INTRODUCTION

The cytoskeleton is composed of microtubuli, microfilaments and intermediate filaments. While the former two components are present in most cell types, intermediate filament expression is much more dynamic. More than 40 different intermediate filament genes have been characterized and they are expressed with very distinct temporal and spatial patterns (for review see Steinert and Liem, 1990; Stewart, 1993). Based on structural criteria, intermediate filament genes, including the nuclear lamins, are currently divided into six classes (Lendahl et al., 1990; for review see Steinert and Liem, 1990), and these six classes fall into two main evolutionary branches (Dahlstrand et al., 1992b; Dodemont et al., 1990; for review see Weber et al., 1991). Little is still known about the specific functions of individual intermediate filament genes, but expression of mutant versions of a keratin gene in transgenic mice resulted in severe defects in skin organisation (for review see Fuchs and Coulombe, 1992). Furthermore, the genetic skin diseases epidermolysis bullosa simplex and epidermolytic hyperkeratosis were recently shown to be caused by mutations in keratin genes (for review see Fuchs and Coulombe, 1992). These data show that one type of intermediate filament is important for organization of a specific tissue type, and it is conceivable that other intermediate filaments may play similar, important roles in other tissues. It is thus of interest to characterize the spatial and temporal expression patterns of various intermediate filaments during tissue development, and to learn how the individual proteins interact with each other in the cell. This may be particularly important for tissues that undergo complex morphogenetic changes during development. In this report we analyse the expression and intracellular distribution of the recently discovered intermediate filament nestin during the course of muscle development.

Nestin comprises the class VI intermediate filaments (Lendahl et al., 1990), and belongs to the same evolutionary branch as neurofilaments and internexin (Dahlstrand et al., 1992b; Dodemont et al., 1990). The expression of nestin has primarily been analysed in the central nervous system (CNS), where it is expressed transiently in CNS stem cells, and is later replaced by neurofilaments and glial fibrillary acidic protein (GFAP) in neurons and astrocytes, respectively (Lendahl et al., 1990). There are however several lines of evidence suggesting that nestin is also expressed in muscle. First, nestin was originally discovered as an epitope expressed in neuroepithelial cells and myotomes. Second, nestin mRNA is found in the developing skeletal muscle of rat embryos (Lendahl et al., 1990). Third, a rhabdomyosarcoma tumor, which is of muscle origin, was positive for nestin immunoreactivity (Dahlstrand et al., 1992a).
Finally, dissection of the rat nestin promoter in transgenic mice revealed a regulatory element that directs expression in developing skeletal muscle (Zimmerman et al., 1994).

Myogenesis, the development of skeletal muscle, is a complex, multistep process. Somite cells become determined to form muscle precursor cells in the myotome. Mononucleate muscle precursor cells proliferate and migrate to their final destinations, cease DNA replication, and subsequently fuse into multinucleate myotubes. This process is influenced by growth factors and myogenic determination factors (Olson et al., 1991). The formation of myotubes is accompanied by the activation of genes encoding muscle-specific proteins. Several of these genes, e.g. actin and myosin heavy and light chains, exist in embryonic, neonatal and adult forms, and are expressed in a sequential order during muscle development (Buckingham, 1985).

It is well established that two intermediate filaments, vimentin and desmin, are synthesized in skeletal muscle cells (Gard and Lazarides, 1980; Osborn et al., 1982). Vimentin and desmin are closely related, both belonging to the class III intermediate filaments. They are, however, relatively distantly related to nestin, which resides on the other main evolutionary branch (see Weber et al., 1991, for review). During early stages of avian and mammalian embryogenesis the intermediate filament network of immature muscle cells was previously reported to be made exclusively of vimentin (Bennett et al., 1979; Gard and Lazarides, 1980; Zehner and Paterson, 1983). Later, myoblasts and early myotubes express both vimentin and desmin. In mouse and human myotubes vimentin disappears shortly after fusion, whereas in chicken myotubes coexpression of vimentin and desmin has been reported (Bennett et al., 1979; Gard and Lazarides, 1980; Zehner and Paterson, 1983). When myoblasts fuse to form myotubes desmin first has a diffuse longitudinal intracellular distribution in the cytoplasm. As the myotubes mature and sarcomeres are organized into visible cross-striations, desmin becomes localized to the Z bands, where it has been found to connect myofibrils to the sarcolemma and to attach actin filaments to the Z bands (Granger and Lazarides, 1979; Holtzer et al., 1982; Tokuyasu et al., 1983). However, it has been shown that muscle differentiation apparently proceeds normally in vitro when the vimentin and desmin networks are disrupted (Schultheiss et al., 1991).

