Desmin and titin expression in early postimplantation mouse embryos

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Summary

The expression of the intermediate filament (IF) constituents desmin, vimentin and keratin, as well as the striated-muscle-specific marker titin, was studied in mouse embryos of 8.0 to 9.5 days post coitum (d.p.c.), using the indirect immunofluorescence technique in combination with polyclonal and monoclonal antibodies. During the development of the embryo, desmin was first detected at 8.25 d.p.c. in the ectoderm, where it was transiently coexpressed with keratin and vimentin. At later stages, the ectoderm contained only keratin and to a certain extent also vimentin IF. At 8.5 d.p.c., desmin was found exclusively in the heart rudiment, and remained present with increasing intensity in the myocardial cells during later cardiogenesis. Striation of desmin in the heart muscle cells was observed in 9.5 d.p.c. embryos. At these stages (8.5–9.5 d.p.c.), triple expression of the IF proteins desmin, vimentin and keratin was evident in these cells. From 9.0 d.p.c. onwards, desmin could be detected in the myotomes as well. Immunoblotting studies of 9.5 d.p.c. mouse embryos confirmed the immunohistochemical data.

Titin was found in the early heart anlage at stage 8.25 d.p.c., when no desmin expression was observed in this tissue. At this stage the titin appeared in a punctate pattern, similar to that observed in cardiac myofibrils of early chicken embryos (Tokuyasu and Maher, 1987; J. Cell Biol. 105, 2781–2793). In 8.5 d.p.c. mouse embryos, this punctate titin staining pattern was still observed, while, at this stage, a filamentous staining reaction could be seen with the desmin antibodies. During further development, cross-striation was detected within myocardial cells using the polyclonal titin antibody from 9.0 d.p.c. onwards, i.e. before such striation could be detected with the desmin antibodies.

From these data, we conclude that titin synthesis may anticipate desmin expression in the developing mouse myocard, although the level of expression of the former protein remains low until 9.0 d.p.c.

Key words: desmin, titin, mouse cardiogenesis, heart muscle, myotome, keratin, vimentin, fibronectin.

Introduction

The protein constituents of intermediate-sized filaments of the cytoskeleton in cells of adult animals have been described to be expressed in a more or less tissue-specific fashion (Quinlan et al. 1985). Keratin normally occurs only in epithelial tissues, while neurofilaments and glial fibrillary acidic protein (GFAP) are mainly restricted to neural cell types (Debus et al. 1983a; Shaw et al. 1986; for review see Holtzer et al. 1982). Desmin has so far been found to be virtually muscle specific (Debus et al. 1983b; for exceptions see Molengraft et al. 1986 and Franke and Moll, 1987), while vimentin occurs in cells of mesenchymal origin, but is also occasionally coexpressed with the other intermediate filament proteins (IFP) (Franke et al. 1982). The expression of IFP in developing embryos has been studied by several investigators (reviewed by Traub, 1985). From these results, it has become obvious that, apart from the lamins, cytoskeletons are the first cytoplasmic IFP to be expressed in early embryogenesis (Jackson et al. 1981). Embryonal cells containing this type of IFP are multipotential in nature and switch on the expression of vimentin when differentiating into mesenchymal cells (Franke et al. 1982; Traub, 1985). Subsequently, differentiation into mature cells results in the expression of the more specific IFP, such as desmin in muscle cells and GFAP in glial cells. However, coexpression of vimentin with these IFP is seen during embryogenesis and even after birth (Osborn et al. 1981; Lazarides et al. 1982; Schnitzler et al. 1981). During myogenesis vimentin and desmin are expressed in sequence in differentiating myocytes (Fischman, 1986). In replicating myoblasts, vimentin is found in early stages of development at which desmin is undetectable. When the myogenic cells withdraw from the cell cycle at an early stage of myofibrillogenesis, desmin expression is initiated (Fischman, 1986). As muscle cell differentiation proceeds, desmin expression becomes more prominent, while its localization becomes more and more restricted
to the outer circumference of the Z-band, both in skeletal and cardiac striated muscle cells (Fischman, 1986; Hill et al. 1986; Danto and Fischman, 1984). Most of the work on IFP expression in muscle morphogenesis has concentrated on avian tissues (Tapscott et al. 1981; Bennett et al. 1981; Tokuyasu et al. 1984), while mammalian myofibrillogenesis has hardly been studied at this level. Since fundamental differences have, however, been noted during myogenesis in avians and mammals (Kaufman and Navaratnam, 1981; Bignami et al. 1984), we have studied desmin and vimentin expression in mouse embryos at 8.0 to 9.5 days post coitum (d.p.c.). It was postulated that during avian in vivo and in vitro myofibrillogenesis desmin is one of the earliest known markers for cells in the myogenic lineage and that proteins such as titin and myosin heavy chain occur later. We also studied the development of titin, which has been identified as a specific component in cardiac and skeletal muscle (Maruyama et al. 1976; Wang et al. 1979; Maruyama, 1986).

