-binding protein (Fowler et al., 1993). It was first isolated from human erythrocyte membrane skeletons where it binds to one end of erythrocyte tropomyosin and blocks head-tail association of tropomyosins along actin filaments (Fowler et al., 1993). Tropomodulin completely blocks elongation and depolymerization at pointed ends of tropomyosin-actin filaments in stoichiometric concentrations where \(K_d \leq 1\) nM (Weber et al., 1994).

α-Actinin initially was discovered in skeletal muscle extracts as a factor promoting the superprecipitation of synthetic actomyosin (Ebashi and Ebashi, 1965), was found to localize in the Z-line of striated muscle (Masaki et al., 1967), and was first purified and characterized in our laboratory (Robson et al., 1970; Suzuki et al., 1976). It is known now that α-actinin is located in the interior of the myofibrillar Z-lines (Granger and Lazarides, 1978; Yamaguchi et al., 1985). The α-actinin molecules from many sources are each a
homodimer with a subunit molecular weight of ~93-103 kDa. The two subunits are assembled in an anti-parallel fashion (Blanchard et al., 1989; Vandekerckhove, 1990).

Other thick filament proteins that bind to myosin are C-protein (one subunit of ~130 kDa) and H-protein (56 kDa by sequence and 74 kDa by SDS-PAGE) (Vaughan et al., 1993). The C-terminal 40 kDa part of H-protein shares 49.6% identity with C-protein. The roles of these proteins remains speculative, but they may link titin to the myosin filament (Craig, 1994).

Proteins in the M-line include: (1) myomesin, an ~185 kDa monomer (Grove et al., 1984); (2) M-protein, an ~165 kDa monomer (Noguchi et al., 1992); and (3) muscle specific creatine kinase, an ~80 kDa dimer (Turner et al., 1973). Skelemin, an ~195 kDa monomer, is also found at the periphery of the myofibrillar M-line in mammalian striated muscle (Price, 1991; Price and Gomer, 1993).

Titin (Wang et al., 1979), also called connectin (Maruyama et al., 1981), is the largest protein known and in vertebrate striated muscle represents a third filament system. Titin is a single polypeptide of about 27,000 amino acids, with a molecular weight of nearly three million (Small et al., 1992; Labeit and Kolmerer, 1995a). Each titin molecule spans from inside the Z-line to inside the M-line, a distance longer than 1 μm (Furst et al, 1988). The entire cDNA sequence of cardiac titin (82 kb) recently has been completed, which was quite an accomplishment (Labeit and Kolmerer, 1995a). The cDNA derived sequence reveals a new type of structural motif (PEVK) that may account for the elasticity within the molecule.
Nebulin (Wang and Williamson, 1980) is a very elongated (1 μm), thin (-1 nm in diameter) molecule that runs in parallel along or in each groove for the full length of the thin filament, i.e., from the free end of the thin filament (nebulin’s N-terminus) to the Z-line (nebulin’s C-terminus) (Chen et al., 1993). Nebulin is only present in skeletal muscle cells. The entire cDNA sequence of human nebulin (20.8 kb) is now known, which encodes for a 773 kDa protein (Labeit and Kolmerer, 1995b). Nebulin may be important in organizing the thin filaments during myofibrillogenesis, by anchoring the thin filament firmly to the Z-line, acting as a template ruler for thin filament assembly to control the thin filament length, and by helping regulate actin-myosin interactions (Jin and Wang, 1991; Labeit et al., 1991; Root and Wang, 1994; Wang et al., 1996).

Intermediate filaments, located primarily around the periphery of the myofibrillar Z-lines, are important components of the vertebrate striated muscle cell that will be described later.

**Contraction of vertebrate striated muscle**

Thick and thin filaments slide past each other during muscle contraction, with both filaments maintaining constant length. The contraction is accompanied by equal reductions in lengths of the I-band and H-zone regions, resulting in contracted muscle being ~15% shorter than its resting length (independently reported by H.E. Huxley and Hansen, 1954; A.F. Huxley and Niedergerke, 1954). These observations led to the ‘Sliding Filament Model of Muscle Contraction’, which proposed that the force of muscle contraction is
generated by a process in which interdigitated sets of thick and thin filaments slide past each other. Contractile force is initiated by actin stimulation of myosin’s ATPase activity. A detailed structural-based model for the interaction between actin and myosin recently has been proposed (Rayment et al., 1993a, 1993b, 1994). This model includes the so-called rigor complex of the myosin S1 head and F-actin, present when the muscle is deprived of ATP, and can explain how ATP hydrolysis is coupled to myosin’s conformational change.

Calcium regulates muscle contraction in a process mediated by troponin and tropomyosin. The troponin-C subunit of troponin binds Ca\(^{2+}\), which causes a long-range allosteric shift through the other two troponin subunits, and causes tropomyosin to move ~10 Å further into the thin filament grooves (Zot and Potter, 1987). This movement of tropomyosin is thought to uncover actin’s myosin-binding site, which switches on muscle contraction. Following a contractile event, Ca\(^{2+}\) ions are pumped back into the sarcoplasmic reticulum, and the series of conformational changes in the thin filament proteins are reversed (for review see Lytton and MacLennan, 1992).

Smooth muscle

Smooth muscles are involuntary and nonstriated, and are located in the digestive tract, reproductive system, vascular system, urinary tract, skin and ducts of glands. Smooth muscle cells are elongated (100-500 μm) and between 3-6 μm in diameter, with a single nucleus located in the center. Cells may be in overlapping bundles, straight, or spiral in shape, depending on the specific tissue. Smooth muscle cells are nonstriated, with no
regularity or cross-banded structure as seen in skeletal or cardiac muscle cells. A common ratio of thin to thick filaments is 13:1, with a weight ratio of actin to myosin of 3:1. Thus, there is proportionally much more actin in smooth muscle than in striated muscle. The thick filaments contain myosin II, and have no C-protein or H-protein. The precise composition of the thin filaments in smooth muscle is controversial because there are probably two types of filaments (for a review see Somlyo and Somlyo, 1992). One thin filament system contains actin, tropomyosin, calponin, and caldesmon in a 28:4:4:1 ratio. These filaments contain no troponin and are in the ‘contractile’ domain. The second filament system is present in the ‘cytoskeletal domain’, and contains actin, tropomyosin, calponin, and filamin (Somlyo and Somlyo, 1992). The thin filaments insert into dense bodies, Z-line analogs. Intermediate filaments (IFs) are very abundant in smooth muscle cells and comprise ~8% of the myofibrillar/structural proteins (Huiatt et al., 1980), which is ~23 times as much as the IFs comprise in skeletal muscle (O’Shea et al., 1981). Intermediate filaments attach to cytoplasmic dense bodies and to membrane associated dense bodies. Some investigators believe that two separate contractile and cytoskeletal domains exist in smooth muscle cells (Small, 1974, 1977), whereas others believe these two domains are not separate, and act as one overall contractile unit (Somlyo and Somlyo, 1992).

Smooth muscle contraction is triggered by Ca\(^{2+}\), which forms a Ca\(^{2+}\)-calmodulin complex that in turn binds to and activates the enzyme myosin light chain kinase (MLCK). The regulatory light chains of myosin are phosphorylated, which allows contraction to
proceed. When the [Ca\(^{2+}\)] falls, through the action of the sarcoplasmic reticulum Ca\(^{2+}\)ATPase, the MLCK is deactivated, the regulatory myosin light chains are dephosphorylated by myosin light chain phosphatase, and the muscle relaxes (Somlyo and Somlyo, 1992). Smooth muscle contraction can also be regulated hormonally, for example, with epinephrine. This hormone modulation inhibits the contractile response causing smooth muscle to relax via calmodulin kinase II, kinase A, and kinase C (Somlyo and Somlyo, 1994). These protein kinases inactivate myosin light chain kinase, and prevent phosphorylation of the myosin regulatory light chains. Studies also have revealed previously unrecognized contractile regulatory processes, such as G-protein-coupled inhibition of myosin light chain phosphatase, regulation of myosin light chain kinase by yet other types of kinases, and by the functional effects of different smooth muscle myosin isoforms (Somlyo and Somlyo, 1994).

**Striated muscle development**

Myogenesis of mammalian skeletal muscle cells can be divided into three stages: (1) myoblast determination, (2) migration, and (3) differentiation into muscle. Precursor cells or myoblasts arise from somites, which are blocks of mesodermal cells found lateral to the neural tube in the embryo (for review see Obinata, 1994). Myoblasts have at least three possible routes, contrary to the classic model of a bimodal switch between cell division and differentiation. A myoblast in the animal cell may also have the option to become a quiescent satellite cell, which is thought to be the inactive stem cell within muscle
Mononucleated myoblasts first arise from somites. A portion of these myoblasts migrate to limb or ventral body-wall regions where they cease division and fuse with one another to form multinucleated muscle fibers. Two helix-loop-helix DNA-binding factors of the Myo D family, myo D and myf.5, were initially thought to control muscle formation; however, mutations in these genes gave rise to mice with surprisingly mild muscle defects (Rudnicki et al., 1992; Braun et al., 1992; Hughes, 1992). However, a disruption by a targeted construct mutation of a third member of the Myo D family, myogenin, yielded mice having little skeletal muscle (Hasty et al., 1993; Nabeshima et al., 1993). Thus, myogenin seems to be required for efficient muscle formation and it may be one of the more important factors controlling muscle formation (Hughes, 1993).

The formation of the sarcomere requires the assembly of thin and thick filaments of appropriate length, and their precise organization into a higher order of structure, the myofibril. Many investigations have demonstrated that most of the myofibrillar proteins exist in multiple isoforms and that the protein isoforms present in young immature muscles, in which myofibrillar assembly takes place, differ from those in adult muscle (for review see Obinata, 1994). The multiple isoforms have been distinguished in each myofibrillar protein using immunocytochemistry, recombinant DNA technology, electrophoretic procedures and protein biochemistry (Fishman, 1986). The expression of myofibrillar protein isoforms within the same muscle changes during the process of muscle development. For example, the embryonic myosin light chain (called L23) of chicken is known to be a constituent of chicken brain myosin along with being common to embryonic
smooth, cardiac, and skeletal muscles cells (Takano-Ohmuro, 1985; Kawashima et al., 1987).

Embryonic muscle cells derived from mesodermal cells already contain actin before terminal muscle differentiation; however, the amount of actin in the developing muscle is much less than in adult muscle tissue. For example, the amount of total actin in embryonic day 10 chick skeletal muscle is one-tenth of that in the adult chicken (Shimizu and Obinata, 1986). The actin isoforms are expressed in higher vertebrates in a tissue-specific manner (Vandekerchove and Weber, 1978a, 1978b). In chicken skeletal muscle, the nonmuscle (β, γ) and cardiac (α) isoforms decline gradually with differential timing during development, and are replaced by increased synthesis of the skeletal (α) isoform (Hayward and Schwartz, 1986; Rubenstein, 1990).

In very young myogenic cells actin exists as a mixture of an unpolymerized pool of monomeric actin (G-actin) and filamentous actin (F-actin), and subsequently as mostly bundled stress-fiber-like structures (Obinata, 1994). As development progresses, the muscle cell cytoplasm becomes filled with myofibrillar sarcomeric structures. The intracellular ionic conditions, at least 100 mM KCl and 5 mM Mg$^{2+}$ in vertebrate muscle cells, strongly favor formation of F-actin from G-actin. Because there is a large pool of monomeric actin in embryonic muscle cells, there may be factors which bind to the actin monomer and prevent polymerization (Obinata, 1994). G-actin binding proteins of low molecular weight (e.g. muscle profilin), which are likely to be involved in actin sequestration and assembly, were isolated from the cytoplasm of embryonic chick skeletal
muscle and their effects on actin polymerization were characterized (Oshima et al., 1989; Abe and Obinata, 1989; Abe et al., 1989).

The protein CapZ may be important in regulating the filament length and attachment of actin filaments to the Z-line during myofibrillogenesis (Schafer et al., 1993). However, it is not clear how CapZ specifically participates in the regulation of actin assembly (Obinata, 1994). The skeletal and smooth muscle isoforms of α-actinin coexist in cultured chicken skeletal muscle cells, with the skeletal muscle isoform appearing only after myoblast fusion (Endo and Masaki, 1984). During myofibrillogenesis, α-actinin is first detectable periodically along actin stress-fibers, but later is located in the Z-line where it presumably anchors actin filaments (Jockusch and Jockusch, 1980). The coordinate accumulation of the three troponin components begins in post-mitotic myoblasts, before the formation of cross-striated myofibrils in embryonic chick skeletal muscle primary cultures, and are assembled at characteristic positions in the very initial phase of myofibrillogenesis (Obinata et al., 1979). Interestingly, very early in development, the troponin complex is functionally active and regulates the actin-myosin interaction in embryonic muscle in a Ca\textsuperscript{2+}-dependent manner. Because the formation of actin-myosin interactions may be important in the initial stage of myofibrillogenesis (Shimada and Obinata, 1977; Abe and Obinata, 1989), it may be possible that the troponin complex helps control the formation of primitive myofibrils in a Ca\textsuperscript{2+}-dependent manner (Obinata, 1994).

Purified myosin is known to polymerize spontaneously \textit{in vitro} in physiological salt solution to form filaments (Huxley, 1963). These synthetic myosin filaments exist in
dynamic equilibrium with a small pool of unpolymerized monomers, and the exchange between myosin filaments and monomers appears to be rapid (Saad et al., 1986). Little is yet known about the assembly process of the nascent myosin polypeptides into thick filaments in developing vertebrate striated muscle (Obinata, 1994). Thick filament assembly also may be modulated by myosin-binding proteins, such as C-protein and H-protein, and also by the M-line proteins, M-protein, creatine kinase and myomesin (Obinata, 1994). Different isoforms of myosin are often spatially segregated within the cell, which may be partly due to cotranslational assembly of cytoskeletal proteins (Isaacs and Fulton, 1987) and local distribution of specific mRNAs (Lawrence and Singer, 1986; Pavlath et al, 1989; Hall and Ralston, 1989).

The process of Z-line organization is poorly understood, although it is important for establishing the spatial arrangement of actin filaments in the myofibril. The main proteins involved in this process most likely would be the integral Z-line proteins α-actinin and CapZ, and possibly the ends of titin and nebulin embedded in the Z-line. The IF proteins constitute an additional important cytoskeletal element in muscle cells. Young myogenic cells contain vimentin and desmin as the major IF protein constituents (Gard and Lazarides, 1980; Tokuyasu, 1984), but with development into mature striated muscle desmin becomes the major IF protein (Tokuyasu et al., 1984). In early developing muscle cells in culture, desmin/vimentin-containing IFs are oriented longitudinally, but then gradually shift their orientation to a perpendicular orientation with the Z-lines of striated
myofibrils (Bennet et al., 1978; Gard and Lazarides, 1980; Lazarides et al., 1982; Tokuyasu et al., 1984).

In adult striated muscle cells, desmin is primarily located at the periphery of the Z-line (Z-disk) with filamin (Granger and Lazarides, 1980; Lazarides, 1982) and a novel intermediate filament protein synemin (Becker et al., 1995). It has been suggested that IFs are responsible for the lateral alignment and organization of nascent myofibrils by crosslinking Z-lines of adjacent myofibrils to each other and possibly of myofibrils to the sarcolemma (Lazarides, 1982; Tokuyasu et al., 1985; Robson, 1995).

**Intermediate filaments**

Intermediate filaments (IFs), ubiquitous components of eukaryotic multicellular organisms, have been studied extensively for over thirty years, and were classified as intermediate (10 nm) filaments nearly thirty years ago, but some evidence for their existence has been known for nearly a century (for a review see Fuchs and Weber, 1994). The first X-ray diffraction patterns of IFs were obtained from wool fibers (albeit the investigators did not know IFs were present), and revealed that keratins, which would later become members of the IF protein family, were abundant in α-helical polypeptides that formed in a coiled-coil fashion. (Astbury and Street, 1931; Pauling and Corey, 1953; Crick, 1953). Electron microscopy made it subsequently possible to recognize the widespread occurrence of IFs (Fuchs and Weber, 1994). Intermediate filaments derived their name because they were intermediate-sized filaments compared to thin actin filaments.
and thick myosin filaments (Ishikawa et al., 1968). They also have a diameter between actin filaments (7 nm) and microtubules (23 nm), which has led to their name being used in this context in nonmuscle cells (Fuchs and Weber, 1994).