The apparent dispensability of vimentin and desmin and the fact that a more distantly related intermediate filament is also expressed in muscle prompted us to characterize the temporal and spatial distribution of nestin in muscle. We have analysed the mRNA expression and protein distribution of nestin, and compared it with that of desmin and vimentin during skeletal muscle development and in vitro myogenic differentiation. Our data demonstrate that nestin is expressed predominantly early in muscle development, but can be detected in the Z bands of postnatal myofibers. In contrast, desmin is more abundant at later, more mature stages of muscle development. When expressed in the same cell nestin appears to copolymerize with both desmin and vimentin, suggesting that during stages of coexpression networks are produced that are mixtures of evolutionarily quite diverged intermediate filaments.

**MATERIALS AND METHODS**

**Cell culture**

The G6 human myoblast cell line was derived from a clone originating from thigh muscle of a 73-day-old, aborted fetus (Jin et al., 1993). Growth medium used for myoblast proliferation was Ham’s nutrient mixture F-10 with 20% fetal calf serum and 0.5% chicken embryo extract. Differentiation medium was Dulbecco’s modified Eagle’s medium with 5% horse serum.

**RNA blot analysis**

Purifications of total RNA, selection of poly(A)+ RNA, gel fractionation, RNA blottings, RNA hybridizations, washing procedures, and determinations of sizes of the transcripts were performed essentially as described earlier (Jin et al., 1991).

**DNA probes**

For northern blot hybridizations the following antisense oligonucleotide probes were used for developing rat muscle:

- Mouse nestin 48mer,
  - (5′-GGTCCCTGGAATCTTGATTCTCTTGTCGACCACCTTTCTGTTGT-3′) (Zimmerman et al., 1994);
- Hamster desmin 48mer,
  - (5′-CTTCAGAACCCTTTGTCCAGGCTGTTTCCTCCTGAAATGACAGCAG-3′) (Quax et al., 1984);
- Mouse vimentin 48mer,
  - (5′-GTCCTCATGACCCTGATCCATCCATCTGCTGCTCCTACACG-3′) (EMBL database).

For G6 cells the following probes were used:

- Human desmin 48mer,
  - (5′-CTTGATCATACCGGCTTCTTGAGATGAGCCACCTCAGAACCCCTTTGCTCAGGG-3′) (Li et al., 1989);
- Human vimentin 48mer,
  - (5′-CAGGAGTGTCCCTTTCTTGATGATATGCAACAGGAGTTGAAATCCACG-3′) (Ferrari et al., 1984).
- As control, a second 50mer from human vimentin was used:
  - (5′-CGTGATGCAGAGAATCTGCTGCTGCTGCTCCTACACG-3′).

Mouse α-actin pAM 91 (Minty et al., 1981) and PstI fragments of mouse fast myosin heavy chain HHC 32 (Weydert et al., 1983) were also used as probes. Human nestin RNA was analysed with a genomic probe containing the first exon of the human nestin gene (Dahlstrander et al., 1992b).

**Protein blot analysis**

Cultured cells were scraped in PBS, lysed in SDS sample buffer, and boiled briefly (Laemmli, 1970). A 2 µg sample from each differentiation state was subjected to electrophoresis in a denaturing polyacrylamide (12%) gel and the electrophoresed proteins were blotted onto nitrocellulose filters (Millipore) by a Bio-Rad electrophrotter. After preadsorption with 5% non-fat dried milk and 20% fetal calf serum in PBS overnight at 4°C, parallel filters were incubated overnight at 4°C with the rabbit anti-nestin antiserum no. 130 (Dahlstrand et al., 1992a) diluted 1:2000 in PBS, a mouse monoclonal anti-desmin antibody (DE-B-5, Boehringer) diluted 1:800 in PBS, or a mouse monoclonal anti-vimentin antibody (Vim 3B4, Boehringer) diluted 1:80 in PBS, and then rinsed three times in PBS. Antibody binding was detected by use of alkaline phosphatase-conjugated second antibodies (Dakopatts) or by second antibodies followed by avidin and biotinylated horseradish peroxidase (Dakopatts), according to the manufacturer’s suggestions. RainbowTM protein molecular mass markers (Amer sham) were used.
**Immunohistochemistry**