Materials and methods

Embryos
The embryos used for this study were from spontaneous matings of mice of the C57Bl/CBA (BCBA) strain. The presence of a vaginal plug indicated a successful mating. The conceptuses were thus presumed to be 0.5 days old at noon (12:00) on the day the vaginal plug was found. At 8.0, 8.25, 8.5, 9.0 and 9.5 days post coitum (d.p.c.) embryos were dissected from the uterus and washed in cold phosphate-buffered saline (PBS; 4°C). Embryos were quickly frozen in liquid N2 after removal from the uterus and immersed in Tissue Tek. The developmental stages are designated according to Thieier (1972).

Indirect immunofluorescence microscopy
5 μm thick cryostat sections were air dried, fixed with methanol at −20°C for 5 min, followed by acetone fixation at −20°C for 1 min. After air drying for 15 min at room temperature, the sections were incubated with the primary antibody for 30 min at room temperature, and washed three times (5 min each) in PBS. Then they were incubated with the secondary antibody for 30 min at room temperature and again washed three times (5 min each) in PBS, for 5 min in distilled water and finally for 5 min in methanol. Sections were mounted in Mowiol (Hoechst, Frankfurt, FRG) containing 2.5% (w/v) NaN3 to retard fading (Johnson and Davidson, 1982). Slides were examined using a Zeiss Universal microscope equipped with epillumination optics.

Gel electrophoresis and immunoblotting
Cytoskeleton preparations from 9.5 d.p.c. mouse embryos were made as follows. After dissection of the embryos from the uterus, a high-salt buffer (1.5 M-KCl, 0.5% Triton X-100, 5 mM-EDTA, 0.4 mM-phenylmethylsulphonylfluoride and 10 mM-Tris–HCl, pH 7.2) extraction was performed for 5 min at 4°C by homogenization of the embryo in a Dounce potter. The pellet obtained by centrifugation for 5 min at 12,000 g was washed with cold (4°C) low-salt buffer (5 mM-EDTA, 0.4 mM-phenylmethylsulphonylfluoride and 10 mM-Tris–HCl, pH 7.2), essentially as described by Moll et al. (1982). After a second washing step in PBS, the cytoskeleton preparation was dissolved by boiling for 5 min in SDS-sample buffer (Laemmli, 1970).

One-dimensional gel electrophoresis was performed in 10% polyacrylamide slab gels containing 0.1% SDS (Laemmli, 1970). To compare the amounts of protein loaded on each lane, gels were stained with Coomassie Brilliant Blue R250 (Gurr, Hopkin and Williams, Chadwell Heath, Essex, UK) as described before (Broers et al. 1986). Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). In the first dimension, isoelectric focusing was performed in 4% polyacrylamide (Biorad Laboratories, California, USA) rod gels containing 1% Biolyte, pH 3.5–10 (Biorad Laboratories, California, USA). For the second dimension, the rod gels were applied directly onto the stacking gel of SDS-polyacrylamide gels.