In the 1980’s it became clear that IFs are composed of proteins that share common structural and sequence features, and that IFs belong in a superfamily of proteins. The IF proteins were first classified by cell type, using biochemical, immunological and structural similarities, into five categories: acidic and basic keratins, desmin, vimentin, neurofilament (NF) proteins, and glial fibrillary acidic protein (GFAP) (Steinert et al., 1976; Lazarides, 1980; Pruss et al., 1981). It became clearer after the first IF protein cDNAs were sequenced (Hanukoglu and Fuchs, 1982, 1983; Marchuk et al., 1985), that the keratins also could be divided into two classes of type I (acidic) and type II (basic) keratins. Additional sequence studies revealed more sequence homologies and led to vimentin (54 kDa), desmin (53 kDa), GFAP (50 kDa) and peripherin (56 kDa) being grouped as type III IF proteins. These type III proteins share greater than 70% overall sequence identity among themselves, but only 25-30% and 35-40% identities with type I and type II keratins, respectively (Geisler and Weber, 1982; Quax-Jeuk en et al., 1983; Thompson and Ziff, 1989). The neurofilament proteins (NF-L, 62 kDa; NF-M, 102 kDa; NF-H, 112 kDa) and α-internexin (56 kDa) have been grouped separately as sequence type IV (Fuchs and Weber, 1994). Electron microscopy studies revealed a meshwork of 10 nm diameter filaments just underlying the nuclear envelope (Aebi et al., 1986), and sequence analysis of these proteins yielded the class of nuclear lamins (McKeon et al., 1986; Fisher et al.,
that now constitutes the sequence type V class of IF proteins. A sequence type VI class has been proposed to accommodate nestin (Lendahl et al., 1990), and I propose herein in Chapter III that tanabin and paranemin (also EAP-300 and IFAPa-400 that may be identical to paranemin) also be grouped into the type VI class with nestin.

Every IF protein has a central conserved domain, that is principally α-helical and forms a coiled-coil with a similar polypeptide. This α-helical ‘rod’ domain provides the building block of the 10 nm filament (Robson, 1989; Fuchs and Weber, 1994). The rod domain is divided up into four blocks of successive helical structures denoted by 1A, 1B, 2A and 2B, with short linker domains, whose sequences suggest they are nonhelical, denoted by L1, L12, and L2 (Steinert and Parry, 1985). The sizes and positions of the four helical domains of the rod and of the linker regions are well conserved throughout different IF protein classes, except for a small number of well-documented exceptions in the lamins, peripherin and filensin. In contrast to the rod domains, the lengths and sequences of the N-terminal head domains and the C-terminal tail domains differ considerably (Quinlan et al., 1994). All IF proteins share considerable sequence identity at the extreme N-terminus and C-terminus of the rod domain. A consensus sequence [(I,V) X (T,A,C,I) Y (R,K,H) X (L,M) L (D,E)], where X can be any amino acid, has been identified at the C-terminus of the rod (Steinert and Roop, 1988; Stewart, 1990; Fliegner et al., 1990; Franke, 1987; Lendahl et al., 1990; and Dodemont et al., 1990).

Purified cytoplasmic IFs can assemble in vitro without binding proteins, which suggests the information needed for assembly of all IFs to first form in-register and parallel dimers.
(Quinlan et al., 1986; Shoeman and Traub, 1993) is present within the α-helical coiled-coil rod domain. Next, tetramers form when dimers bind in an anti-parallel and staggered fashion (Quinlan, 1994). The following steps remain unclear, but tetramers (protofilaments) presumably join at each end to form longer protofilaments (Stromer et al., 1981; Ip et al., 1985a, 1985b), and two protofilaments align side by side to form a protofibril. Four protofibrils align side by side and, in turn, make up the 10 nm diameter filament (Fuchs and Weber, 1994; Heins and Aebi, 1994).

Keratins are the largest and most complex group of IF proteins with at least 31 keratins ranging in size from 40-68 kDa, which are composed of 17 acidic type I keratins (pI = 4-6) and 14 basic type II keratins (pI = 6-8) found in epithelial cells and hair (Quinlan et al., 1994). Pairs of type I and type II keratins are expressed differentially in most epithelial cells at different stages of development (Fuchs and Green, 1980; Wu et al., 1982). Keratins assemble in vitro as obligatory heteropolymers (Steinert et al., 1976) in a 1:1 ratio containing one type I and one type II keratin (Hatzfield and Frank, 1985; Hatzfield et al., 1985). Filaments generated from different keratins have distinct physical properties, suggesting the expression of specific heteropolymers in vivo may be important for tissue-specific structural requirements of tensile strength, flexibility and dynamics (Fuchs and Weber, 1994). A K5 (type I)/K14 (type II) heterodimer is the major keratin pair broadly expressed in stratified squamous epithelia (Nelson and Sun, 1983), whereas the K8 (type I)/K18 (type II) heterodimer is the major keratin pair produced by nearly all simple epithelia (Wu et al., 1982).
A new cytoplasmic IF protein found in lens tissue that is most similar to the type I keratins, is phakinin (47 kDa), and contains the ~310 amino acid rod domain but no tail (Merdes et al., 1993; Klymkowski, 1995). Another new lens-specific, cytoplasmic IF protein, filensin (100 kDa), acts like a peripheral membrane protein (Brunkener and Georgatos, 1992). Sequence analysis revealed that filensin lacks 29 amino acids in the rod domain and, on its own, cannot form IFs in vitro; however, filensin and phakinin copolymerize at a 1:3 ratio to form normal looking IFs in vitro (Gounari et al., 1993). Thus, two IF proteins coassemble de novo in cultured cells and form a novel type of beaded IF (Goulielmos et al., 1996). Filensin shares the greatest similarities with nestin, type III and type IV IFs (Fuchs and Weber, 1994). Filensin and phakinin may be candidates for a separate class of IFs, specific for the eye lens (Quinlan et al., 1994).

Type III IF proteins were the first to be studied by immunofluorescence in tissue culture, mostly in fibroblasts or myoblasts (Quinlan et al., 1994). All type III IF proteins can form homopolymeric IFs. They can also heteropolymerize in vitro and in vivo with other type III proteins (Steinert et al., 1981; Quinlan and Franke, 1982) or with the neurofilament protein NF-L (Monteiro and Cleveland, 1989) into IFs. Type III IF proteins cannot, however, coassemble with keratins into IFs (Osborn et al., 1980). These IFs are frequently coexpressed, especially at early stages of differentiation; for example, vimentin and desmin are coexpressed in embryonic chick skeletal muscle cells (Gard and Lazarides, 1980). Vimentin is the most widely expressed IF protein and is produced by mesenchymal cell types and by a variety of transformed cell lines and tumor cells (Osborn, 1983).
addition, vimentin is expressed in many types of endothelial cells and haematopoetic cells, and is the major IF protein in lens cells (Quinlan et al., 1994).

Desmin is more restricted in its expression and is found in smooth muscle associated with both cytoplasmic and membrane-associated dense bodies, and in cardiac and skeletal muscle cells primarily around the periphery of the myofibrillar Z-lines and between Z-lines of peripheral myofibrils and the sarcolemma (for a review see Stromer, 1995, and references therein). Desmin is one of the earliest tissue-specific structural proteins to be expressed after muscle differentiation has been triggered (Quinlan et al., 1994). GFAP is expressed in glial cells and astrocytes associated with the nervous system. GFAP has also been found in interstitial cells of the pituitary and Schwann cells of the peripheral nervous system (Quinlan et al., 1994). Peripherin is a more recently described type III IF protein and got its name because it is expressed by peripheral neurons of the dorsal root ganglia, sympathetic ganglia, cranial nerves, and ventral motor neurons (Portier et al., 1983).

A number of recently identified IF proteins have been reported. Synemin was recently discovered to be an IF protein rather than an intermediate filament-associated protein (IFAP) because it contains the ~310 amino acid rod domain characteristic of cytoplasmic IFs (Becker et al., 1995). Synemin (230 kDa by SDS-PAGE) copurifies with desmin and vimentin (Granger and Lazarides, 1980; Sandoval et al., 1983) and colocalizes with desmin at the periphery of myofibrillar Z-lines (Granger and Lazarides, 1980; Price and Lazarides, 1983). It appears from the consensus IF sequence pattern of synemin that it most closely resembles type III IF proteins. Xenopus neuronal intermediate filament
(XNIF) is a neuronal IF protein (Chamas et al., 1992). Gelfiltin and plasticin are recently described neuronal cytoplasmic IF proteins found in the optic nerve of the goldfish (Glasgow et al., 1992, 1994a, 1994b). Plasticin has been classified as a type III IF protein, but it is still unknown whether XNIF and gelfiltin are members of the neuronal type IV or vimentin-like type III IF protein groups (Klymkowsky, 1995).

Neurofilaments and microtubules form the major cytoplasmic structural units of the neuronal cell body. Type IV neurofilament proteins are coexpressed and form in axons and dendrites (Quinlan et al., 1994). The neurofilaments are composed primarily of three proteins, NF-L (light), NF-M (medium), and NF-H (heavy) that have predicted sizes, based upon sequence, of 62, 102 and 112 kDa, respectively. They differ significantly in the lengths of their glutamic acid and lysine-rich carboxy terminal tails (Geisler et al., 1982; Lewis and Cowen, 1985; Levy et al., 1987; Myers et al., 1987; Lees et al., 1988; Fuchs and Weber, 1994). α-Internexin (66-70 kDa), is an additional type IV protein also expressed in neurons where it seems to play a more prominent role in embryonic development than the NF triplet proteins (Pachter and Liem, 1985; Napolitano et al., 1985). α-Internexin is able to form homopolymers of 10 nm filaments in vitro (Ching and Liem, 1993). The NFs are obligate heteropolymers in vivo (Heins et al., 1993; M.K. Lee et al., 1993).

Type V IF proteins, universally expressed in higher eukaryotes, compose the nuclear lamina, which is a fibrous network on the inner surface of the nuclear membrane. This structure seems to provide a framework for the nucleus and to help organize chromatin
(Fuchs and Weber, 1994). In a few cell types, the lamina appears as an orthogonally arranged net of IFs that seem to interconnect nuclear pore complexes (Aebi et al., 1986). The type V proteins are the lamins B1-B2 (63-68 kDa), lamin A (70 kDa) and lamin C (60 kDa) (Fuchs and Weber, 1994). Early vertebrate embryos possess only lamins B1-B2 (Vorburger et al., 1989; Peter et al., 1989), while somatic cells synthesize lamins A and the truncated mammalian lamin C, in addition to B-type lamins (McKeon et al., 1986; Fisher et al., 1986; Peter et al., 1989; Stick, 1992). Expression of A-type lamins often occurs at the same time with major changes in tissue differentiation (Rober et al., 1989; Lehner et al., 1987). All lamins possess sequences that signal their dispatch to the nucleus (Loewinger an McKeon, 1988). Once they get to the nucleus, the assembly of lamin IFs seems to be influenced by the chromatin (Glass and Gerace, 1990; Yuan et al., 1991).

Nestin is an IF protein that does not clearly classify as one of the five IF classes previously described. It was proposed that nestin be classified into a new type VI class of IF proteins (Lendahl et al., 1990; Dahlstrand et al., 1992; Quinlan et al., 1994). Nestin was originally identified by the monoclonal antibody 'Rat.401' (Hockfield and McKay, 1985), and was shown to be expressed predominantly in central nervous system stem cells of the neural tube (Frederiksen and McKay, 1988). Nestin, however, is not limited to neural tissue cells. It is also expressed in myogenic cells during both skeletal (Lendahl et al., 1990; Sejersen and Lendahl, 1993; Zimmerman et al., 1994; Sjöberg et al., 1994; Kachinsky et al., 1994) and cardiac myogenesis (Kachinsky et al., 1995). The human
nestin cDNA sequence predicts a 177 kDa protein with a large nonhelical carboxy tail that resembles the type IV NF proteins (Lendahl et al., 1990).

Tanabin is a protein that was first described as being concentrated in the growth cones of Xenopus neurons (Hemmati-Brivanlou et al., 1992). Tanabin contains the conserved IF rod domain and shares 45% identity with the rod domain of nestin (Hemmati-Brivanlou et al., 1992). This close relationship between tanabin and nestin suggests they both should be classified as type VI IF proteins. It is unlikely, however, that tanabin is the Xenopus version of nestin, since the long C-terminal tails, although similar in length, are quite different based on degree of sequence homology. Tanabin has a predicted molecular weight (200 kDa) and isoelectric point (4.05), which are similar to nestin’s 177 kDa and 4.24, respectively (as I calculated from the human nestin sequence in Dahlstrand et al., 1992, and the frog tanabin sequence in Hemmati-Brivanlou et al., 1992). Based upon these similarities and a high degree of rod domain sequence homology, tanabin should be considered a nestin-like cytoplasmic class VI IF protein.

Paranemin was first described as a high molecular weight protein (a closely spaced doublet at ~280 kDa by SDS-PAGE) in embryonic chick skeletal muscle that copurified with vimentin and desmin (Breckler and Lazarides, 1982). Immunofluorescence localization detected paranemin in all developing muscle cells, regardless of their type, simultaneously with desmin, vimentin, and synemin (Price and Lazarides, 1983). Paranemin, desmin, vimentin, and synemin all have the same spatial distribution in both early myotubes, where they are associated with cytoplasmic filaments, and in late
myotubes, where they are associated with myofibril Z-lines. In adult chicken, paranemin is expressed in cardiac muscle at the Z-lines, conducting (Purkinje) fibers, smooth muscle cells in the aorta, capillary endothelial cells in skeletal and smooth muscle, Schwann cells in peripheral nerves of skeletal muscle, and in a subpopulation of fibroblasts (Breckler and Lazarides, 1982; Price and Lazarides, 1983). However, paranemin was reportedly removed during differentiation of both fast and slow skeletal muscle and smooth muscle. As we show herein in chapters I and II, a small amount of paranemin is present at the Z-lines and intercalated disks of adult chicken cardiac muscle, at the Z-lines of adult chicken skeletal muscle and at the Z-lines of adult porcine cardiac and skeletal muscle.

I also show herein (Chapter III) that paranemin contains the conserved ~310 amino acid IF rod domain, which is 63.3% identical to amino acid sequence in the rod domain of tanabin and 45.5% identical to the rod domain of nestin. Thus, I have discovered that paranemin is an IF protein rather than an IFAP. Paranemin is similar in size to tanabin and nestin, with a predicted molecular mass of 178 kDa, a short amino terminal head domain, and a very long C-terminal tail. Paranemin's predicted isoelectric point (4.17) is also very similar to those of tanabin and nestin. It is unlikely that paranemin is the chicken homologue of nestin, because the long tail domains only share 21.8% identity. I propose in Chapter III that, because of the high degree of rod domain sequence homology and biochemical similarities between paranemin, nestin, and tanabin, they should all be classified as type VI IF proteins.
During the studies described in Chapter III, other interesting sequence homologies were discovered by computer searches via the BLASTx program of the BLAST e-mail server (Altschul et al., 1990) operated by the National Center for Biotechnology Information. Nearly identical sequence homology of parts of my paranemin sequence were found with partial sequences of EAP-300 (Kelly et al., 1995) and IFAPa-400 (Simard et al., 1992). Thus, these two proteins are probably identical to paranemin.

IFAPa-400, avian intermediate filament-associated protein, was first described as a high molecular weight protein (>400 kDa by SDS-PAGE) in myogenic and neurogenic structures in the chick embryo by using monoclonal antibody 51H2 (Vincent and LaHaie, 1988). Later, it was reported that IFAPa-400 was expressed in chick embryonic cells derived from the neuroectoderm (Chabot and Vincent, 1991), and persistently expressed in smooth muscle cells of elastic arteries and in Purkinje fibers (Vincent et al., 1991).