Skeletal muscle from the thigh of a 15.5 days post-coitum (dpc) rat embryo and from the thigh of a 15-day-old postnatal rat was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Sections were deparaffinized for 3 x 10 minutes in xylene, rehydrated, and incubated for 5 minutes with 3% hydrogen peroxide, followed by a 20 minute incubation with 3% bovine serum albumin in PBS. Adjacent sections were subsequently stained with the anti-nestin antiserum (diluted 1:1000 in PBS), or with the antibodies to desmin (diluted 1:200 in PBS), or to vimentin (diluted 1:50 in PBS). The immunostaining was visualized by use of avidin and biotinylated horseradish peroxidase (Dakopatts). Double-immune staining of postnatal muscle was performed as outlined below. To visualize the morphology of the tissue, additional sections were stained with eosin/hematoxylin.

**Immunocytochemistry**

G6 cells grown on coverslips were fixed for 10 minutes with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS for 3 minutes, rinsed three times in PBS, incubated for 30 minutes with 3% bovine serum albumin in PBS, and subsequently incubated overnight at room temperature with the anti-nestin antiserum (diluted 1: 500 in PBS), or with antibodies to desmin (diluted 1: 200 in PBS) or to vimentin (diluted 1: 20 in PBS). Following three 5-minute washes in PBS, the coverslip was incubated with an appropriate second antibody (FITC goat anti-rabbit IgG, Boehringer (605210), diluted 1:100 or RTITC goat anti-mouse IgG, Boehringer (605140), diluted 1:100) for 2 hours at room temperature and washed 3x 5 minutes in PBS. For double-immune staining samples were first incubated with the anti-nestin antiserum and its appropriate second antibody, followed by either desmin or vimentin antiserum and the appropriate second antibody, with washes in PBS for 3x 5 minutes between each incubation. Control experiments where either of the first antibodies was omitted resulted in loss of signal corresponding to the omitted antibody, demonstrating that no cross-hybridization occurred. Immunoreactive material was visualized in a fluorescence microscope and photographed with Kodak 400 ASA T-max film.

**RESULTS**

**Analysis of mRNA levels of nestin, vimentin and desmin during rat muscle development**

To characterize the expression of nestin during muscle development, and to compare it with the expression patterns of desmin and vimentin, poly(A)+ RNA from a 13.5 dpc embryo and from rat thigh muscle from various stages of development was analysed by the northern blotting technique with probes specific to the rat nestin, vimentin and desmin genes (Fig. 1). In all three cases a single, specific hybridization signal was obtained, corresponding to the expected mRNA size for each intermediate filament gene. The sizes of the nestin, vimentin and desmin mRNAs were 6.7, 2.0 and 2.6 kb, respectively.

Desmin and vimentin transcripts were easily detected in the 13.5 dpc embryos, whereas nestin transcripts were barely visible. In prenatal muscle tissue from 15.5 dpc rat thigh muscle nestin and vimentin transcripts were more abundant, while desmin RNA was almost undetectable. At later stages, the expression of desmin gradually increased to reach maximal levels in the adult rat, with kinetics similar to the increase in myosin heavy chain expression.

Nestin and vimentin transcripts were found in early postnatal thigh muscle, but the levels became downregulated by postnatal day 21, and were strongly reduced in the muscle from a 5-month-old rat. The relative levels of nestin mRNA were transiently reduced around birth, producing a biphasic expression pattern, whereas vimentin mRNA levels were more uniform during the same time period.

**Immunohistochemical analysis of intermediate filament proteins in pre- and postnatal rat skeletal muscle**

Given the expression of nestin in both pre- and postnatal muscle, we next decided to analyse the distribution of the nestin protein at an early and a late stage of muscle development, and to compare it with that of vimentin and desmin. Sections from fixed, embedded thigh muscle from a 15.5 dpc rat embryo and from a 15 day postnatal rat were produced. Cross-sections of embryonic thigh muscle, together with surrounding mesodermal and epidermal tissue, were easily identified by eosin/hematoxylin staining on such sections (Fig. 2).