For immunoblotting experiments, the electrophoretically separated polypeptides were transferred to a nitrocellulose sheet (Schleicher and Schüll Membrane Filters BA 85, Dassel, FRG) by blotting for one hour at 100 V in a cold (4°C) buffer containing 25 mM-Tris–HCl, 192 mM-glycine, 0.02% SDS and 20% methanol (pH 8.3) (Towbin et al. 1979). The blots were incubated for 90 min with PBS containing 0.05% Tween 20 (Sigma). All reagents were diluted in this solution, which was also used for the washing steps. After incubation overnight with undiluted culture supernatants of the primary antibodies RD301, RV202, CK18-2 or BV1118, the blots were washed three times for 10 min and incubated for 1 h with peroxidase conjugated rabbit anti-mouse IgG (DAKOpatts, Glostrup, Denmark) diluted 1:400. The blots were washed again three times for 10 min in 0.05% Tween 20/PBS and once in PBS alone for 10 min and stained with 4-chloro-1-napthol (Merck, Darmstadt, FRG) and 0.12% hydrogen peroxide (Merck, Darmstadt, FRG). After staining the blots were rinsed for 5 min with water.

Antibodies
The following antibody preparations were used in this study:
1. A polyclonal antisem to chicken gizzard desmin (pDes). Preparation and characterization of this rabbit antisem have been described elsewhere (Ramaekers et al. 1985). For indirect immunofluorescence microscopy, this serum was diluted 1:50 in PBS.
2. Two mouse monoclonal antibodies to desmin (DEB5 and DER11; DAKOpatts, Glostrup, Denmark; Debus et al. 1983). These antibodies were used as undiluted culture supernatants in the immunohistochemical assays.
3. A mouse monoclonal antisem to desmin (RD301), giving a weak, although specific, reaction on muscle cells in adult and embryonic mouse when used in the indirect immunofluorescence assay, was used for the immunoblotting assays. This antibody has been described before (Pieper et al. 1989).
4. An affinity-purified polyclonal antisem to bovine lens vimentin (pVim). Purification and characterization of this polyclonal rabbit antisem have been described in detail elsewhere (Ramaekers et al. 1983).
5. A mouse monoclonal antibody (BV1118) of the IgM subclass, reactive with vimentin of human, bovine, rabbit, hamster, mouse and chicken origin (C. Viebahn, unpublished data). This antibody, when tested in the indirect immunofluorescence assay, stains tissues of mesenchymal origin such as fibroblasts, endothelial cells, some smooth muscle cells, glomeruli in the kidney, but does not stain most adult epithelial cells. In cultured cells (BHk, HeLa), a filamentous staining pattern is obtained with antibody BV1118. No significant reaction is found in cultured cells such as T24, RT4
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and MCF-7, known to be vimentin negative (Heuveljaar et al. 1989). In one- and two-dimensional immunoblotting assays of cytoskeletal extracts from bovine lens, BHK (Fig. 1), and Hela cells, this monoclonal antibody shows a positive reaction with a protein band migrating at the molecular weight level and isoelectric pH of vimentin. This antibody was used as undiluted culture supernatant in the immunohistochemical assays.

6. A mouse monoclonal antiserum to vimentin (RV202), giving a weak, although specific, reaction on stromal cells and other mesenchymal tissues in adult and embryonic mouse in the indirect immunofluorescence assay, was used for immunoblotting experiments. This antibody has been described in detail elsewhere (Ramaekers et al. 1987; Viebahn et al. 1988; Pieper et al. 1989).

7. An affinity-purified polyclonal antiserum to human skin keratins (pKer). This rabbit antiserum is described elsewhere (Ramaekers et al. 1983). For indirect immunofluorescence microscopy, this antiserum was diluted 1:10 in PBS.

8. A mouse monoclonal antibody (CK18–2) specifically recognizing cytoe八十 18 was raised against human mesenchymal cells. This antibody was used as undiluted culture supernatant (Broers et al. 1986).

9. An affinity-purified polyclonal rabbit antiserum to titin, isolated from Physarum polycephalum (Kind gift from Dr. D. Gassner, Bonn, FRG), was used in a 1:25 dilution. Preparation and specificity of this antiserum have been described by Gassner (1986). When tested on adult mouse tissues using the indirect immunofluorescence assay, this antiserum reacted with striated (skeletal- and cardiac-) muscle cells. No reaction was found in smooth muscle cells or in nonmuscle cells.

10. A polyclonal antiserum against human fibronectin was purchased from DAKO (Glostrup, Denmark) and used at a dilution of 1:120.