EAP-300, embryonic avian polypeptide (300 kDa), was first described by using a monoclonal antibody (A2B11) that immunolabeled ganglion cells in the embryonic chick neural retina, and bound to a 260 kDa protein (Cole et al., 1986). Later studies showed that EAP-300 is a transiently expressed protein in the developing chick nervous system, where it was found associated with barrier structures together with claustrin (320 kDa), an extracellular matrix heparin sulfate proteoglycan (McCabe et al., 1992; McCabe and Cole, 1992; Burg and Cole, 1994). More recently, EAP-300 was reported to be developmentally regulated in the myocardium and the cardiac neural crest during chick embryogenesis (McCabe et al., 1995). Because I have shown by sequence analysis that paranemin, EAP-
300 and IFAPα-400 are most likely identical proteins, paranemin may fulfill some or all of the functions previously ascribed to these two proteins (Chapter III herein). As a result, paranemin is a novel IF family protein rather than an IFAP, and may have an important role in modulating IF function in developing central and peripheral nervous systems, and in developing and mature cardiac and skeletal muscle cells.

**Intermediate filament-associated proteins (IFAPs)**

The designation as an IFAP is given to proteins that possess one or more of the following properties: codistribute in cells with IFs, occur at IF anchorage sites, copurify with IFs in vitro, and bind to IFs or IF subunit proteins (Foisner and Wiche, 1991). Paranemin has been considered to be an intermediate filament-associated protein (IFAP) (Steinert and Roop, 1988; Robson, 1989; Foisner and Wiche, 1991) since its discovery (Breckler and Lazarides, 1982) and initial localization (Price and Lazarides, 1983). However, since it contains the rod domain characteristic of IF proteins, it should no longer be considered an IFAP (Chapter III herein).

Plectin, one of the more studied IFAPs, was initially identified as a prominent component of vimentin IF preparations (Wiche, 1989; Foisner and Wiche, 1991). It is broadly expressed in different cell types, binds to most, if not all, IF proteins, and localizes to the IF cytoskeleton as well as desmosomes and hemidesmosomes (reviewed in Fuchs and Weber, 1994). The complete sequence of plectin revealed it is a 466 kDa polypeptide chain, with a three domain structure based upon a centrally located α-helical
coiled-coil region (Wiche et al., 1991). Expression of plectin mutant cDNA in cultured cells indicates a role of the C-terminal domain in IF association (Wiche et al., 1993). It was recently reported that a mitosis-specific phosphorylation involving p34<sup>cdc2</sup> kinase may regulate plectin's crosslinking activities and associations with IFs during the cell cycle (Foisner et al., 1996). IFAP 300 is another high molecular weight IFAP that initially was characterized as a vimentin crosslinker in preparations of baby hamster kidney cells (Yang et al., 1985). This protein was subsequently shown to have several properties in common with plectin and, therefore, it was suggested that IFAP 300 and plectin were identical (Herman et al., 1987; Wiche, 1989). More recently, however, it has been reported that IFAP 300 differs from plectin in several respects, including some differences at the primary structure level (Skalli et al., 1994).

Desmoplakins I (240-285 kDa) and II (210-225 kDa) are two major related proteins located in the innermost portion of the desmosomal plaques, where they are thought to help attach IFs to the cell membrane (Green et al., 1990). Deletion mutagenic studies on desmoplakin suggest that IFs bind to the tail region of the desmoplakin molecules (Stappenbeck et al., 1993).

Filaggrins, produced by cleavage of a precursor profilaggrin molecule, are a family of low molecular weight, basic, histidine-rich proteins of mammalian epidermis that have been shown to laterally crosslink keratin filaments into macrobundles (Presland et al., 1992; Markova et al., 1993). Trichohyalin (240 kDa) is another crosslinking IFAP, and is
produced and retained in the cells of the hardened inner-root sheath and medulla of the hair follicle and granular layer of the epidermis (Fietz et al., 1993; S-C. Lee et al., 1993).

It seems likely that most of the IFAPs play roles in establishing and maintaining IF networks, and perhaps in governing the dynamic changes that occur in IF networks during cell differentiation and growth (Fuchs and Weber, 1994).

**Similarities of intermediate filament proteins to transcription factors**

Intermediate filament proteins possess slight similarities to transcription factors. Characteristics of transcription factors include their coiled-coil dimerization motif and a flanking DNA binding region (Jones, 1990; Lamb and McKnight, 1991). Two transcription factor molecules align, via formation of coiled-coils, because of the parallel-oriented, α-helical sequence elements, which in turn permits the recognition of symmetric DNA sequence motifs. These neighboring DNA-binding regions impart sequence specificity for these interactions (Traub and Shoeman, 1994). Some IF proteins have regions that resemble those of transcription factors of the bZIP family (Cohen and Curran, 1990; Kerppola and Curran, 1991) and bHLH family (Garrell and Campuzano, 1991; Kadesch, 1992) in that transcription factors all possess a coiled-coil dimerization interface which is flanked on the N-terminal side by a DNA binding site that is usually a cluster of basic amino acid residues (Traub and Shoeman, 1994). Members of the bZIP family include GCN4 (O'Shea et al., 1991; Ellenberger et al., 1992), members of the C/EBP subfamily (Landschulz et al., 1989; Williams et al., 1991), Fos-Jun (Turner and Tjian, 1989;
Pathak and Sigler, 1992) and ATF-CREB (Brindle and Montminy, 1992). Examples of the bHLH family include the myogenic factors myo D and myogenin (Tapscott and Weintraub, 1991; Edmondson and Olsen, 1993), and Myc (Blackwood and Eisenmann, 1991).

Interestingly, vimentin contains a leucine zipper motif, which is located in the N-terminal half of the α-helical core domain of vimentin and matches three to four out of five leucines of the zipper domains of members of the CREB and Fos-Jun protein groups (Traub and Shoeman, 1994). Paranemin’s putative leucine zipper-like motif is in its C-terminal tail, and has a repeat region of about 39 consecutive heptad repeats, the most common being LQEEHGD, TQEEHGD, LQVEHGD, and LQVEHED (Chapter III herein). However, when leucine is in position \( a \) of the heptad, glutamate is usually found in position \( d \); and if leucine is in position \( d \), histidine is usually found in position \( a \), so this is not like a “normal” leucine zipper or the heptads usually associated with formation of α-helical coiled-coil structures. Even though there are not hydrophobic residues in the alternate hydrophobic heptad positions, perhaps paranemin may still form some type of coiled-coil structure and function like some other leucine zippers. Not all leucine zippers contain the ‘4-3’ hydrophobic repeat. For example, leucine zipper repeats in Myc and Fos contain charged residues at the alternate site (O’Shea et al, 1989). These leucine zippers may have a different conformation, or the hydrophobic contacts may be provided by side chain methylene groups, which is thought to be the case in some regions of myosin (McLachlan and Karn, 1983; O’Shea et al, 1989). It remains to be experimentally proven if the leucine zipper-like motif in paranemin is functional. Although highly speculative,
perhaps the IF proteins vimentin and paranemin somehow interact with transcription factors in the nucleus and bind DNA for transcription initiation and elongation (Traub and Shoeman, 1994; Chapter III herein).

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PARANEMIN IS A GLYCOPROTEIN LOCALIZED AT Z-LINES OF 
ADULT CARDIAC AND SKELETAL MUSCLE

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Summary: Paranemin is a poorly characterized ~280 kilodalton protein previously identified in embryonic chick skeletal muscle, but not purified [Breckler, J., and Lazarides, E. (1982) J. Cell Biol. 92, 795-806]. We have purified paranemin from the same tissue source. Purified paranemin stained an intense blue with the cationic carbocyanine dye 'Stains-all', indicating it is a phosphoprotein and/or a glycoprotein. Periodic acid treatment of paranemin, followed by biotinylation of oxidized carbohydrate and streptavidin-alkaline phosphatase detection, demonstrated that paranemin is a glycoprotein. A monoclonal antibody (4D3) to paranemin was produced and labeled at the Z-lines of adult chicken and porcine cardiac and skeletal muscle myofibrils.

Introduction

Intermediate filaments (IFs) comprise one of the three major cytoskeletal filament networks present in most eukaryotic cells (1-3). Although much is known about the cellular distribution and the protein structure of members of the IF protein super-family (1-8), our understanding of some of their biological functions is only recently becoming apparent (8,9). A significant number of proteins, often present in very small quantities, copurify with major IF proteins in vitro and colocalize with the major IF protein(s) composing the
IFs of a given cell type (1,2,10). It has been suggested that these IF-associated proteins (IFAPs) may play a significant role as regulators or modifiers of IF function and in the integration of IFs into the cell cytoskeleton (1,10).

The protein paranemin was discovered and described by Breckler and Lazarides (11) as an ~280 kDa IFAP associated with the desmin and vimentin IFs in avian embryonic skeletal muscle. Paranemin was identified in all types of myogenic chick cells, and in several adult avian tissues including smooth muscle cells of the aorta, and cardiac, but not skeletal, striated muscle cells (12). Because of our overall interest in understanding proteins such as synemin (13) and paranemin that might link IFs and myofibrillar Z-line-type structures (14-16), we have examined specific properties of purified paranemin. We show that paranemin is a glycoprotein and is located at the myofibrillar Z-lines of mature avian and porcine cardiac and skeletal muscle cells.

**Materials and Methods**

**Protein purification.** A crude high-speed supernatant of a homogenate of whole 14 day-old embryonic (E14) chick skeletal muscle was prepared as described (11), and used to purify paranemin by sequential column chromatography on hydroxyapatite (HA Ultragel, Sepracor) and DEAE-cellulose (DE-52, Whatman) columns. Protein fractions were analyzed by SDS-PAGE (5% stacking gel over 8% separating gel) (17). Protein concentrations were determined by the method of Bradford (BioRad) (18). Full details of the preparation will be presented elsewhere.
Western blotting. SDS-polyacrylamide gels were transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Cell (BioRad). After blocking, the blots were probed for 2 hr at 23°C with a monoclonal antibody (4D3 culture supernatant) specific for paranemin. The secondary antibody, goat anti-mouse IgG-horseradish peroxidase (No. A2554, Sigma), diluted 1:5,000 in PBS-Tween, was incubated for 30 min and labeling was visualized by using a chemiluminescence method as described by the supplier (RPN 2108, Amersham).

Amino acid analysis. Chromatographically-purified paranemin was subjected to SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes (Micron Separations) (19), and extensively washed with several changes of deionized water for 24 hr. The 280 kDa paranemin band was excised and hydrolyzed in vacuo in 6 N HCl at 150 °C for 1, 2, and 3 hr (20). Amino acid composition was determined by an Applied Biosystems Amino Acid Analyzer, using a phenylisothiocyanate based system, at the Iowa State University Protein Facility.

Stains-all staining. Purified paranemin and samples of the IF proteins desmin (21) and synemin (13) from adult avian smooth muscle were run on SDS-PAGE and stained with the cationic carbocyanine dye, Stains-all (Eastman Kodak), to determine if paranemin was phosphorylated and/or glycosylated (22-25). The procedure outlined by Cutting (25) was followed for staining. The Stains-all stained gel was photographed using Kodak Technical Pan 2415 film, both without and with a Nikon R60 red filter. Stains-all was then washed out of the same gel with water, restained with Coomassie blue, and photographed again.
Carbohydrate detection. SDS-polyacrylamide gels were transferred to nitrocellulose and positive detection of carbohydrate moieties by membrane blotting, as outlined by Bayer et al. (26), was done as described by the supplier's directions (GlycoTrack, Oxford GlycoSystems), in which carbohydrate was oxidized with periodic acid, biotinylated with biotin-hydrazide solution, labeled with streptavidin-alkaline phosphatase, and visualized with nitroblue tetrazolium/5-bromo, 4-chloro, 3-indoyl phosphate substrate.

Indirect immunofluorescence microscopy. Myofibrils from adult chicken heart and thigh, and pig heart and mixed neck muscles were prepared as described by Goll et al. (27). A monoclonal antibody (4D3) was developed against paranemin essentially as described by Hemken et al. (28). Myofibrils were labeled with 4D3 (undiluted culture supernatant), followed by labeling with goat anti-mouse IgG-FITC (No. F4018, Sigma). Labeled myofibrils were examined with a Zeiss Photomicroscope III equipped with epifluorescence optics, and photographed with a 40x planapochromat objective using Kodak Technical Pan 2415 film.

Results

A preparation of purified paranemin from E14 chick skeletal muscle is shown in Figure 1. The homogenate of whole embryonic muscle, which contains a very small amount of paranemin (11), is shown in lane 2. The crude high speed supernatant (lane 3), which was prepared according to (11) and slightly enriched in paranemin, was used to prepare the purified paranemin used herein (lane 4). A Western blot of a duplicate gel of the purified
paranemin, probed with monoclonal antibody 4D3, is shown in Figure 1, lane 5. Only the 
~280 kDa paranemin band is labeled in crude muscle extracts (results not shown).

Results of the amino acid analysis of purified paranemin, which has not been 
previously reported, are shown in Table 1. The mole percent of Ile is low compared to that 
present in many proteins (29,30). The mole percents of Glx and Asx are very high, in 
comparison to those of His, Arg, and Lys, and are consistent with the acidic pI of 4.1-4.5 
obtained by two-dimensional electrophoretic analysis of paranemin in crude samples of 
embryonic chick skeletal muscle (11).

Stains-all staining of purified paranemin, and samples of purified synemin and desmin, 
are shown in Figure 2. The identical gel is shown in all panels. Paranemin stained 
intensely dark-blue, indicating that paranemin is either a phosphoprotein or a glycoprotein 
(22-25), while synemin and desmin stained pink. The identical gel was stained with Stains-
all and photographed without (panel A) and with a red filter (panel B). Because only 
paranemin stained dark-blue, it is seen in panel B; however, synemin and desmin are not 
seen because they stained pink. After the Stains-all photographs were taken, the Stains-all 
was washed out of the gel. It was then stained with Coomassie blue and photographed 
(panel C). The intensity of paranemin's staining with Stains-all (see Fig. 2A and 2B, lane 
1) is especially notable considering the relatively small amount of paranemin on the gel 
(Fig. 2C, lane 1).

Preliminary studies using wheat germ agglutinin and RCA120 lectin (Boehringer 
Mannheim) blotting experiments (31) suggested that paranemin may be a glycoprotein
Paranemin was identified as a glycoprotein by using a glycoprotein detection kit from Oxford GlycoSystems (Figure 3). Purified paranemin in lane A is positive for carbohydrate, whereas samples of purified desmin (lane B) and actin (lane C) from avian smooth muscle, serve as negative controls. The glycoprotein ovalbumin (lane D) serves as a positive control. A duplicate gel, but stained with Coomassie blue, is seen in lanes E-H.

The immunolocalization of paranemin in adult chicken and porcine cardiac and skeletal muscle myofibrils is shown in Figure 4. Monoclonal antibody, 4D3, to paranemin specifically labels the Z-lines (Z) in both cardiac and skeletal myofibrils. Corresponding phase contrast (a, c, e, g) and fluorescent (b, d, f, h) micrographs of avian cardiac (a, b) and skeletal (c, d), and porcine cardiac (e, f) and skeletal (g, h) muscle myofibrils are shown. Frozen sections of adult chicken and porcine cardiac and skeletal muscle also were labeled at the Z-lines with monoclonal antibody 4D3 (results not shown).

Discussion

Breckler and Lazarides (11) demonstrated that paranemin copurified with the IF proteins vimentin and desmin, but did not separate paranemin from those two IF proteins or other contaminants. We prepared a crude high-speed supernatant from a homogenate of whole muscle from E14 chick skeletal muscle using their procedure as outlined (11). Further chromatography steps were used to prepare purified samples of paranemin herein for the production of monoclonal antibodies and characterization studies. We prepared a
monoclonal antibody (4D3) to paranemin to verify the identity of paranemin throughout purification by Western blotting and to localize paranemin by indirect immunofluorescence.

A striking property of paranemin was its intense staining by Stains-all, which indicated that paranemin was either phosphorylated or glycosylated (22-25). Treatment with alkaline phosphatase (No. 4252, Sigma) and lambda protein phosphatase (New England Biolabs), however, did not alter the mobility or the intensity of Stains-all staining of the paranemin band. Both enzymes have activities toward serine, threonine, and tyrosine residues (32,33) and removed phosphate from casein (No. 12840, United States Biochemical) (results not shown). We demonstrated, by using a sensitive carbohydrate detection method, that paranemin was a glycoprotein. Cloning and sequencing studies done in our laboratory have revealed a predicted glycosylation site in paranemin (results not shown). The specific carbohydrate bound to paranemin and its function are unknown.