**Fig. 1.** RNA blot analysis of nestin, vimentin and desmin expression in developing skeletal muscle. A 9 μg sample of poly(A)+ RNA was analysed in each lane, from the following tissues and stages: whole 13.5 dpc embryo (d 13 w.e.); crude thigh muscle tissue from 15.5 dpc (d 15 m.); 17.5 dpc (d 17 m.); 18.5 dpc (d 18 m.); 20.5 dpc (d 20 m.); and from newborn (d 1 m.); 7 days postnatal (d 7 m.); 14 days postnatal (d 14 m.); 21 days postnatal (d 21 m.); and from a 5-month-old rat (Adult m.). The same filter was sequentially hybridized with probes to rat nestin (Nes), vimentin (Vim), desmin (Des), and myosin heavy chain (MHC). Sizes are denoted in kb to the right.
Adjacent sections from the 15.5 dpc muscle were stained with antisera specific to nestin, vimentin and desmin (Fig. 2). Staining with the polyclonal anti-nestin antiserum showed immunoreactivity in developing muscle fibres, and in endothelial cells (Fig. 2). The staining was evenly distributed in most muscle fibres in the thigh muscle. Staining of endothelial cells has previously been observed during human brain development (Tohyama et al., 1992), and in adult human brain and brain tumors (Dahlstrand et al., 1992a; Tohyama et al., 1992). The monoclonal antibody to vimentin produced a more widespread pattern. Developing skeletal muscle was stained, as well as the surrounding connective tissue and dermis, but no staining was observed in endothelial cells (Fig. 2). Finally, the monoclonal anti-desmin antibody produced a distinct and evenly distributed staining pattern in the muscle fibres, with little or no staining of other mesodermal or epidermal tissues (Fig. 2).

In the postnatal day 15 thigh muscle, a stage when nestin mRNA levels were reduced but still detectable (Fig. 1), longitudinal sections were analysed with the three different antibodies (Fig. 3). The anti-nestin antiserum stained a subset of muscle fibers in a banded pattern (Fig. 3a). The desmin antibody produced a similar banded staining of the myofibers (Fig. 3b). By double immunofluorescence it was shown that nestin and desmin antisera stain the same banded structure (Fig. 3c,d). Since it has previously been established that desmin is localized at the Z bands (Granger and Lazarides, 1979), we conclude that nestin in young myofibers is also located at Z bands. We frequently observed that desmin staining (Fig. 3d) in muscle fibrils was more widespread than nestin staining (Fig 3c). This was also seen when alternate sections were stained with nestin and desmin, respectively (data not shown). This is probably due to a broader expression pattern for desmin, as suggested by the RNA data, but we can not formally exclude the possibility that the accessibility for the anti-nestin antiserum was different than for the anti-desmin antibody. In contrast to the nestin and desmin experiments, we could not detect any immunoreactivity using the anti-vimentin antibody (data not shown), in accordance with previous observations in mouse and man (Bennett et al., 1979; Gard and Lazarides, 1980; Zehner and Paterson, 1983).

Intermediate filament expression and intracellular location during in vitro differentiation of the myogenic cell line G6

The extensive temporal overlaps in expression and the colocalization of nestin and desmin in Z bands prompted us to analyse the intracellular localization of the proteins in more detail. We found the human myoblast cell line G6, derived
from a 73-day-old aborted fetus (Jin et al., 1993), to be particularly suitable for this purpose. First, G6 myoblasts differentiate spontaneously under low serum conditions to form multinucleate myotubes, and the morphological differentiation is accompanied by changed expression patterns of the muscle determination genes Myf3-6 and induction of creatine phosphokinase, α-actin and myosin heavy chain (Jin et al., 1993). Second, upon differentiation both typical elongated myotubes and very flattened myotubes are formed, and the latter are particularly suitable for a detailed morphological study of the intermediate filament network in the early myotube.