As secondary antibodies fluoresceine (FITC)-conjugated goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgG were used in a dilution of 1:60. All FITC-conjugated antisera were obtained from Nordic Immunochimicals (Tilburg, The Netherlands).

In order to prove that the correct protein constituents are recognized in mouse embryos, studies were performed with 9.5 d.p.c. embryos. Fig. 2 shows that with the monoclonal antiserum to vimentin, desmin and keratin 18, these individual components can be detected in cytoskeletal preparations from 9.5 d.p.c. embryos.

Fig. 1. Characterization of monoclonal antiserum BV1118 by one- (A, C, E) and two-dimensional (B, D, F) immunoblotting on cytoskeletal preparations of bovine lens (A, B) and BHK-cells (C–F). Note that antiserum BV1118 reacts exclusively with vimentin (v) and its breakdown products (A–D), both from calf and hamster. Reincubation of the immunoblots with the monoclonal desmin (d) antiserum (RD301) (E, F) confirms the vimentin-specific reaction of antiserum BV1118.

Fig. 2. Immunoblotting study on cytoskeletal preparations from 9.5 d.p.c. mouse embryos (lanes A–D). Immunoblots were incubated as follows: lane A with the monoclonal desmin antiserum (RD301); lane B with the monoclonal vimentin antiserum (RV202); lane C with RD301 and subsequently with RV202; lane D as lane C, but thereafter incubated with the monoclonal keratin 18 antiserum (CK18–2). For comparison, lane E depicts an immunoblot of a chicken gizzard desmin preparation incubated with RD301 and lane F a cytoskeletal preparation of cell line RT4 incubated with CK18–2, to show the position of desmin (d) and keratin 18 (k), respectively. v, vimentin.

Results

The results obtained in the immunohistochemical studies, performed on mouse embryos of 8.0, 8.25, 8.5, 9.0, and 9.5 d.p.c., are summarized in Table 1 and depicted in Figs 3–9.

Presomite embryo (Theiler stage II)

At 8.0 d.p.c., the polyclonal antiserum pKer shows a strongly positive reaction in the visceral endoderm. An extremely intense reaction with this antiserum was also observed in the parietal endoderm, while the mesoderm and ectoderm were virtually negative. Antibody CK18–2 reacted in a similar fashion but weaker. The vimentin antiserum (pVim) showed a pronounced reaction in the mesoderm, which was particularly obvious in
the tangentially sectioned areas of the embryos. The desmin polyclonal antiserum was negative in all embryonic tissues, but did stain the extraembryonic smooth muscle tissue of the uterus. Fibronectin was detected extracellularly in the embryonic mesoderm and in nonembryonic tissues, but not in the visceral endoderm and ectoderm. The polyclonal titin antiserum (pTitin) was also tested at 8.0 d.p.c. and found to be negative.

Heart rudiment (Theiler stage 12)
In the mouse, heart development starts in the midline as an unpaired anlage (Kaufman and Navaratnam, 1981) rostral from the neural plate and caudal from the embryonic-extraembryonic junction (Theiler, 1972). This early stage of heart formation was studied in a 8.25 d.p.c. embryo (Fig. 3) in which the heart-forming region can be seen to have bent ventrally in order to form the foregut pocket. In the sagittal sections through this region, we have observed no reaction, or at best an extremely weak staining reaction, with the desmin polyclonal antiserum (pDes) (see Fig. 3A). However, the basal plate of the neuroectoderm overlying the foregut pocket dorsally showed a positive staining reaction with the desmin antiserum while the mesoderm and endoderm were negative. Using a titin polyclonal antiserum (pTitin), single positive dots were observed in the heart rudiment (Fig. 3B). The mesoderm, the ectoderm, the endoderm and the foregut pocket were negative. The vimentin monoclonal antiserum (BV1118) (Fig. 3C) and the polyclonal antiserum against vimentin were positive in the mesoderm and the neuroectoderm as well as in the heart anlage. In particular with the monoclonal vimentin antiserum (Fig. 3C), a strong filamentous staining was found in the heart anlage. Note that there is also a strong positive staining reaction in the neuroectoderm overlying the foregut pocket, indicating coexpression with desmin in these cells. An intense reaction with the monoclonal antibody against cytokeratin 18 (CK18–2) (Fig. 3D) was observed in the visceral endoderm, the foregut pocket and the visceral layer of the yolk sac at this stage. Except for the amnion epithelium, both ectoderm and neuroectoderm are still negative for the monoclonal and polyclonal keratin antibodies. However, mesodermal cells anterior to, and in, the heart anlage show a distinct positive reaction for keratin (Fig. 3D). Fibronectin showed the outlines of the basement membrane of the endoderm and the amnion epithelium, and was detected in the mesenchyme (Fig. 3E), as well as in the heart anlage. The endoderm and ectoderm are negative with this antiserum.