Price and Lazarides (12) observed localization of paranemin at the Z-lines of adult myocardium, but no labeling of paranemin in adult skeletal muscle cells. We observed immunolabeling of paranemin, by monoclonal antibody 4D3, at Z-lines in both cardiac and skeletal muscle myofibrils from adult avian and porcine animals. The results presented here, together with previous results from embryonic and adult cardiac tissue (11,12), suggest that paranemin may have important cytoskeletal roles in assembly of myofibrils in developing muscle and at the Z-line structures in both mature cardiac and skeletal muscle cells.
Acknowledgments

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References


Table I  
Amino Acid Analysis of Paranemin

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<td>Arg</td>
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<td>Ile</td>
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<td>Leu</td>
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<td>Phe</td>
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Amino acid results are expressed in mole percent with standard errors (n=2) determined (34) on two different preparations with six measurements from each preparation. N.D. = Not determined.
Figure 1. Purification of paranemin from 14 day-old embryonic (E14) chick skeletal muscle. Adult chicken cardiac myofibril protein markers (lane 1) indicated in kilodaltons are myosin heavy chain (205), α-actinin (100), actin (42), and tropomyosin (33). Lane 2, whole muscle homogenate from E14 chick skeletal muscle; lane 3, crude high speed supernatant before loading onto gel filtration column; lane 4, purified paranemin from DEAE-cellulose column; lane 5, Western blot of purified paranemin using monoclonal antibody 4D3. S=stacking gel, P=paranemin.
Figure 2. "Stains-all" staining of paranemin, synemin, and desmin. Purified samples of paranemin (lane 1), synemin (lane 2), and desmin (lane 3) are shown in panels A, B, and C. Results after staining with Stains-all, and photographed with no filter are shown in panel A. Results of same gel as shown in A, but photographed with a Nikon R60 red filter, are shown in panel B. Results of the same gel, but after removal of Stains-all and subsequent staining with Coomassie blue, are shown in panel C. 280=paranemin, 230=synemin, 53=desmin.
Figure 3. Carbohydrate detection in purified paranemin. Positions of biotinylated standards in kilodaltons are shown at the left and include: phosphorylase B (97), catalase (58), and alcohol dehydrogenase (40). Lanes A-D, carbohydrate detection; lanes E-H, corresponding gel to that shown in A-D, but stained with Coomassie blue. Purified paranemin (lanes A, E), purified desmin (lanes B, F), purified actin (lanes C, G), and purified ovalbumin (lanes D, H) are shown.
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Figure 4. Immunofluorescence localization of paranemin in adult chicken and porcine cardiac and skeletal muscle myofibrils. Paranemin monoclonal antibody 4D3 specifically labels Z-lines (Z) in both cardiac and skeletal muscle myofibrils. Corresponding phase contrast (a, c, e, g) and fluorescent (b, d, f, h) micrographs of avian cardiac (a, b) and skeletal (c, d), and porcine cardiac (e, f) and skeletal (g, h) muscle myofibrils are shown. Bar=10 μm.
A method has been developed for the preparation of purified paranemin from embryonic (chick) skeletal muscle. A crude paranemin-containing fraction, primarily containing paranemin and the intermediate filament (IF) proteins desmin and vimentin, was prepared by a modification of a previously outlined procedure [Breckler and Lazarides (1982) J. Cell Biol. 92, 795-806]. Embryonic chick skeletal muscle was homogenized in a buffer containing 130 mM KCl, pH 7.5, at 4°C, and the homogenate was centrifuged for 90 min at 145,000 g. The crude high speed supernatant, which contained filamentous components, was used for successive column chromatographic steps, first on gel filtration in a solution containing 100 mM NaCl, pH 7.5, and then on hydroxyapatite and DEAE-cellulose in 6 M urea-containing solutions, pH 7.5, at 4°C. The pooled fractions of purified paranemin contained routinely more than 95% 280,000-Dalton protein with no detectable contamination by vimentin or desmin. The present study demonstrates that the very small amount of paranemin in embryonic skeletal muscle can be isolated in sufficient quantity and purity to permit studies on its properties and functions. One- and two-dimensional Western blots were used to identify paranemin throughout the purification procedure. Double-label confocal immunofluorescence showed colocalization of paranemin
with desmin at the myofibrillar Z-lines of adult cardiac and skeletal muscle cells and at cardiac intercalated disks.

**Introduction**

The term "intermediate-sized" filament was first used by Ishikawa *et al.* (1968) for describing a newly observed system of filaments, approximately 10 (8-12) nm in diameter, between myofibrils in myogenic skeletal muscle cells grown in culture. It is now known that almost all vertebrate cells contain, in addition to microtubules and microfilaments, this less-characterized cytoplasmic filament system (Steinert and Roop, 1988; Robson, 1989; Fuchs and Weber, 1994; Klymkowsky, 1995). Tissue specificity was the primary basis used to initially classify IFs into five classes (Lazarides, 1980, 1982), but IFs subsequently have been classified into as many as six classes or types based on sequence homology (Steinert and Roop, 1988; Fuchs and Weber, 1994; Traub and Shoeman, 1994; Klymkowski, 1995). Previous studies identified paranemin as an IF-associated protein (IFAP) (Breckler and Lazarides, 1982; Price and Lazarides, 1983) because it copurified with large amounts of vimentin and desmin in the initial steps of preparation of a crude paranemin-containing fraction and colocalized with these two known IF proteins in all myogenic cells and adult cardiac muscle cells (Price and Lazarides, 1983).

Our laboratory has recently shown that paranemin is a glycoprotein (Hemken *et al.*, 1996a) that possesses the ~310 amino acid rod domain characteristic of all cytoplasmic IFs (Hemken *et al.*, 1996b). Thus, paranemin is a member of the IF protein family rather than an IFAP. The latter study also reported nearly identical sequence homology of portions of
paranemin with the reported partial sequences of EAP-300 (Kelly, et al., 1995) and IFAPa-400 (Simard et al., 1992). Significant sequence homology within the paranemin rod domain to the rod domains of the putative class VI IF proteins, frog tanabin (Hemmati-Brivanlou et al., 1992) and human nestin (Lendahl et al., 1990) also was found (Hemken et al., 1996b).

Many of the properties of paranemin remain unclear. To help answer this question, and as part of our continuing studies on the structural composition and properties of the myofibrillar Z-line and Z-line-associated structures (Robson et al., 1970; Robson and Zeece, 1973; Dayton et al., 1976; Yamaguchi et al., 1978, 1982, 1985, 1988; Zeece et al., 1979; Huiatt et al., 1980; Goll et al., 1991; Chou et al., 1994; Becker et al., 1995), it was necessary to develop a method to purify paranemin. Here we describe the purification of the developmentally-regulated skeletal muscle protein paranemin from embryonic skeletal muscle, verify its identity by one- and two-dimensional SDS/PAGE and corresponding Western blot analysis, and show colocalization of paranemin with desmin at the Z-lines of isolated adult avian cardiac and skeletal muscle myofibrils and intact frozen tissue sections. Paranemin also colocalizes with desmin in LR White cross-sections of 14 day-old embryonic chick skeletal muscle.
Materials and Methods

Source of muscle tissue and preparation of crude paranemin extract

The following steps were all done at 0-4 °C in the cold room or on ice with precooled solutions prepared with double-deionized distilled water. The adjustment of the buffer pH was done at the temperature at which it was to be used. Fourteen day-old chick embryos were obtained from the Hy-Vac Corporation, Gowrie, IA. Approximately 100 g (wet weight) of thigh and breast muscles were removed from 12 dozen eggs, and homogenized in a Waring blender by three 10 s bursts, then in a Teflon homogenizer three times, 5 min each, in 200 ml of a homogenization buffer containing 130 mM KCl/5 mM EDTA/20 mM Tris-HCl, pH 7.5 (Breckler and Lazarides, 1982). The resulting homogenate was centrifuged for 15 min at 31,000 g. The supernatant was filtered through glass wool and then centrifuged for 90 min at 145,000 g. The supernatant (~10 mg/ml) was divided into four 50-ml aliquots for gel filtration chromatography.

Column chromatography

Gel filtration chromatography was done with Biogel A5-m (BioRad) in buffer containing 100 mM NaCl/0.1 mM EDTA/20 mM Tris-HCl, pH 7.5 (Breckler and Lazarides, 1982) in a 2.6 cm x 110 cm column equilibrated with 3 column vol. of the same solution. The column was eluted at a flow rate of 24 ml/h, and 6.5 ml fractions were collected in tubes already containing one drop of 50 mM TAME. Constant volumes were loaded onto gels from every second fraction and analyzed by SDS/PAGE and Western blotting. Paranemin-enriched fractions near the column void volume were collected,
dialyzed against 6 M urea/1 mM DTT/0.2 mM PMSF/10 mM Tris-HCl, pH 7.5, and subjected to hydroxyapatite chromatography with HA-Ultragel (Sepracor) by procedures adapted from Huiatt et al. (1980) and O'Shea et al. (1981) used for desmin and from Sandoval et al. (1983) used for avian smooth muscle synemin.

The hydroxyapatite was suspended in 6 M urea/1 mM DTT/0.2 mM PMSF/10 mM Tris-HCl, pH 7.5, packed into a 2.6 cm x 33 cm column, and equilibrated with 3 column vol. of the same buffer. The protein sample (130 ml of pooled gel filtration void volume fractions 26-30, Fig. 1; 0.47 mg/ml) was applied to the column and eluted at a flow rate of 24 ml/h, with a linear 0-250 mM potassium phosphate gradient, programmed on a Gradifrac System (Pharmacia Biotech.), in the presence of 6 M urea/1 mM DTT/0.2 mM PMSF/10 mM Tris-HCl, pH 7.5. Three-ml fractions were collected and one drop of 50 mM TAME was then added to each tube. Constant volumes of every other fraction were loaded onto gels and analyzed by SDS/PAGE and Western blots.

Ion-exchange chromatography was done with DEAE-cellulose (Whatman, DE-52). The column material was suspended in 6 M urea/1 mM DTT/5 mM EDTA/0.2 mM PMSF/20 mM Tris-HCl, pH 7.5, packed into a 2.6 cm x 20 cm column, and equilibrated with 3 column vol. of the same solution. The hydroxyapatite-partially-purified paranemin (pooled column fractions 64-78, Fig. 2; 45 mls at 0.2 mg/ml), was dialyzed against the equilibration buffer and applied to the column. Elution was done at a flow rate of 24 ml/h, with a linear 0-2 M NaCl gradient in the presence of 6 M urea/1 mM DTT/5 mM EDTA/0.2 mM PMSF/20 mM Tris-HCl, pH 7.5. Three-ml fractions were collected, and
constant volumes of every other fraction were analyzed by SDS/PAGE and Western blots, and pooled as appropriate. The sample of purified paranemin was dialyzed in the equilibration buffer and stored at -70°C for at least 4 weeks without detectable degradation.

**One- and two-dimensional electrophoresis**

One-dimensional electrophoresis was done with SDS/polyacrylamide slab gels in Mini-Protean II units (BioRad) at 20 mA per gel in the Tris/glycine buffer system described by Laemmli (1970). The concentrations of acrylamide (bisacrylamide/acrylamide, 1:37, w/w) were 5% in the stacking gel and 8% in the separating gel. Duplicate gels were run, with one stained with Coomassie blue, and the duplicate gel transferred to nitrocellulose, and probed for paranemin with mouse monoclonal antibody 4D3 to embryonic 14 d chick paranemin (Hemken et al., 1996a, 1996b).

The first dimension, isoelectric focusing (IEF), was done by using a modification of the O'Farrell (1975) technique and a Mini-Protean II 2-D Cell (BioRad) as suggested by the manufacturer. The first dimension tube gels (4% acrylamide total monomer, 9.2 M urea/2.0% Triton X-100/0.4% pH 3.5-9.5 ampholytes/1.6% pH 5-7 ampholytes/0.01% ammonium persulfate/0.1% TEMED) were poured into capillary tubes (7.5 cm x 1 mm diameter) and allowed to polymerize overnight. Samples were prepared by adding an equal volume of sample buffer (9.2 M urea/2.0% Triton X-100/0.4% pH 3.5-9.5 ampholytes/1.6% pH 5-7 ampholytes) and increasing the concentration of urea to 9.2 M by addition of urea crystals. The tube gels were prefocused for 15 min at 500 V, with 20 mM
NaOH in the upper chamber and 10 mM phosphoric acid in the lower chamber. The protein samples were applied, followed by 20 μl of overlay buffer (9 M urea/0.2% pH 3.5-9.5 ampholytes/0.8% pH 5-7 ampholytes/0.01% [w/v] Bromophenol blue), and focused for 4 hr at 750 V. The pH of duplicate first dimension tube gels was determined every 0.5 mm. The second dimension 8% SDS/PAGE, without a stacking gel, was done with the acidic end of the tube gel toward the left (see Fig. 5) as described above for one-dimensional electrophoresis. A duplicate gel run with each sample was transferred to nitrocellulose and probed for paranemin with monoclonal antibody 4D3.

Western blotting

One- and two-dimensional gels were transferred to nitrocellulose by the following procedure. One sheet of blotting paper (BioRad) soaked in transfer buffer (15% methanol/25 mM glycine/192 mM Tris-HCl, pH 8.3) was placed on the cathode side of a tank transfer system (Trans-Blot Cell, BioRad), followed by the nitrocellulose membrane, the gel, and one more sheet of blotting paper soaked in transfer buffer. Transfers were done at 60 V for 3 hr, followed by 90 V for 30 min. Immunoblotting was done as follows: the nitrocellulose membrane was stained with 0.1% napthol blue black (Amido Black) in destaining solution (45% ethanol/10% acetic acid) for 1 min, and destained to reveal the transferred polypeptide bands. The membrane was incubated for 30 min in 5% non-fat dry milk (blocking solution). Blots were then incubated with culture supernatant containing monoclonal antibody 4D3 (anti-paranemin) for 2 hr at 23°C, or overnight at 4°C, and washed three times for 3 min each with PBS containing 0.5% Tween 20, pH 7.5
(PBS/Tween). The blots were then incubated for 1 hr at 23°C with horseradish peroxidase-labeled goat anti-mouse IgG (No. A2554, Sigma) diluted 1:5000 in PBS/Tween, and washed three times for 15 min each with PBS/Tween. Antibody binding was visualized by chemiluminescence as described by the supplier (ECL, Amersham).

**Adult chicken cardiac and skeletal muscle myofibril preparation**

All steps were done in a cold room at 4°C using pre-chilled solutions. Chickens were sacrificed by cervical dislocation and decapitation. Immediately after exanguination, the heart papillary muscle and mixed thigh skeletal muscles were removed, cut into approximately 2-4 mm diameter strips and tied to wooden applicator sticks at slightly longer than rest length. Strips were soaked overnight at 4°C, with stirring in standard salt solution (SSS) consisting of 100 mM KCl/2 mM MgCl₂/1mM EGTA/1 mM sodium azide/20 mM potassium phosphate, pH 6.9 (Goll et al., 1977). The strips were finely minced with a scalpel, weighed, and homogenized in a Warring blender for 20 s in 10 vol of SSS. The myofibrils were collected by centrifugation at 1400 g for 10 min. The pellet was resuspended in 10 vol of SSS containing 0.5% Triton X-100 (Sigma), hand homogenized with 10 strokes of a teflon homogenizer, strained to remove connective tissue, and centrifuged at 1400 g for 10 min. This step was repeated twice with Triton X-100-free SSS. The pellet was resuspended in a minimum volume of SSS. An equal volume of glycerol was added, and the suspension was stored at -20°C.
Indirect immunofluorescence and confocal microscopy of adult chicken cardiac and skeletal muscle myofibrils and tissue sections

Monoclonal antibody 4D3 to paranemin was prepared essentially according to the procedures described in Hemken et al. (1992). Myofibrils were placed on 18 mm diameter #1 coverslips and washed three times with washing buffer (75 mM KCl/2 mM MgCl₂/2 mM EGTA/20 mM imidizole-HCl, pH 7.2) to remove unbound myofibrils. Eight-μm thick frozen sections, from intact adult chicken cardiac and skeletal muscle, were prepared using an IEC CTF Microtome-Cryostat. One-μm thick LR White sections, from 14 day-old embryonic chick skeletal muscle, were prepared using an LKB Ultrotome III. The myofibrils and tissue sections were labeled with mouse monoclonal anti-paranemin 4D3 (undiluted culture supernatant) and rabbit polyclonal anti-skeletal muscle desmin diluted 1:500 in blocking solution (20% horse serum, 1% polyethylene glycol [MW=20,000, Sigma] in washing buffer), incubated at 4°C overnight, and washed three times, 5 min each, with washing buffer. Monoclonal antibody 4D3, diluted 1:50 in blocking solution, was detected with FITC-labeled goat anti-mouse IgG (No. F4018, Sigma). The desmin rabbit antibodies, diluted 1:50 in blocking solution, were detected with TRITC-labeled goat anti-rabbit IgG (No. T6778, Sigma). Both secondary antibodies were incubated for 1 hr, and then washed three times, 15 min each, with washing buffer. The samples were mounted in FITC-Guard (Testog, Inc., Chicago, IL) and examined with a Zeiss Photomicroscope III equipped for epifluorescence. Myofibrils were photographed with a
40x planapochromat objective and Technical Pan 2415 film. Confocal microscopy was done with a Noran Odyssey confocal microscope (Madison, WI), equipped for epifluorescence and a 63x oil planapochromat objective, at the Iowa State University Confocal Microscope Facility.