Fig. 3. Immunohistochemical analysis of nestin and desmin in postnatal skeletal muscle. Longitudinal sections from postnatal day 15 rat thigh muscle were analysed with antibodies to nestin (a) and desmin (b). Immunoreactive material was visualized by a peroxidase reaction. Double immunofluorescence analysis of nestin (c) and desmin (d) in the same section. Bars, 50 µm.
We first analysed the G6 cells for expression of the three intermediate filaments. In northern blot experiments mRNAs of sizes expected for nestin, vimentin and desmin were observed in both undifferentiated and differentiated cells, using probes for the three human genes (Fig. 4). Vimentin RNA levels were reduced during differentiation. As control, the same filter was rehybridized with a probe detecting \( \alpha \) and \( \beta \)-actin. Differentiation resulted, as expected, in downregulation of \( \beta \)-actin and upregulation of \( \alpha \)-actin RNAs. The downregulation of vimentin and \( \beta \)-actin was more pronounced after six days in differentiation medium, both in the G6 cell line and in cells from the E6 line, another myogenic clone derived from the same fetus as G6 (Fig. 4). During the same period \( \alpha \)-actin and myosin heavy chain (MHC) mRNA levels were elevated, as expected. Interestingly, the reduction of \( \beta \)-actin mRNA levels parallels that of vimentin in both experiments. To rule out any possibility of cross-hybridization between vimentin and \( \beta \)-actin the filter was rehybridized with a different probe for vimentin. An identical pattern was obtained with the second vimentin probe (data not shown). To control for tissue specificity of intermediate filament expression a fibroblast cell line, G11, derived from the same human fetus as G6, was analysed. Expression of vimentin and \( \beta \)-actin was observed, but not of nestin, desmin and \( \alpha \)-actin, showing that nestin and desmin are not promiscuously expressed in all cell lines (Fig. 4).

To prove that the different antisera did not cross-react we performed immunoblot experiments on proteins from the G6 cell line. The anti-nestin antiserum produced a predominant approximately 200 kDa band from the various stages of in vitro differentiation, and in addition bands of slightly lower molecular masses (Fig. 5). The antibodies to desmin and vimentin produced a set of prominent bands in the 50-60 kDa range, and very weak bands in the 100-200 kDa range (Fig. 5). The reasons for the presence of the additional bands in the 50-60 kDa range observed for the desmin, and in particular for the vimentin antisera, are not known. They could possibly represent partial proteolysis or post-translational modifications, as previously observed (Granger and Lazarides, 1979), but may also be caused by cross-reactivity to other intermediate filament proteins in the same size range. In any case, the western blot experiment strongly argues against the possibility that the anti-

---

**Fig. 4.** RNA blot analysis of intermediate filament expression during in vitro differentiation of a human myogenic cell line. A 5 \( \mu \)g sample of poly(A)* RNA was analysed in each lane. (a) G6 cells cultured at low density or high density in growth medium, and G6 myoblasts cultured for 1, 2 or 3 days in differentiation medium (denoted Diff 1d, Diff 2d, and Diff 3d, respectively). (b) G11 fibroblasts (G11 Fb), G6 myoblasts in growth medium (G6 Mb), G6 myotubes after 6 days in differentiation medium (G6 Mt), E6 myoblasts in growth medium (E6 Mb), and E6 myotubes 6 days in differentiation medium (E6 Mt). Sizes are denoted in kilobases to the right.

**Fig. 5.** Identification of intermediate filament proteins in western blots. A 2 \( \mu \)g sample of protein from the G6 cell line under proliferating myoblast conditions (P), or after 8, 15, 24, 48 and 72 hours in differentiation medium was western blotted with antisera to nestin (Nes), desmin (Des) and vimentin (Vim). Migration of molecular mass markers of indicated size in kDa is shown on the left margin.
Fig. 6. Intracellular localization of intermediate filaments by double immunofluorescence experiments. Undifferentiated G6 cells were fixed and stained with a rabbit polyclonal anti-nestin antiserum (a) and a mouse monoclonal anti-vimentin antibody (b); or with the anti-nestin antiserum (c) and a mouse monoclonal anti-desmin antibody (d). G6 cells after 3 days in differentiation medium were stained with the anti-nestin antiserum (e) and the anti-vimentin antibody (f); or with the anti-nestin antiserum (g) and the anti-desmin antibody (h). Note the perinuclear distribution of the intermediate filaments in the undifferentiated cells and the visualization of long fibrils in the sac-like myotubes in the differentiated cells. Nestin and vimentin or desmin networks are indistinguishable in the majority of cells. Bar, 10 µm.
nestin antiserum cross-reacts with lower molecular mass intermediate filaments.