A slightly more advanced stage of heart development is shown in Fig. 4 in horizontal sections of a 8.5 d.p.c. mouse embryo. Cells of the presumptive endocard can now be seen between the endoderm and the developing myocard. Desmin is now exclusively observed in the developing heart (Fig. 4A), and could not be detected in the neuroectoderm or any other region of the embryo. Titin could be detected in the developing heart region, again in a punctate distribution (Fig. 4B). Vimentin was coexpressed with desmin in this region, and showed a strong filamentous immunofluorescence staining reaction in the myocardial cells (Fig. 4C), but virtually no reaction in the endoderm. Underlying the endoderm, a few vimentin positive, but desmin negative, cells can be identified as the first endocardial cells. The neuroectoderm was still positive for vimentin (results not shown). Both the polyclonal and monoclonal keratin antisera stained cells of the heart anlage (Fig. 4D) as well as the overlying endoderm, while the neuroectoderm was negative.

Tubular heart (Theiler stage 13)
The next stage during heart development is the formation of a tubular heart in which the endocard is almost completely surrounded by myocard. Fig. 5 shows oblique sections through such a 8.5 d.p.c. embryonic heart. The formerly punctate staining pattern of titin antibodies is now replaced by a more filamentous staining pattern (Fig. 5B). The desmin-staining reaction remains filamentous, comparable to the foregoing developmental stage (Fig. 5A). With the monoclonal and polyclonal vimentin antisera (Fig. 5C), a strong positive reaction was observed in the myocard and the endocard. Also with the keratin antisera we have found a positive reaction in the myocard of the tubular heart (result not shown).

Segmented heart (Theiler stage 14)
At this stage, the anlagen of the different parts of the heart can be distinguished, i.e. the anlagen of the ventricular and the atrial compartments (Fig. 6). As in 8.5-day-old embryos, in 9.0 d.p.c. mouse embryos desmin expression seems to be restricted to the heart tissue, except for the myotomes (see below). The cells forming the myocardial wall are strongly positive with the polyclonal desmin antiserum (Fig. 6A), while the endocardial tissues are distinctively negative (asterisks in Fig. 6A). The monoclonal desmin antibody DEB5

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Fig. 3. Immunofluorescence micrographs of sagittal frozen sections from 8.25 d.p.c. embryos of the mouse (Theiler stage 12) showing the heart rudiment (HR), the foregut pocket (FP) and the neuroectoderm (NE). The sections were incubated with: (A) the polyclonal antiserum against desmin (pDes); (B) the polyclonal antiserum against titin (pTitin); (C) the monoclonal antiserum BV1118 against vimentin; (D) the monoclonal antiserum CK18–2 against cytokeratin 18 and (E) the polyclonal antiserum against fibronectin (pFN). Bars indicate 25 μm.
cross-striation was found (Fig. 7B) with this polyclonal
titin antiserum, while there was no cross-striation
found with the desmin (Fig. 7A) and the vimentin antibodies
at this stage. The endocardial cells as well as the
myocardial cells are specifically stained with the vimen-
tin antiserum (Fig. 6C). Keratin expression seems to be
drastically reduced in the heart anlage at this stage,
although part of the cells still showed a weak punctate
to fibrillar staining reaction with the monoclonal keratin
antiserum CK18–2 (Fig. 6D). Keratin-positive reac-
tions of differentiating pericardial cells are also ob-
served in the primitive oral epithelium, the thyroid
anlage, the lateral plate of the mesoderm and the
erpidermal ectoderm with the monoclonal keratin anti-
body (CK18–2). During further differentiation of myo-
cardial cells (e.g. in 9.5 d.p.c. embryos), coexpression
of desmin and vimentin is evident. With both antibodies
cross-striation can now be observed in individual cells at
this stage (Fig. 8A, B) in addition to the cross-striations
seen with the titin antibody. Keratin is still weakly
expressed in some myocardial cells of 9.5 d.p.c. mouse
embryos (results not shown).