Results

Chromatographic purification of paranemin

The main objective of this study was to devise a reproducible method for purifying paranemin from embryonic avian skeletal muscle cells. A high molecular weight protein (~280 kDa) was detected in the whole muscle homogenate (see Fig. 4A, lane 1) and in the crude high speed supernatant (Fig. 1, lane a), together with the intermediate filament proteins vimentin and desmin (Breckler and Lazarides, 1982) with molecular weights of 54 and 53 kDa, respectively, and many other proteins. A constant volume of fractions from each column was loaded onto each lane of the gels shown in order to compare the amount of paranemin in each of the respective fractions. Void volume fractions from the gel filtration column contained a complex of primarily paranemin, vimentin and desmin (Fig. 1, lane b). Although paranemin was present throughout the first peak, a much greater number and amount of contaminating proteins appear near the apparent molecular weight of paranemin (280 kDa) (Fig. 1, lane c) in these fractions, and were not used further. In subsequent fractions, only trace amounts of paranemin appear (Fig. 1, lane d) between the first and second peak. Fractions in the second peak contained only lower molecular weight
proteins (Fig. 1, lane e), and the last peak contained no proteins over a molecular mass of ~40 kDa that stained with Coomassie blue. It should be noted that rather large volumes of starting material could be loaded onto the gel filtration column because only the first few fractions following the void volume were used for further purification.

Hydroxyapatite chromatography was very effective in removing small amounts of many proteins near the apparent molecular weight of paranemin, and near the molecular weights of vimentin and desmin (Fig. 2). Fractions with the greatest amount of paranemin and the least amounts of contaminating proteins were in the small peak (Fig. 2, lane c) eluted just before the last, and largest peak (Fig. 2, lane d), which contained approximately the same amount of paranemin as in Fig. 2, lane c, but also contained much greater amounts of vimentin and desmin. DEAE-cellulose column chromatography removed both the remaining vimentin and desmin (Fig. 3, lane b) and the trace of actin seen below vimentin and desmin in the proteins loaded onto the column (Fig. 3, lane a) before elution of the purified paranemin (Fig. 3, lane c). The last large peak eluted from the column contained no proteins over ~40 kDa that stained with Coomassie blue. The pooled fractions of purified paranemin are shown in Fig. 3 (lane e).

Western blot and one- and two-dimensional gel analysis of paranemin

As shown by SDS/PAGE (Fig. 4, left panel) and Western blotting (Fig. 4, right panel), monoclonal antibody 4D3 specifically labeled paranemin throughout the entire purification procedure, from the total embryonic muscle homogenate (lane a) to the final DEAE-cellulose-purified paranemin (lane e). Two-dimensional gel electrophoretic analysis is
presented in Fig. 5, and showed that the gel filtration pool contained paranemin, and considerable amounts of vimentin and desmin (Fig. 5 a), with vimentin, pI range of 5.3 to 5.5, present in greater amounts than desmin, pI range of 5.5 to 5.6. In the hydroxyapatite pool, vimentin and desmin are present in smaller but similar amounts (Fig. 5 c) in comparison to the paranemin (Fig. 5 c, d) At least four isovariants were detected in the pooled purified paranemin (Fig. 5 e, f), with a pI range of ~4.1 to 4.5. The pI values obtained for the 280 kDa paranemin (Fig. 5 b, d, f, between the vertical arrows) is nearly identical to that reported for gel filtration-purified paranemin by Breckler and Lazarides (1982) and Price and Lazarides (1983). The focusing range of paranemin remained between the pI values of ~4.1-4.5 throughout the purification procedure.

Immunolocalization of paranemin in mature avian cardiac and skeletal muscle myofibrils and muscle tissue sections

Paranemin is localized by conventional fluorescence microscopy at the Z-lines in both cardiac (Fig. 6 a, b) and skeletal (Fig. 6 c, d) muscle myofibrils. Double-label confocal immunofluorescence of adult chicken myofibrils with monoclonal antibody 4D3 and polyclonal antibodies against desmin shows that paranemin colocalizes with desmin at the Z-lines of both cardiac and skeletal muscle myofibrils (Fig. 7). Labeling of paranemin (Fig. 7 a, c) and desmin (Fig. 7 b, d) is shown in three consecutive 1 μm thick optical sections through the myofibrils. In skeletal muscle myofibrils, paranemin appears to label more continuously along the Z-lines (Fig. 7, c) than does desmin, which shows more of a punctate pattern of labeling along the Z-lines (Fig. 7, d). Confocal double-labeling of tissue sections shows that paranemin colocalizes with desmin at the adult cardiac muscle
intercalated disk (Fig. 8 a, b; small arrows) and at Z-lines in both adult cardiac (Fig. 8 a, b) and skeletal (Fig. 8 c, d) muscle. In embryonic 14 d chick skeletal muscle (Fig. 8 e, f), paranemin colocalizes with desmin in a peripheral layer of myofibrils that have assembled in these early myotubes.

Discussion

We report here the purification of paranemin, its one- and two-dimensional electrophoretic mobility, and its colocalization with desmin at the intercalated disks of mature cardiac muscle and the Z-lines of mature cardiac and skeletal muscle cells. Paranemin is much less abundant in muscle tissue than desmin. The yield of paranemin, using the procedures outlined herein, typically was approximately 2 mg of purified protein from 100 g of wet embryonic skeletal muscle tissue, compared to 6 mg of purified desmin obtained from 100 g of ground porcine skeletal muscle (O'Shea et al., 1981), where desmin comprises approximately 0.35% of ‘washed’ myofibrils, and compared to 150 to 200 mg of purified desmin obtained from ground turkey gizzard (Huiatt et al., 1980), where desmin comprises approximately 8% of ‘washed’ avian smooth muscle myofibrils. Paranemin is highly sensitive to proteolysis and, thus, the proteolysis of paranemin had to be inhibited by including PMSF, EDTA and TAME in the buffers and fractions.

We purified paranemin from the same tissue, 14 day-old embryonic chick skeletal muscle, studied by Breckler and Lazarides (1982) and Price and Lazarides (1983), because we wanted to be certain that we were purifying the same protein, paranemin. Many
proteins in embryonic skeletal muscle, most from low abundance, migrate with mobilities near that of paranemin. The procedures we have developed to purify paranemin from embryonic skeletal muscle can now be adapted to further study paranemin in other muscle sources.

Previous work reported that the isoelectric point of paranemin in crude muscle extracts was in the range of 4.0 to 4.5 (Breckler and Lazarides, 1982), and we found a similar range for paranemin of 4.1 to 4.5. Both ranges agree well with the calculated pI of 4.17 from the full-length cDNA sequence (Hemken et al., 1996b). Paranemin did focus within the same range throughout its purification, indicating that the purification procedure did not create any significant charge modifications. Gel filtration-purified and hydroxyapatite-purified paranemin did not focus nicely into discrete variants as did desmin (Huiatt et al., 1980; O’Shea et al., 1981). This may reflect, in part, its much larger size and the inherent difficulty in focusing, or perhaps that it is glycosylated (Hemken et al., 1996a).

Results from our lab have indicated that the partial sequences reported for EAP-300 (Kelly et al., 1995) and IFAPa-400 (Simard et al., 1992) have nearly identical nucleotide sequence homology to regions in the sequence of paranemin; therefore, we have suggested that these proteins are identical to paranemin (Hemken et al., 1996b). A recent report (Kelly et al., 1995) has also shown that EAP-300 and IFAPa-400 are highly homologous if not identical.

Localization of paranemin in our experiments is in general agreement with that reported with adult cardiac muscle by Breckler and Lazarides (1982) and Price and Lazarides
(1983), except we also found paranemin, albeit a somewhat smaller amount, at the Z-lines of adult skeletal muscle cells. It has also been reported that EAP-300 (McCabe et al., 1995) and IFAPa-400 (Vincent et al., 1991) are present in developing chick nervous tissue, skeletal and cardiac muscle, and Purkinje fibers of the adult chicken heart (Vincent and LaHaie, 1988; Chabot and Vincent, 1990; Cossette and Vincent, 1991; McCabe et al., 1992; McCabe and Cole, 1992). No purification procedure has been reported for IFAPa-400, and an immunopurification procedure for EAP-300 using monoclonal antibody A2B11 resulted in only partially purified protein (McCabe et al., 1992). That procedure appeared to yield a smaller amount of EAP-300, with several remaining contaminating proteins. In comparison, the paranemin purification scheme described herein results in highly purified protein and a greater yield.

Little is yet known about how vimentin and desmin IFs become anchored at the Z-lines in developing muscle cells, or how desmin IFs are attached to the periphery of myofibrillar Z-lines in mature striated muscle cells. The more continuous, nonpunctate, labeling along the Z-lines shown herein with a paranemin monoclonal antibody, in comparison to that obtained with desmin, may give clues for a role of paranemin in muscle cells at the Z-lines. The well defined IF rod domain of paranemin (Hemken et al., 1996b) may allow paranemin to be incorporated into vimentin and/or desmin (via interactions with their rod domains) IF systems located around the myofibril. An attractive hypothesis is that the long tail of paranemin, which contains the epitope region for monoclonal antibody 4D3 (Hemken et al., 1996b), bridges the IF/Z-line gap and interacts with integral Z-line
proteins to functionally link the IFs to the myofibrillar Z-lines. The ability to prepare highly homogeneous paranemin will permit further investigations of these and other intriguing problems in order to determine paranemin's cellular role(s).

Abbreviations used: IF, intermediate filament; IFAP, intermediate filament-associated protein; TAME, tosyl-L-arginine methyl ester; DTT, dithiothreitol; IEF, isoelectric focusing; TEMED, N,N',N''-tetramethylethylenediamine; SSS, standard salt solution; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate

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References


Figure 1 Elution profile of crude paranemin from a gel filtration (GF) column and SDS/PAGE analysis

Letters on the column profile correspond to gel lanes in the inset. (Lane a) The crude high speed supernatant of an embryonic 14 d chick skeletal muscle homogenate loaded on the column; (lane b) void volume fraction 28 containing primarily paranemin (large arrow-head), and the closely spaced IF proteins vimentin/desmin (small arrow-head); (lanes c-f) remaining profile fractions not used for further purification.
Figure 2 Elution profile of gel filtration-purified paranemin from a hydroxyapatite (HA) column and SDS/PAGE analysis

Letters on the column profile correspond to gel lanes in the inset. (Lane a) pooled GF fractions 26-30 (Figure 1) were loaded; (lane b) fraction 10; (lanes c, d) fractions 74 and 82, respectively, containing paranemin (large arrow-head), and IF proteins vimentin/desmin (small arrow-head).
Figure 3 Elution profile of hydroxyapatite-purified paranemin from a DEAE-cellulose column and SDS/PAGE analysis

Letters on the column profile correspond to gel lanes in the inset. (Lane a) pooled HA fractions 64–78 (Figure 2) were loaded; (lane b) fraction 32 containing IF proteins vimentin and desmin (small arrow-head); (lane c) fraction 36 containing purified paranemin (large arrow-head); (lane d) fraction 42 from last peak eluted; (lane e) pooled fractions 36-40 = purified paranemin.
Figure 4 Western blot analysis of fractions obtained during purification of paranemin

Monoclonal antibody 4D3 was used to identify paranemin by Western blot analysis throughout purification. Molecular mass markers (adult chicken cardiac myofibrillar proteins in kDa): myosin heavy chain (205), α-actinin (100), actin (42), and tropomyosin (34), are indicated on the left. Paranemin (P) is indicated by the arrow on the right. Paranemin purification SDS/PAGE survey stained with Coomassie blue is shown in the left panel and a duplicate gel for Western blot analysis is shown in the right panel. (lane a) Total embryonic muscle homogenate; (lane b) high speed supernatant; (lane c) GF pool; (lane d) HA pool; (lane e) DEAE-cellulose-purified paranemin.
Figure 5 Two-dimensional gel electrophoresis and corresponding Western blot analysis of fractions obtained during purification of paranemin

Isoelectric focusing was done from right (basic) to left (acidic) as indicated by the arrow at the top, and SDS/PAGE was done from top to bottom as indicated by the arrow on the right. (Panels a, c, e) two-dimensional electrophoresis gels and (panels b, d, f) Western blot analysis of respective duplicate gels; (a, b) pooled fraction of GF-purified paranemin; (c, d) pooled fraction of HA-purified paranemin; (e, f) pooled fractions of DEAE-purified paranemin (= purified paranemin) (P), with purified samples of synemin (S) and desmin (D) added. The pair of vertical arrows in panels b, d, and f mark the approximate ends of the paranemin band. V=vimentin.
Figure 6 Immunolocalization of paranemin in adult chicken cardiac and skeletal muscle myofibrils with monoclonal antibody 4D3

Corresponding phase contrast and fluorescence micrographs of cardiac (a, b) and skeletal (c, d) muscle myofibrils; arrows indicate the Z-lines (Z); Bar = 10 μm.
Figure 7 Confocal double label immunolocalization of paranemin and desmin in adult chicken muscle myofibrils

Cardiac (a, b) and skeletal (c, d) myofibrils were labeled with paranemin specific monoclonal antibody 4D3 (a, c) and rabbit anti-desmin polyclonal antibodies (b, d). Three consecutive 1 μm optical sections, from top to bottom, taken from the middle of the myofibrils are shown for both cardiac and skeletal myofibrils; arrows indicate the Z-lines. Bar = 10 μm.
Figure 8 Confocal double label immunolocalization of paranemin and desmin in sections of avian cardiac and skeletal muscle

Frozen sections (8 μm thick) of adult chicken cardiac (a, b) and skeletal (c, d) muscle, and LR White-embedded cross-section (1 μm thick) of embryonic 14 d chick skeletal (e, f) muscle were labeled with paranemin specific monoclonal antibody 4D3 (a, c, e) and rabbit anti-desmin polyclonal antibodies (b, d, f). Small arrows in a and b point out an intercalated disc; large arrows in a - d indicate the end of a transverse Z-line; large arrows in e and f indicate myofibrils observed in cross-section at the periphery of a developing myotube. Bar = 10 μm for a - f.
MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF PARANEMIN, A NOVEL TYPE VI INTERMEDIATE FILAMENT PROTEIN

SEQUENCE REVEALS EAP-300 AND IFAPα-400 ARE HIGHLY HOMOLOGOUS, IF NOT IDENTICAL, TO PARANEMIN

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In this study we report the full-length nucleotide and deduced amino acid sequence of paranemin, a novel intermediate filament (IF) family protein, which may be classified as a type VI IF protein. We determined the cDNA sequence of paranemin by immunoscreening a λgt22 cDNA library from embryonic chick skeletal muscle with a monoclonal antibody specific for paranemin (4D3) and by hybridization screening. Northern blot analysis reveals a single transcript of 5.3 kb, which is much smaller than predicted from the size of paranemin (280 kDa) by SDS-PAGE. Monoclonal antibodies, 4D3 and 4C7, were used to verify the identity of the paranemin cDNA sequence by immunoblotting proteins expressed by a paranemin phage clone. We also confirmed the identity of the paranemin sequence by amino acid sequencing, by Western blotting of a β-galactosidase-paranemin fusion protein and by comparison of the predicted and measured amino acid composition. Paranemin contains the conserved IF rod domain (308 amino acids), which is 63.3% identical to the rod domain of tanabin and 45.5% identical to the rod domain of nestin. The partial cDNA sequences of two proteins, namely EAP-300 and IFAPα-400, which, in turn, overlap each other by 402 nucleotides, are almost identical to a region of cDNA sequence of paranemin.
Introduction

Intermediate filaments (IFs), together with microfilaments and microtubules, comprise the three major cytoskeletal networks in nearly all differentiated eukaryotic cells (Steinert and Roop, 1988; Robson, 1989; Fuchs and Weber, 1994; and Klymkowsky, 1995). The precise biological roles of most intermediate filaments (IFs) have yet to be elucidated (Fuchs and Weber, 1994; Traub and Shoeman, 1994; Klymkowsky, 1995), even though much is known about their cellular distribution, structure, and assembly (for reviews, see Stewart, 1990; Parry and Steinert, 1992; Shoeman and Traub, 1993; Fuchs and Weber, 1994). The IFs may be grouped into six distinct types or classes based upon their sequence (Fuchs and Weber, 1994; Traub and Shoeman, 1994).