Access to non-cross-reacting antibodies produced in different species made it possible to analyse the intermediate filament network in G6 cells by double-label immunofluorescence cytochemistry for nestin and either of the other two intermediate filaments (Fig. 6a-h). In undifferentiated cells all three antibodies revealed a typical intermediate filament pattern, with strong perinuclear staining and filaments radiating out towards the periphery of the cell (Fig. 6a-d). Most myoblasts displayed the three intermediate filaments, and the patterns of nestin and vimentin (Fig. 6a,b) or nestin and desmin (Fig. 6c,d) were indistinguishable in the vast majority of cells, including areas with a more elaborate filamentous network. However, a variation in the relative staining intensity between the individual intermediate filament proteins was noted in some cells. After in vitro differentiation a large proportion of the myoblasts differentiated and fused into multinucleated myotubes (Fig. 6e-h). In some of the resulting myotubes, large flat sac-like structures were observed. Immunocytochemical staining revealed the presence of nestin, vimentin and desmin in the myotubes, including the flat structures. The filaments were much longer in the multinuclear cells, and sometimes ran almost in parallel in the flat areas, while the perinuclear organization was less pronounced in multinuclear cells (Fig. 6e-h). The filament pattern could be analysed in detail in the flat areas and, again, nestin appeared to colocalize with vimentin (Fig. 6e,f) and desmin (Fig. 6g,h), producing indistinguishable patterns.

DISCUSSION

Myogenic differentiation is closely associated with complex changes in morphology and cellular organization. It is therefore reasonable to assume that changes in the composition of the cytoskeleton are an inherent feature of this process. To investigate this in more detail, we have analysed the expression pattern and intracellular localization of the recently discovered intermediate filament nestin during skeletal muscle development. Our results show that nestin is expressed predominantly early, but with considerable temporal and spatial overlap with both vimentin and desmin, and that the three proteins produce cytoplasmic, filamentous networks that are indistinguishable by conventional immunocytochemistry.

Distinct expression patterns during muscle development

During muscle development the expression patterns for the three intermediate filaments are distinct. Nestin mRNA is most abundant during early stages, and sharply downregulated in the adult muscle, whereas desmin mRNA levels increase throughout muscle development. Vimentin mRNA levels are also highest during the early stages, but the expression profile is monophasic in contrast to nestin, which has a biphasic mode of expression, with reduced mRNA levels around birth. This has previously been observed also for PDGF β-receptor transcripts (Jin et al., 1993), and may reflect the effect of dramatic but transient hormonal changes around the time of birth.

The expression pattern can thus be viewed as a transition from one type of intermediate filament to another, i.e. from nestin and vimentin during the early stages to desmin in the fully differentiated muscle. Developmental successions of intermediate filaments are also found in skin, where different keratin genes are expressed at particular stages of keratinocyte maturation (Fuchs and Coulombe, 1992), and in the developing CNS. Interestingly, in the early CNS nestin and vimentin are coexpressed in the mitotically active neuroepithelium of the neural tube and, upon differentiation to neurons and glial cells, nestin is downregulated and replaced by neurofilaments and GFAP, respectively (Lendahl et al., 1990). Thus, the transient expression of both nestin and vimentin, succeeded by the expression of another intermediate filament in the differentiated cell type, is a common feature for both muscle and CNS development. It appears, however, that the period of coexpression between nestin and the subsequent intermediate filament is more extended in muscle than in CNS development.

Recent experiments have addressed how the regulatory regions mediating the CNS and muscle expression of nestin are organized. Various regions from the nestin gene were fused to a reporter gene and analysed for expression in transgenic mice. Two distinct regulatory regions were identified: the second intron is sufficient to reproduce the developing CNS expression pattern, while the first intron directed lacZ expression to the myotomes of the somites in the embryo. The sequences in the first intron contain two so-called E box motifs (ACACGTGG and GCAGCTGG), suggesting that nestin transcription may be regulated by binding of myogenic transcription factors of the helix-loop-helix type.

Indistinguishable filamentous networks

Nestin produces a cytoplasmic filamentous pattern indistinguishable from that of vimentin and desmin in the myogenic cell line G6. Considering the evolutionary relationship between the genes this finding may not have been expected a priori. It has previously been shown that closely related intermediate filament proteins, belonging to the same class, can copolymerize, whereas more distantly related proteins may not. Thus, the class III intermediate filament proteins vimentin and GFAP copolymerize (Sharp et al., 1982), and vimentin and desmin are found in the same filaments in cultured cells (Quinlan and Franke, 1982; Töll et al., 1986). However, vimentin and keratins, which belong to different classes, form quite distinct cytoplasmic structures (Franke et al., 1979). The class VI intermediate filament nestin is evolutionarily less closely related to vimentin and desmin, and in fact belongs to the other main branch of the cytoplasmic intermediate filament gene family, together with internexin and the neurofilaments (Dahlstrand et al., 1992b). In the central α-helical domain, which is critical for polymerization of intermediate filaments (see Stewart, 1993, for review), vimentin and desmin are 74 % conserved at the amino acid level. In contrast, nestin is only 28 and 30 % conserved to desmin and...
vimentin, respectively, in this domain; yet the filament patterns are indistinguishable.