Intermediate filaments in mature striated muscle are arranged in a collar-like arrangement around the myofibrillar Z-lines, where they appear to connect adjacent myofibrils together, and possibly help link the peripheral layer of myofibrils to costameric sites along the muscle cell membrane (Yagyu et al., 1990; Price, 1991; Klymkowsky, 1995).

We have detected small amounts of paranemin in adult chicken and pig skeletal muscle at the myofibrillar Z-disks (Hemken et al., 1996a, 1996b), where it previously had not been detected (Price and Lazarides, 1983). Paranemin, ~280 kDa by SDS-PAGE, was first identified in embryonic chick skeletal muscle (Breckler and Lazarides, 1982). Its immunolocalization indicates a developmentally regulated expression in myogenic chick cells, and a more restricted expression in adult chicken muscle cells (Breckler and
Lazarides, 1982; Price and Lazarides, 1983). Paranemin has been considered to be an
intermediate filament-associated protein (IFAP) (Steinert and Roop, 1988; Foisner and
Wiche, 1991) because it copurifies with the type III IF proteins vimentin and desmin
(Breckler and Lazarides, 1982; Hemken et al., 1994, 1996a, 1996b) and colocalizes with
desmin at the periphery of myofibrillar Z-lines (Price and Lazarides, 1983; Hemken et al.,
1994, 1996a, 1996b). Thus, paranemin may be an important regulator of vimentin and
desmin function. As shown herein, paranemin contains the ~310 amino acid rod domain
characteristic of IF proteins. Therefore, paranemin should be considered a member of the
IF protein family rather than an IFAP. By sequence comparisons with other IF proteins,
we also have found that paranemin shares high homologies to tanabin and nestin.

In this report we show that parts of the sequence of paranemin are almost identical to
the known partial cDNA sequences of two other proteins, EAP-300 and IFAPa-400. EAP-
300 was first described by a monoclonal antibody (A2B11) that immunolabeled ganglion
cells in the embryonic chick neural retina and bound to a $M_r=260,000$ protein (Cole et
al., 1986). Later studies showed that EAP-300 is a transiently expressed protein in the
developing chick nervous system, where it is found in barrier structures together with
claustrin ($M_r=320,000$), an extracellular matrix heparin sulfate proteoglycan (McCabe et
al., 1992; McCabe and Cole, 1992; Burg and Cole, 1994). These barriers, which are
considered inhibitory for neuron migration or axon growth, include the glial roof plate or
dorsal septum of the spinal chord, the glial knot of the diencephalon, the marginal layer of
the embryonic day 2 spinal chord, and the midline of the mesencephalon (McCabe and Cole, 1992; McCabe et al., 1992).

IFAPα-400 was first observed using a monoclonal antibody (F51H2) and described as a high molecular weight ($M_r > 400,000$) cytostructural protein in myogenic and neural tube derived structures of the chick embryo (Vincent and LaHaie, 1988). IFAPα-400 was reported to be transiently expressed in chick embryonic cells derived from the neuroectoderm (Chabot and Vincent, 1990), expressed during the replacement of vimentin by desmin in muscle cell differentiation (Cossette and Vincent, 1991), and persistently expressed in the smooth muscle cells of elastic arteries and in cardiac Purkinje fibers (Vincent et al., 1991).

Experimental Procedures

Preparation of Monoclonal Antibodies—Monoclonal antibodies were prepared following procedures described in Hemken et al. (1992). Fusions with SP2/0-Ag14 (ATCC CRL 1581) myeloma cells were performed using a modification of published protocols (McKearn et al., 1979). Paranemin was purified from embryonic day-14 chick skeletal muscle according to Hemken et al. (1996b).

Characterization of Monoclonal Antibodies by ELISA—ELISAs were performed according to Hemken et al. (1992). Purified paranemin (0.5 μg in 100 μl) was used as the antigen and coated onto 96-well, flat-bottom non-tissue culture treated microtitration plates (ICN Linbro, ICN Biomedicals Inc.). PBS-Tween (0.5% Tween-20 in 150 mM NaCl, 20
mM sodium phosphate, pH 7.4) was used to wash the plates three times. Undiluted culture supernatant from individual hybridoma wells was added and incubated at 37°C for 2 h. Plates were washed three times with PBS-Tween, then incubated with alkaline phosphatase-labeled sheep anti-mouse IgG (Sigma A3563) for 30 min at 37°C. PBS-Tween was used to wash the plates nine times, with 2 min incubations between each wash, at 23°C. The substrate p-nitrophenyl phosphate (Sigma 104-0) was added and color was allowed to develop in the dark for 30 min at 23°C. The dye released was quantified by measuring the absorbance at 405 nm relative to control wells incubated with undiluted SP2/O culture supernatant in a Bio-Kinetic Reader (Bio-Tek Instruments).

Preparation of Polyclonal Antibodies—New Zealand white specific-pathogen free-rabbits were injected several times, subcutaneously over the back and intramuscularly in the thigh, with purified paranemin prepared in the following manner. Paranemin bands from SDS-PAGE gels, each containing 1 mg of protein, were minced and homogenized in 1 ml aliquots of PBS and emulsified with equal volumes of adjuvant. Titers were determined using Western blotting (see later).

Amino Acid Sequencing—Purified samples of paranemin from embryonic day-14 chick skeletal muscle were electrophoresed by SDS-PAGE, transferred to PVDF membrane (Micron Separations, Inc.) and stained with 0.1% Coomassie blue R in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 5-10 min (Matsudaira, 1987). Paranemin bands were excised and washed extensively with HPLC grade water. Deblocking was necessary to obtain sequence, so trifluoroacetic acid was used to deblock
the protein (Wellner et al., 1990). For N-terminal sequence analysis, the sample was then loaded on a 477A Protein Sequencer/120A Analyzer (Applied Biosystems Inc., Division of Perkin Elmer) at the Iowa State University Protein Facility.

**SDS-Polyacrylamide Gels and Western blotting**—SDS-PAGE and Western blotting were performed essentially as described previously (Hemken et al., 1992, 1996b), with the following modifications. Purified paranemin and synemin (Becker et al., 1995), and paranemin partially digested with m-calpain (concentration of 0.9 U/mg for 10 min; m-calpain was kindly provided by Dr. Elisabeth Lonergan, Auburn University) in a buffer containing 10 mM Tris-HCl (pH 7.4), 2 M urea, 5 mM CaCl₂ and 10 mM β-mercaptoethanol, were run on SDS-polyacrylamide gels (5% stacking over 8% separating) and transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad). Transfer was performed at 90 V for 2 hr in transfer buffer (15% methanol, 192 mM Tris, 25 mM glycine, pH 8.3) (Towbin et al., 1979; Burnette 1981). After blocking in 1% ovalbumin, 0.5% Tween 20, 5 mM sodium azide in PBS for 30 min (blocking solution), blots were probed with monoclonal antibodies (4D3, 4C7, or 3B12 culture supernatant) or polyclonal antibodies to paranemin. Secondary antibodies, diluted in blocking solution according to the manufacturer's recommendations, included alkaline phosphatase-labeled sheep anti-mouse IgG (No. A3563, Sigma) and goat anti-rabbit IgG (No. A-9919, Sigma), and horseradish peroxidase-labeled goat anti-mouse IgG (No. A2554, Sigma). Antibody labeling in Fig. 1 was visualized by using the substrate nitro blue tetrazolium/5-bromo-4-
chloro-3-indolyl phosphate, and labeling in Fig. 3 was visualized by chemiluminescence (ECL, Amersham).

RNA isolation and construction of cDNA Library—Total RNA was isolated from 14 day-old embryonic chick skeletal muscle cells by extraction with guanidinium thiocyanate at low temperature (Han et al., 1987). Poly(A)^+ RNA was purified by chromatography on an oligo-dT cellulose column (No. 20009, type 3, Collaborative Biomedical Products) (Aviv and Leder, 1972). Ten µg of poly(A)^+ RNA was used to construct an oligo dT-primed directional cDNA expression library in lambda gt22A using the Lambda SuperScript system as described by the supplier (No. 18256, GIBCO BRL). The library contained 2.6x10^6 independent plaque-forming units.

Isolation of cDNA Clones for Avian Paranemin—The lambda gt22 cDNA library was initially immunoscreened, using monoclonal antibody 4D3 to paranemin, following the methods described in the ProtoBlot detection system (Promega Corp.). One hundred thirty two positive plaques were identified and, of these, 20 were purified to homogeneity. For sequencing, the two clones having the largest inserts, 9 (3.2 kb) and 40 (2.7 kb), were subcloned into pBluescript II SK (+) vector (Stratagene) according to standard protocols (Sambrook et al., 1989). The cDNA library was rescreened by hybridization using a 205 bp BsrX I restriction enzyme-generated cDNA probe from the 5' end of clone 9 and a 2.2 kb cDNA probe generated by 5'RACE (described later). Reverse-transcription of a Poly(A)^+ RNA sample was performed with the use of a gene-specific primer spanning paranemin cDNA positions 2148 to 2165. The PCR amplification of the target cDNA was
performed using a paranemin specific primer spanning cDNA positions 2130 to 2147 and the anchor primer. Both cDNA probes were used for hybridization screening of the λgt22 library according to the methods described in the Digoxigenin DNA Labeling and Detection Kit (No. 1093-657, Boehringer Mannheim). A total of 200 positive plaques were chosen, 100 from each probe. PCR was used to screen lambda phage mixes of all 200 positive plaques for sequence overlapping clone 9 using a gene-specific primer spanning paranemin cDNA positions 2130 to 2147 and the λgt11 Upstream Amplimer Primer (No. 5412-1, Clontech). Twenty clones that contained a 2.2 kb product by PCR screening were purified to homogeneity. Clones 3, 24, 89 and 169 were subcloned into pBluescript II SK (+) vector for sequencing.

**Northern Blot Analysis**—Northern blots were performed as described by Wiche et al. (1991), with the following modifications. Poly(A)^+ RNA samples from 14 day-old embryonic chick skeletal muscle cells were denatured by heating to 95°C for 2 min in a loading buffer containing 50% formamide (deionized), and 2.3 M formaldehyde (Davis et al., 1986), with subsequent cooling on ice. RNA and synthetic RNA markers, treated in parallel, (No. 15620-016, GIBCO BRL) were separated on a vertical 1% agarose gel in 2.2 M formaldehyde (deionized), 5 mM sodium acetate, 1 mM EDTA, and 10 mM sodium phosphate, pH 7. The transfer of large RNA species was facilitated by soaking the gel in alkali to partially hydrolyze the RNA (Sambrook et al., 1989). The gel was neutralized in 0.1 M Tris-HCl, pH 7.5, and the RNA was transferred to nitrocellulose by capillary blotting in 20× SSC. The filter was prehybridized for 2 hr at 42°C in 50% formamide
(deionized), 5x SSC, 10x Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% BSA), 0.1% SDS, 30 mM sodium phosphate, pH 7, 0.1% sodium pyrophosphate, and 100 µg/ml heat-denatured salmon sperm DNA. For hybridization, the cDNA probes were radiolabeled with [α-32P]dCTP to specific activity of ~2 x 10^8 cpm/µg (Feinburg and Vogelstein, 1983) and diluted with fresh prehybridization solution to a specific activity between 1 x 10^6 and 1 x 10^7 cpm/ml. The hybridization was performed overnight at 42°C, and unbound probe was removed by washing the filter at a final stringency of 0.2x SSC and 0.1% SDS at 50°C for 30 min.

**Expression of cDNA-encoded Proteins and Western Blotting**—Purified and nonpurified phages of clone 9 were tested with monoclonal antibodies 4C7 and 3B12, and with rabbit polyclonal antiserum to paranemin. Bacterial lawns were infected with approximately 500 plaque forming units for each 150 mm plate. Incubations and visualization of antibody binding were done identically to immunoscreening of the library with mAb 4D3. To prepare a β-galactosidase and paranemin fusion protein for subsequent SDS-PAGE analysis and Western blotting, crude lysates from recombinant lysogens in Escherichia coli Y1089 were prepared as described (Huynh et al., 1985).

**DNA Sequencing and Computer Analysis**—Nucleotide sequencing was conducted at the Iowa State University DNA Sequencing and Synthesis Facility using automated sequencers (373A and 377 DNA Sequencers, Applied Biosystems Inc., Division of Perkin Elmer). Both strands of overlapping clones 9 and 89 were sequenced at least one time. Sequence at both 5' and 3' ends and at least three internal regions were also obtained for clones 3, 24,
40, and 169. Clones spanned the following paranemin cDNA positions: 3 (-24 to 2853), 24 (-14 to 5261), 89 (-14 to 2164), 169 (-9 to 2574), 9 (2035 to 5255) and 40 (2575 to 5255).

Analysis of the predicted nucleotide and predicted amino acid sequences was done using the GCG software package (Program Manual for the Wisconsin Package, Version 8, September, 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Devereux et al., 1984). Dot matrix comparisons were performed with the programs COMPARE and DOTPLOT (Maizel and Lenk 1981). Multiple sequence alignment was done using the program PILEUP. Sequences of the rod domains from the following IF proteins were aligned: frog tanabin (Hemmati-Brivanlou et al., 1992); human nestin (Lendahl et al., 1990); human keratin 14 (Marchuk et al., 1985); chicken synemin (Becker et al., 1995); chicken vimentin (Zehner et al., 1987); mouse NF-M (Levy et al., 1987); and chicken lamin A (Peter et al., 1989). The percent identity was calculated by individual alignments of each rod domain using the program GAP. The intermediate filament signature was identified with the program MOTIFS. Secondary structure predictions were analyzed according to the methods of Chou and Fassman (1978) and Garnier et al., 1978) via the programs PEPTIDESTRUCTURE and PLOTSTRUCTURE.

A restriction enzyme map and isoelectric point, of selected paranemin sequences, were predicted using the programs MAPSORT and ISOELECTRIC, respectively. Basic Local Alignment Search Tool (BLAST) searches were performed using the NCBI BLAST e-mail server (Altshul et al., 1990) to search both nucleotide (PDB, GBUupdate, GenBank,
EmblUpdate, and EMBL), and peptide (PDB, SwissProt, PIR, SPUpdate, GenPept, and GPUpdate) sequence databases.

**Confirming the 5' End cDNA by 5' RACE and PCR Analysis**—The 5'RACE system for rapid amplification of cDNA ends (No. 18374-025, GIBCO BRL) was used to synthesize cDNA corresponding to the 5' end of the paranemin transcript (Frohman et al., 1988). Reverse-transcription of a Poly(A)^+ RNA sample was performed with the use of gene-specific primers spanning paranemin cDNA positions 1011 to 1028 and 2130 to 2147. After removal of excess dNTP and primers, the cDNA was tailed with dCTP and terminal deoxynucleotidyl transferase. Amplification of target cDNA was performed with Taq DNA polymerase (Promega), the anchor primer, and nested gene-specific primers spanning paranemin cDNA positions 455 to 472 and 1011 to 1028. PCR reactions of clones 3 and 24 were performed with the same nested gene-specific primers used for the amplification of the target cDNA and the M13 forward primer (No. 18257-041, GIBCO BRL). The 5'RACE and PCR products were electrophoresed on a 1% agarose gel for comparison analysis (See Fig. 4).

**Results**

A cDNA expression library in λgt22 was constructed from poly(A)^+ RNA isolated from 14 day-old embryonic chick skeletal muscle using an oligo dT-primer. This library, which contained about 2.6×10^6 independent plaque forming units, was screened for the expression of paranemin epitopes by using the monoclonal antibody 4D3, which had been
prepared earlier using purified paranemin from 14 day-old embryonic chick skeletal muscle (Hemken et al., 1996a, 1996b). This antibody detected only a band corresponding to paranemin's size on a Western blot of whole muscle extract from 14 day-old embryonic chick skeletal muscle (Fig. 1A).

Two monoclonal antibodies, 4D3 and 4C7, and the rabbit polyclonal antibody reacted specifically with paranemin, with an apparent molecular weight of 280 kDa (Fig. 1); however, monoclonal antibody 3B12 recognized both paranemin and synemin (Fig. 1A). The monoclonal antibody 4D3, used to screen the library, was tested against the 14 day-old embryonic chick skeletal whole muscle revealing specificity for only paranemin (Fig. 1A).

Out of 2.6 million plaques immunoscreened, 132 gave a positive signal. Phages from the 20 plaques with the strongest signal were further purified by rescreening at lower densities, until positive phage clones could be isolated. The two largest cDNA inserts, of clones 9 (3.2 kb) and 40 (2.7 kb), were then sequenced (Fig. 2A).

The 5'RACE was initially used to generate a 2.2 kb probe, using a primer at the 5' end of clone 9 (see Experimental Procedures, Isolation of cDNA Clones for Avian Paranemin), to rescreen the λgt22 library (data not shown). Hybridization screening using this probe and a 205 bp BstXI probe (Fig. 2A) generated from the 5' end of clone 9 yielded approximately 200 positive plaques. PCR analysis of the 200 phage mixes identified 20 clones, which extended 2.1 kb beyond the 5' end of clone 9 and were the same size (2.2 kb) as the 5' RACE product (data not shown). These 20 clones were purified to homogeneity and, of these, clones 3, 24, 89, and 169 were used for sequencing (Fig. 2A).
To confirm that the phages of clone 9 were indeed expressing cDNA of paranemin, we tested blots of phage plaques of clone 9 with a panel of monoclonal antibodies and rabbit polyclonal antibodies to paranemin. Monoclonal antibodies, 4D3 and 4C7, and rabbit polyclonal antibodies clearly bound to proteins contained in plaques of clone 9 (data not shown). However, monoclonal antibody 3B12, which crossreacts with synemin (Fig. 1A), did not bind to clone 9 phage plaques. Apparently, monoclonal antibody 3B12 recognizes an epitope of paranemin that is located upstream of the 3.2 kb sequence of clone 9.

Monoclonal antibodies 4D3 and 4C7 recognize different epitopes on paranemin, as shown by a Western blot of a partial m-calpain digest of paranemin (Fig. 1B). A series of six closely spaced doublets were labeled with 4D3, and three closely spaced doublets were labeled on a duplicate blot with 4C7, indicating that these two monoclonal antibodies are binding to two different epitopes of paranemin. Thus, at least two different epitopes, within the sequence of clone 9 of paranemin, are recognized by 4D3 and 4C7.

The size of the mRNA for paranemin from embryonic chicken skeletal muscle was estimated by Northern blot analysis to be 5.3 kb (Fig. 2B). As probes, we used two restriction enzyme cDNA fragments of clone 9, one that is located in the overlap region with the cDNAs for EAP-300 and IFAPa-400 (Hae III fragment), and the other one that is derived from the 5' end (BsrX I fragment), which does not have any overlap to those cDNAs.

To determine the size of the immunoreactive protein in the plaques of clone 9, β-galactosidase fusion protein was expressed in E. coli Y-1089 and analyzed on a Western
blot (Fig. 3). The monoclonal antibody 4D3 specifically labeled a band of 220 kDa (Fig. 3B, lanes 3-5), and no labeling was seen in lanes containing wild type Y-1089 bacterial proteins (control; Fig. 3B, lanes 1 and 2). Assuming an average molecular weight of 110 daltons for an amino acid residue in paranemin, we calculated from the size of the fusion protein, that the cDNA insert of clone 9 contained an open reading frame equivalent to about 950 amino acids. This is slightly less than the length predicted from the size of the cDNA insert (3.2 kb), indicating the presence of a short non-coding region on this clone.

After obtaining cDNA clones that appeared to contain the 5' end of paranemin by hybridization screening, 5' RACE and PCR analyses were used to confirm that the paranemin cDNA clones 3 and 24 indeed contained the 5' end of paranemin. In two comparisons for each clone (Fig. 4, lanes 2-5 and 7-10), the sizes of the 5'RACE and PCR products are almost the same size, with the 5'RACE product slightly smaller, indicating that these clones do contain the 5' end of paranemin (Fig. 4). The slight difference in size is due to the amplification of 90 bp of pBluescript II SK (+) vector compared to approximately 36 bp of a modified anchor primer, which was synthesized without a 12 bp cloning site at its 5' end.

The complete nucleotide sequence and deduced amino acid sequence of paranemin are shown in Fig. 5. A striking heptad repeat region is located approximately in the center of the molecule, from paranemin nucleotide positions 2231 to 3025 (also see Fig. 9). N-terminal amino acid sequence was attempted; however, during the deblocking procedure,
the paranemin molecule was apparently cleaved in the repeat region and, as a result, internal sequence was obtained (see underlined sequence in Fig. 5).

A comparison of the predicted and measured amino acid compositions (Table I) indicates close agreement, especially Glx, Ser, His, Arg, Thr, Val, Ile, Leu, Phe and Lys. The Met, Cys, and Trp composition had not been experimentally determined (Hemken et al., 1996a). The calculated pI and molecular weight of paranemin, based on the cDNA sequence, are 4.17 and 178,161, respectively.

To examine the relationship of paranemin to the rod domain of other intermediate filament proteins, we aligned the deduced amino acid sequence of paranemin (Fig. 5) to those of the rod domains of seven other intermediate filaments proteins, which were chosen to represent other types of IF proteins. The overall sequence identity within the rod domain was determined to be in the range of 63.3% (tanabin) to 23.7% (keratin 14). The highest degree of conservation was found at both ends of the rod domain. Paranemin amino acid sequence had zero mismatches with the IF protein consensus signature, [(I,V) X (T,A,C,I) Y (R,K,H) X (L,M) L (D,E)], where X can be any amino acid (Steinert and Roop, 1988; Stewart, 1990; Fliegner et al., 1990; Franke, 1987; Lendahl et al., 1990; and Dodemont et al., 1990), at the C-terminal extremity of the rod domain. Valine in the first position of the pattern was only found in type VI IF proteins, whereas Ile is found in all other types (Fig. 6).

The overall amino acid identities between paranemin and nestin, a class VI IF protein, and between paranemin and tanabin, a putative class VI IF protein, are 25.2% and 27.4%,
respectively. The percent identity in the tail domains of these proteins is significantly lower than in the rod domains, with 63.3% (rod) and 17.7% (tail) between paranemin and tanabin, and 48.5% (rod) and 21.8% (tail) between paranemin and nestin.

The comparison of the sequence of the full-length cDNA sequence of paranemin (5.3 kb) with partial sequences for EAP-300 (1.4 kb) and IFAPa-400 (1.7 kb), found by searches in GenBank, revealed that these three sequences are almost 100% identical in their overlapping regions (Fig. 7).

Secondary protein structure predictions of paranemin revealed mostly α-helices with interspersed regions of turns and β-sheets (Fig. 8). The paranemin sequence is very hydrophilic (Fig. 8), most likely reflecting the acidic character of the sequence, which has a predicted isoelectric point of 4.17. The comparison of the full-length paranemin cDNA sequence with itself shows a major block of 39 consecutive heptad repeats (Fig. 9), with the most common being GDLQEEH, GDTQEEH, GDLQVEH, and EDLQVEH (heptad repeat sequence is shown in bold face in Fig. 7).

Discussion

The observed sequence identity and the immunoblotting results provide strong evidence that EAP-300 and IFAPa-400 are paranemin. Paranemin and partial EAP-300 sequences share 476/481 amino acids (99.0% identity) and 1436/1443 nucleotides (99.5% identity), whereas paranemin and IFAPa-400 sequences share 428/431 amino acids (99.3%
identity) and 1708/1711 nucleotides (99.8% identity). The near 100% identity between EAP-300 and IFAPa-400 was reported by Kelly et al. (1995). Minor differences observed in the sequences may be due to allelic variations, point mutations, or tissue specificity (paranemin, muscle; EAP-300, brain; IFAPa-400, brain). Also, the reverse transcription of the RNA during the construction of the cDNA library may have introduced slight deviations in the sequence. However, we have obtained identical sequences from a full-length clone and from overlapping clones, and we have confirmed those sequence differences by sequencing both strands of the paranemin cDNA.

The size of the paranemin transcript was much smaller than we expected from the size of paranemin as observed by SDS-PAGE. Simard et al. (1992) suggested that the size of the IFAPa-400 message was greater than 10 kb, which was based on only two ribosomal RNA markers. However, we find that the transcript size is 5.3 kb, when compared to six synthetic RNA markers. This message size of the paranemin transcript is large enough to code only for an ~190 kDa protein, which is much less than the ~280 kDa estimated for paranemin by SDS-PAGE (Breckler and Lazarides, 1982; Hemken et al., 1996a, 1996b).

The aberrant migration of paranemin in SDS gels may be due to the large number of negative charges (Table 1; Hemken et al., 1996a), which may cause SDS to bind poorly to proteins (Bryan, 1989a). For example, other proteins such as caldesmon (Graceffa et al., 1988) and calpastatin (Takano et al., 1988) also are rich in acidic amino acids and exhibit anomalous behavior by SDS-PAGE. The estimated molecular weights determined by SDS-PAGE for smooth muscle caldesmon, [120,000 (Marston and Smith, 1985; Fujii et al.,
122

1987); 125,000 (Ball and Kovala, 1988); 140,000 (Shirinsky et al., 1988); and 150,000 (Sobue et al., 1981)], are much higher than the estimated molecular weight based on the cDNA-derived sequence, [82,000 (Bryan, 1989b)]. Another explanation for this discrepancy in size may be due in part to the glycosylation of paranemin (Hemken et al., 1996a), which may cause paranemin to migrate slower in SDS-polyacrylamide gels.

We identified the paranemin cDNA clones by immunological screening of a phage expression library using monoclonal antibody 4D3. To confirm that we have obtained paranemin cDNA clones and not those of an immunologically-related protein, we tested the proteins expressed by our paranemin clone 9 with a panel of monoclonal antibodies and polyclonal antiserum to paranemin. The polyclonal antiserum and two of these monoclonal antibodies (4D3 and 4C7), which recognize different epitopes in paranemin (Fig. 1B), reacted positively with proteins expressed by clone 9. Further lines of evidence, namely the internal amino acid sequence obtained from purified paranemin by Edman degradation (underlined sequence in Fig. 5), the close agreement between the predicted and measured amino acid compositions (Table 1), and the predicted and measured isoelectric point (Hemken, et al., 1996b), argue that the sequence presented here is indeed paranemin sequence.

Because paranemin contains an IF rod domain, it seems plausible that this region interacts with the rod domains of other IF proteins such as vimentin or desmin (Meng et al., 1996) in the backbone of IFs and that it links heteropolymeric IFs to other cytoskeletal components via its long C-terminal tail domain. Preliminary data (negative staining of
samples of purified paranemin dialyzed into an IF-forming buffer) indicated that paranemin does not assemble into uniform 10 nm diameter filaments by itself (data not shown).

The consecutive heptad repeat region in paranemin's tail domain is intriguing. Secondary structure predictions show that this motif could be α-helical. It has been proposed that the heptad repeat region in EAP-300 (Kelly et al., 1995), that is highly homologous, if not identical to paranemin, contains multiple leucine-zipper-like motifs. The leucine zipper contains a ‘4-3’ heptad repeat of hydrophobic and nonpolar residues that pack together in an α-helical coiled-coiled (Landschulz et al., 1988); however, not all of the proposed leucine zippers contain the 4-3 hydrophobic repeat. For example, leucine repeats in Myc and Fos contain charged residues at the alternate site (O'Shea et al., 1989). These leucine repeats may have hydrophobic contacts provided by side chain methylene groups, or a different conformation, which is thought to be the case in part of the rod domain of nematode myosin (McLachlan and Karn, 1983). However, it remains to be proven whether paranemin contains a leucine zipper domain and whether paranemin binds DNA under any conditions.

Localization of paranemin by Price and Lazarides (1983) indicated that paranemin was expressed in all myogenic muscle tissues, but only remained in heart muscle of the adult chicken. Our results, however, have shown that paranemin also is present in adult skeletal muscle (Hemken et al., 1994, 1996a, 1996b). We have observed by confocal microscopy a small amount of paranemin present in frozen sections and isolated myofibrils from adult skeletal muscle (Hemken et al., 1994, 1996a, 1996b). The differences in results may be
explained by the fact that we used a different antibody (monoclonal antibody 4D3) and a
different technique (confocal microscopy), which may be more sensitive than the methods
of Price and Lazarides (1983). EAP-300 and IFAPa-400 have been localized in embryonic
chick skeletal and cardiac tissue and in adult cardiac tissue, but neither EAP-300 nor
IFAPa-400 were detected in adult chicken skeletal muscle (McCabe et al., 1992; McCabe
and Cole, 1992; Cossette and Vincent, 1991). Embryonic chick neuronal tissues that have
been shown to express EAP-300 include the brain, spinal chord, and somites (McCabe et
al., 1992; McCabe and Cole, 1992; McCabe et al., 1995; Chabot and Vincent, 1990).
Because we have shown that EAP-300 and IFAPa-400 are most likely identical proteins,
paranemin may also fulfill functions previously ascribed to those two proteins, although
differences in post-translational modification in non-muscle tissue are possible. In
summary, paranemin has been shown to be a novel protein of the IF family, and it may
have an important role in modulating IF function in developing neurons and in developing
and mature cardiac and skeletal muscle cells.

The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate
buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis;
PVDF, polyvinylidene fluoride; HPLC, high performance liquid chromatography; kb, kilobase (s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.
Acknowledgments

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References


Table I
Comparison of predicted and measured amino acid composition
Calculated molecular weight = 178,161.