The intermediate filament network undergoes a dramatic reorganization from the primarily perinuclear organization in G6 myoblasts to the long parallel fibers in multinuclear myotubes. The fact that nestin, vimentin and desmin produce a common type of network before in vitro differentiation, and another, different form after differentiation, suggests that the reorganization takes place in a concerted manner for the three intermediate filaments. It is reasonable to assume that this is also the case in vivo, since nestin is expressed in the same cells as vimentin and desmin during early muscle development, and since nestin and desmin are found in the Z bands of postnatal myofibers.

**A dynamic intermediate filament network in developing muscle**

The described findings highlight a longstanding question: why are different intermediate filaments expressed at different times during development of tissues like muscle, CNS (Lendahl et al., 1990) and skin (Fuchs and Coulombe, 1992), when they form very closely related, if not identical, filamentous networks? One possibility is that the individual intermediate filaments do not confer qualitative differences on the filamentous network per se, but that the members expressed early are particularly suited to establish the filamentous structures. This could be achieved by faster polymerization kinetics or better de novo polymerization characteristics. In muscle, this may be the role of nestin and vimentin, whereas desmin, found in the differentiated myotubes, may instead form a more robust and long-lived network, better suited to be maintained in differentiated cells. It is interesting to observe that the order of intermediate filament expression in developing CNS, i.e. transient nestin and vimentin expression, also fits this model, and that neurofilaments and GFAP may play special roles in the differentiated CNS cells.

An alternative view is that the various intermediate filaments in fact provide qualitative differences for the filamentous structure, and that cells at different developmental stages have different requirements for their cytoskeleton. At present, we cannot distinguish between these two models, and the question of detailed functions for the different intermediate filaments has been difficult to approach. During myogenic differentiation in vitro desmin and vimentin appear to be dispensable, and myotubes are formed in the presence of disrupted desmin and vimentin networks (Schultheiss et al., 1991), which suggests that the functions may only be revealed in vivo. The best evidence for intermediate filament function comes from the linkage between mutations in keratins and certain genetic skin diseases and experiments with mutated keratin genes introduced into transgenic mice (see Fuchs and Coulombe, 1992, for review). In addition, overexpression of neurofilament genes in transgenic mice produces a motoneuron phenotype similar to that found in patients with amyotrophic lateral sclerosis (Côté et al., 1993; Xu et al., 1993). Similar experiments in transgenic mice with nestin, vimentin and desmin may reveal more about their biological functions, interplay in vivo, and possible roles in neuromuscular disorders. Given the data on keratins it is tempting to speculate that aberrant organization of nestin and desmin may contribute to the pathology of certain myopathies. In support of this, several cases of myopathy have been reported to be associated with accumulation of large, dense desmin-containing sarcoplasmic bodies, not integrated into the normal intermediate filament network (Edström et al., 1980; Pellissier et al., 1989; Prelle et al., 1992; Rappaport et al., 1988).

Our data thus suggest that nestin is a bona fide component of the dynamic intermediate filament network in skeletal muscle and, although we still know very little about its function, the data provide a more complete picture of the transitions in expression during muscle development. In addition, the transient nestin expression pattern may be helpful in the diagnosis of pathogenic conditions accompanied by muscle regeneration. Intermediate filaments have been widely used in diagnosis of tumors and other pathological conditions because of their tissue-specific expression and distinct cytoplasmic location (Osborn and Weber, 1989). Transient expression of nestin in developing CNS and its reappearance in CNS tumors make nestin an interesting CNS tumor marker (Dahlstrand et al., 1992a; Tohyama et al., 1992), and it will be interesting to learn if nestin also reappears in myopathogenic situations.

We thank Mrs Gabriella Dombos for excellent technical assistance. This work was supported by grants from the Swedish Cancer Society and Karolinska Institutets fonder (T.S., U.L.), the Swedish Child Cancer Fund (T.S.) and by the Swedish Medical Research Council, Margaret and Axel Ax:son Johnsons Stiftelse, Knut and Alice Wallenbergs Stiftelse, Magn. Bergvalls Stiftelse, and Stiftelsen Lars Hiertas Minne (U.L.).

**REFERENCES**


Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. *Neuron* (in press).

(Received 22 June 1993 - Accepted 23 August 1993)