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<th>Amino acid</th>
<th>Mole % predicted</th>
<th>Mole % measured</th>
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<th>Mole % predicted</th>
<th>Mole % measured</th>
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<td>Asp</td>
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<td>Ala</td>
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<td>—</td>
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<td>—</td>
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<td>N.D.</td>
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<td>33.188</td>
<td>35.2</td>
<td>Basic</td>
<td>12.328</td>
<td>12.2</td>
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The predicted amino acid composition was calculated using PEPTIDESORT and the measured amino acid composition was reported in Hemken et al. (1996a). N.D., not determined.
Fig. 1. Specificity of anti-paranemin monoclonal and polyclonal antibodies for avian paranemin. A, (a-d) Western blots of paranemin samples purified from 14 day-old embryonic chick skeletal muscle and purified synemin from avian gizzard smooth muscle were tested against monoclonal antibodies (a) 4D3, (b) 4C7, (c) 3B12, and (d) polyclonal anti-14 day-old embryonic chick skeletal muscle paranemin. (e) Whole tissue homogenate from 14 day-old embryonic chick skeletal muscle was tested with monoclonal antibody 4D3, which was used to screen the expression library. B, Western blots of two strips cut from the same lane of a sample of calpain-digested purified paranemin, were tested with monoclonal antibodies (a) 4D3 and (b) 4C7. Large arrows indicate paranemin, with an apparent molecular mass of 280 kilodaltons, and the small arrow indicates the position of synemin (230 kDa). Migration of molecular mass markers (No. SDS-6H, Sigma) are at the left.
Fig. 2. Clone scheme of overlapping cDNAs for paranemin, EAP-300, IFAPa-400, and of the cDNA fragments used for Northern blot analysis. A, Diagram showing the relative location of the aligned nucleotide sequences of paranemin (5285 bp), and partial sequences of EAP-300 (1443 bp) (Kelly et al., 1995) and IFAPa-400 (1711 bp) (Simard et al., 1992). The cDNA fragments produced by restriction enzymes, BstX I(205 bp) and of Hae III (864 bp), from clone 9, were used for hybridization analysis. The scheme includes cDNA clone numbers 24 (5275 bp), 3 (2898 bp), 169 (2574 bp), 89 (2164 bp), 9 (3205 bp), and 40 (2666 bp). B, DNA hybridization analysis of poly(A)$^+$ RNA from 14 day-old embryonic chick skeletal muscle with the restriction enzyme-generated probes $BstX$ I and $Hae$ III indicated at the top of each line. Lanes a and c, 3 μg poly(A)$^+$ RNA were loaded; Lanes b and d, 6 μg. The single transcript size for paranemin, in kilobases, is indicated by the arrow. RNA-ladder markers in kilobases are indicated at the left.
Alignment of Paranemin cDNA Clones and Northern Blot Analysis

A

EAP-300
IFAPa-400
#24
#3
#169
#89
#9
#40
Probes

B

BstXI
HaeIII

<table>
<thead>
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<th>1 kb</th>
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</thead>
</table>

9.49   7.46  4.40  2.37  1.35  0.24

kb a b c d

5.3 kb
Fig. 3. Western blot analysis of the β-galactosidase-paranemin fusion protein. Control Y1089 bacteria (lane 1, supernatant; lane 2, pellet) and λgt22-clone 9 lysogenic Y1089 bacteria (lanes 3, 4, and 5, supernatants) were subjected to SDS-PAGE (5% stacking [S] gel and 8% separating gel). (A) Coomassie blue staining pattern and (B) Western blot, using monoclonal antibody 4D3, of a duplicate gel transferred to nitrocellulose. Protein loads were approximately 20 μg in lane 1, 40 μg in lane 2, 5 μg in lane 3, 10 μg in lane 4, and 20 μg in lane 5. Arrows indicate the β-galactosidase-paranemin fusion protein. Lane M, molecular mass markers (No. SDS-6H, Sigma) in kilodaltons.
Fig. 4. Comparison of 5’RACE and PCR of clones 3 and 24 for confirmation of the 5-prime end of paranemin. PCR (lanes 2, 4, 7, 9) and 5’RACE (lanes 3, 5, 8, 10) of clones 3 (lanes 2-5) and 24 (lanes 7-10). For PCR of clones 3 and 24, two gene specific primers spanning from paranemin cDNA positions 455 to 472 and 1011 to 1028, and the M13 forward primer (No. 18257-014, GIBCO BRL) were used. For amplifying the 5’RACE products by PCR, the same paranemin specific primers were used in addition to the anchor primer (No. 18388-017, GIBCO BRL) (see “Experimental Procedures” for details). A mix of lambda DNA/Hind III and φX174 DNA/Hae III nucleic acid markers (Promega) are in lanes 1 and 6 with the size in kb indicated at the left.
Fig. 5. Nucleotide sequence of avian muscle paranemin cDNA and its deduced amino acid sequence. The positive numbers of nucleotides start at the first residue of the coding sequence. Amino acids are designated by the one-letter code. Underlined regions represent the matching avian muscle paranemin sequence obtained by automated Edman degradation of peptides (parentheses indicate equivocal amino acid assignments). The initiation codon, stop codon, and polyadenylation signal sequences are double underlined. The nucleic acid sequence of paranemin is available from GenBank under accession number ______.
Fig. 5 (cont.)

117 AAGGTGCTGACCACCCAAGACAAGAGCTCCAGTCCAGGAGTCCAGAACATTCTAGCTTGAG

KVLTPKSKSALFQKISS 392

117 GTCTCTCCAGCCAGACAAGAGCTCCAGTGCTGCCAGGGGCGAGGAGCCAGGATCTCTGGAC

VLQAPRSWEAPAAPSPTPVPVV 412

1237 TCCCCAGACCCAGCGCTGGGAGGAGCTGGGAGCTGCTGCCCCCAGCCCCACTGTGCCCCAG

SEPAGSREPVEHCAGK 432

1297 GAGTCCCCATGCTGACCCCACTGTGCTGCAACACGCTGAGGAGGACAGCAGGATGGTG

VLQAPRSWEPAAPSPTVPV 452

1357 GCTTCAAGGAGGATGCAAGATGCTGAGGCCAAAGAAGTGCCCACACTCCAGTCAGGCCAC

ALKEMQDDAEAKEVPTLSAT 472

1417 CAGAGTACCAGGGATGGGGATCTTGAAGCCACCATGGAGGAGGAGGAGGCTGCAGGAAC

QSTROGDLEATHEEEAEAGT 492

1477 CAGGGTTGGTGCTGCTGGAGTAAACCCTGCCCAGGTCGGTTTCTGCAGCAAC

QGVGVAEGETVSPPGLCFCSN 512

1537 GAGCCCTACATTGTTAAGTGCCACCCAGAGTGATGTAGAGAGCCAGGAGGAGATGTGGGAG

EEPASATQSDVESQEEMWEE 532

1597 GAGGAGAGGAGCAAAGAGGAGATGCTGAACCCACTGAGCTCCATGGAGAGCCAGGAGCC

EERSKEEMLNPLSSMESQEP 552

1657 GGGGAGAGCCCTGGGGAGGGGTCACAAGGAGGTCCAGGCTGCAGGTGGGCAAGGAGGAC

GGEPWGGVTRRSRLQVGKE 572

1717 ATGGAGGCCAGCAAGGAGGAGCGACTTCAGACTCCAGGAGAAGGAGGAGGAGCCAGGAAATC

MEATSTEALHISEKKKEOREI 592

1777 TGGAGCCCTCAGGGAGGAGATGAGGATGTGCTGCAGGCTGAGGCAGGAGAAATGCAA

WSPSREDDEEECEEFPDDEEREEMQ 612

1837 GAAGAAGGTCCTGCAAGGAGAATCTGCTGCTGCTGCTCTGTGGGAGGCAAC

EEGSGLQEMIEIEAACAVPVGSHE 632

1897 CAGTCTTTGCCAACAGGAATCCTGAGAGGACTTTCTGTGAGGAGGAGGAGAGAGCAGGAGTCT

PVLPPTGIHLQEDFLEREQES 652

1957 GAGCACCAGGAGACGCTCCCTGGGTGAGGCTGCTGCTGCTCTGTGGGAGGCAAC

EHEQGETSLGELGAAGAEGEREQ 672

2017 GAGGTCTGTCAGGGAGCTCTGCTCTGAGCTTACAGAGCAGGCTCCAGCAGATGAGGGGAGG

EVCGQELKASSIEEAAMPAAEG 692

2077 TCATCGGGTCTGAGGAGAGAATCCACAGGAGCAAGGAGGAGGACAGAGCAGGAGGAGGAGGAT

SSGSGEDTTTGRRESTGARD 712

2137 GAGGAGAGGAGAGAATCAAGGAGGAGGAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG

EGEDEDKGMREALGEDDPQAG 732

Fig. 5 (cont.)
Fig. 5 (cont.)
Fig. 5 (cont.)
Fig. 6. **Optimal alignment of the rod domain of paranemin with the rod domains of other IF proteins.** Sequence identity to paranemin is indicated by *dashes*, and *dots* represent gaps inserted by the alignment program PILEUP with the GapWeight=6 and GapLength=0.1. Hydrophobic residues in the heptad repeat are indicated by *asterisks*. The start of helical domain 2A for paranemin was difficult to define. The regular pattern of heptad repeats is interrupted once in domain 2B by the presence of a "stutter". The intermediate filament signature (*double underline*), [(I,V) X (T,A,C,I) Y (R,K,H) X (L,M) L (D,E)] where X can be any amino acid (Steinert and Roop, 1988; Stewart, 1990; Fliegner et al., 1990; Franke, 1987; Lendahl et al., 1990; and Dodemont et al., 1990), was identified in paranemin with zero mismatches using the GCG program MOTIFS. The percent identity was calculated by individual alignments of each rod domain using the program GAP with the same GapWeight and GapLength as used with PILEUP. The 42 amino acid insertion in region 1B of the lamin A sequence was not included in the calculation of percent identity.
L2—Helical Domain 2B
Paranemin  WQVAE DNQSQLQLRHELEKELVGLKVRKEMLEELSGQQEQLHQGEAEKFQQL
Nestin  ARAVQ GAREVR-E-QQ-QA--RGG--LE--RRA--QR--EGR--RLRAT-----
Keratin 14  QSGKS EISELRRTMQN--I--QSQLSM--AS--N--EETKGRYCMQLAQLI-E
Synemin  ELLR-  ESR-CEQH-ED-HROGQE-CGLR-R--QE-LAMODRHA--V--EY-R
Vimentin R-AKQ EANFYRR--IQS--TC--VDA----GSN--S--RQMREMEENFAV--ANY-D
NF-M RSKA-  ETAEYRR--QSKSI--ESVRGT--5--RQ--SDIEERHNNHDLSSY-D
Lamin A GAAH- EL---THIRDS-SA--SQ--QKQLAAK--AK--REVLEALSREREGRR

Paranemin  AIEALEQEKQLVQIAVLEDRQQLMHLKMSLSLEVATYRTLLEAESTRL
Tanabin  KA----------D-RG--E-----------------------------63.3%
Nestin  -V----------G----------G----------A------------------NS--48.5%
Keratin 14  M-GSV-EQLAQ-RCMEM-QNQYKTI-LDV-TR-EQ-I-----R---G-DAH-23.7%
Synemin  I-D--E---F-TMS-TDY-R-Y-E-LQV-AG-I--IE--A---SGK-NQW30.4%
Lamin A  LLAEK-R-MAERMARQQ-Q-DEY-E-LDI-LA-DM-INA---K---G-EE--26.3%

Fig. 6 (cont.)
Fig. 7. The comparison and alignment of predicted amino acid sequences of cDNAs encoding paranemin, and partial sequences of EAP-300 (accession no. X80877) and IFAPa-400 (accession no. S52130). Nearly identical coding sequence was identified between paranemin and the partial sequences of EAP-300 (Kelly et al., 1995) and IFAPa-400 (Simard et al., 1992). Identities are shown as dashes. Mismatched EAP-300 amino acid residues are at positions 859 (Q), 946 (D), 1269 (Q), 1308 (A), and 1309 (E), and mismatched IFAPa-400 amino acid residues are at positions 1333 (V), 1382 (I), and 1415 (S). Amino acids are designated by their one-letter code, the stop codon is marked by an asterisk, and continuous pseudo heptad repeats are in bold.
<table>
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<th>Paramein</th>
<th>Sequence</th>
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**Note:** The sequences are likely from a protein or DNA strand, and the asterisk (*) indicates the end of the sequence.
p.

Elucidation of the (residues having the composition NXR or NXS, when X is not D, W, or T). The high content of charged residues, indicating one predicted structure sheet. The Kyte-Doolittle (KD) plot predicts that parahemoglobin is very hydrophobic because of the sequence of parahemoglobin is mostly α-helical with interspersed regions of p-helices and β-sheets. Robinson (GOR) and PSIPRED (PSI-PRED) predict secondary structural features of α-helices and β-sheets with three structural models based on Chou-Fasman (CF) and Garnier-Osguthorpe.

Figure 8. Predicted secondary structural features of α-helices and β-sheets.
Fig. 9. Self comparison dot matrix profile of the predicted paranemin protein sequence. The homology scoring using the GCG programs COMPARE and DOTPLOT, based on the method of Pearson and Lipman (1988), was plotted with a window of 21 residues, stringency of 14.0, and with a total of 59,127 points. A major block of repeating sequences is located approximately in the middle of the molecule (also see bold sequence in Fig. 7) and other minor blocks of repeating sequences are seen.
OVERALL SUMMARY

The major objectives of the work presented here were to purify, characterize, and clone and sequence muscle paranemin. Gel-purified paranemin was injected to immunize BALB/C mice for the production of monoclonal antibodies to paranemin. A specific monoclonal antibody to paranemin, 4D3, was used to identify paranemin by one- and two-dimensional Western blotting throughout the purification scheme. In the first and second papers in this dissertation, the biochemical purification of paranemin was done for the first time. Embryonic chick skeletal muscle was homogenized in a buffer containing 130 mM KCl, pH 7.5, at 4°C, and the homogenate was centrifuged for 90 min at 145,000 g. The high speed supernatant was used for successive chromatographic steps, first on gel filtration in 100 mM NaCl-containing solution, pH 7.5, and then on hydroxyapatite and DEAE-cellulose in buffers containing 6 M urea, pH 7.5. Purified paranemin was routinely more than 95% 280,000-dalton protein, by SDS-PAGE and Coomassie blue staining, with no detectable contamination by other proteins. Relatively small amounts of paranemin (~2 mg) can be isolated in sufficient quantities to permit further studies on its properties and function.

Purified paranemin was stained with Stains-all and gave an intense blue color, which indicated that it is a phosphoprotein and/or glycoprotein. Periodic acid treatment of paranemin, followed by biotinylation of oxidized carbohydrate and streptavidin-alkaline phosphatase detection, was used to determine that paranemin is a glycoprotein. In support of this result, a strong glycosylation site is predicted in the deduced amino acid sequence of
paranemin, which is described in the third paper. Two-dimensional gel analysis revealed purified paranemin had 3-4 major isovariants, with pI's between 4.1-4.5. The amino acid analysis of purified paranemin showed a high ratio of acidic to basic amino acids, which is consistent with paranemin's pI.

Immunofluorescence localization of paranemin in the first and second papers, using the monoclonal antibody 4D3, revealed labeling at Z-lines in avian and porcine species in both adult chicken cardiac and skeletal muscle myofibrils. Confocal double-label immunofluorescence microscopy of frozen tissue sections showed colocalization of paranemin with desmin at the Z-lines of adult cardiac and skeletal muscle cells and at the intercalated disks of adult cardiac muscle.

We determined the cDNA sequence of paranemin by first immunoscreening a λgt22 cDNA library from 14 day-old embryonic chick skeletal muscle with a monoclonal antibody specific for paranemin (4D3) and by hybridization screening in the third paper of this dissertation. The molecular cloning and nucleotide sequence of paranemin is reported for the first time. Northern blot analysis revealed a single transcript of 5.3 kb, which is much smaller than predicted from the size of paranemin (280 kDa) by SDS-PAGE. Monoclonal antibodies, 4D3 and 4C7, were used to verify the identity of the paranemin cDNA sequence by immunoblotting proteins contained in plaques of clone 9. We also confirmed the identity of the paranemin sequence by amino acid sequencing of a polypeptide isolated from paranemin, by Western blotting of a β-galactosidase-paranemin fusion protein, and by comparison of the predicted amino acid composition with that
measured on purified paranemin. Paranemin contains a conserved IF rod domain (308 amino acids), which is 63.3% identical in amino acid sequence to the rod domain of frog tanabin and 45.5% identical to the rod domain of human nestin. The reported partial sequences of two proteins, namely EAP-300 and IFAPa-400, are almost identical to regions of the cDNA sequence of paranemin. Because of this identity, paranemin may fulfill some or all of the functions previously ascribed to these proteins; however, differential types and degrees of post-translational modification in other cells may lead to some differences in precise function(s). In view of its sequence homology to tanabin and nestin, which are often classified as class VI proteins, paranemin should be considered a member of the novel type VI IF protein family.

Paranemin may play a very important role in modulating IF function in developing neurons and in developing and mature cardiac and skeletal muscle cells. The rod domain of paranemin probably binds to other IFs via their rod domains, and the large tail domain of paranemin may extend from the surface of the IFs and bind to integral Z-line proteins or to cell-matrix adhesion plaque proteins at the cell membrane. The multiple leucine zipper-like repeat motifs in the tail of paranemin also may be important in regulating transcription or binding to other cytoskeletal proteins. The results obtained in my dissertation provide a solid foundation for future studies designed to determine paranemin’s precise cellular role(s).
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