Myotome
In the mouse, somites begin to form at Theiler’s stage
12 (Theiler, 1972). The somites at this stage are dis-
tinctly negative for desmin, titin and keratin, but
positive for vimentin. Differentiation of the somite into
dermatome, myotome and sclerotome starts at day 9.0
d.p.c. (Ede and El-Gadi, 1986; Theiler stage 14) and
this is accompanied by a positive staining reaction with
the desmin and titin antiserum in the most cranial
myotomes of the embryo (Fig. 9). Desmin staining
shows a filamentous staining pattern (Fig. 9A), while
titin antibodies give rise to both a punctate and a
filamentous staining pattern (Fig. 9B). The vimentin
antibodies stain all three somite-derived tissues (derma-
tome, myotome and sclerotome) (Fig. 9C), whereas
these tissues are distinctly negative with all the keratin
antibodies tested in this study (Fig. 9D).

Discussion
Desmin has been described to be a muscle-specific
intermediate filament (IF) component in adult ver-
tebrates (Hill et al. 1986), while titin is a marker in adult
striated muscle (Wang et al. 1979). Both components
may also serve as early markers of the anlagen of these
tissues during embryogenesis (Tokuyasu et al. 1984;
Tokuyasu and Mahler, 1987). In early stages of mam-
malian myofibrillogenesis, however, the intermediate
Fig. 5. Immunofluorescence micrographs of frozen sections from 8.5 d.p.c mouse embryos (Theiler stage 13), showing the tubular heart. Sections were incubated with (A) the monoclonal desmin antiserum (DEB5); (B) the polyclonal titin antiserum (pTitin) and (C) the monoclonal vimentin antiserum (BV1118). Bar indicates 50 µm.

filament protein (IFP) vimentin is expressed in the developing muscle cells, either without or in combination with desmin. Van Muijen et al. (1987) and Kuruc and Franke (1988) have recently shown that human myocardial cells may even coexpress three different types of IF-proteins, i.e. keratins, vimentin and desmin (see also Hüttfeld and Brandtzæg, 1985).

The main studies, so far, on the expression of muscle-specific components during embryogenesis have concentrated on the chicken system (for a review see Fischman, 1986). Recently, Tokuyasu and Maher (1987) have described the distribution of titin in chicken cardiac premyofibrill stages. Hill et al. (1986) studied the interaction between titin and desmin in postmitotic mononucleated myoblast and concluded that the spatial organization of both components was not coupled. To a much lesser extent, such experiments have been performed in the mouse system (for an example, see Sassoon et al. 1988). Studies on the formation of IF components during early mouse embryogenesis by Jackson et al. (1980, 1981) and Franke et al. (1982) have shown that no desmin expression can be detected before day 8.0 d.p.c.

In the present study, a punctate staining pattern of titin antibodies was found as the earliest sign of myogenic differentiation in the mouse heart at Theiler’s stage 12 (8.25 d.p.c.). Still at the same stage, only a few hours later, desmin expression was also initiated in the heart rudiment, showing a filamentous staining pattern, while titin still showed a punctate staining pattern. Again a few hours later when the heart changed to a tubular organization at Theiler’s stage 13, the titin pattern changed to a fibrillar intracellular distribution with the desmin-staining pattern remaining the same as in the foregoing stage. Striation of titin became apparent in the segmented heart at 9.0 d.p.c. (Theiler stage 14). A few hours later at 9.5 d.p.c., but still the same stage according to Theiler, cross-striation of desmin and vimentin was observed. Myogenesis in somites (i.e. in the myotome) was similarly accompanied by a punctate titin staining pattern (Theiler stage 14) which was followed by a change to a fibrillar-staining pattern. But here a delay between the appearance of titin and desmin could not be detected, possibly due to the speed of differentiation in myotome cells.

To our knowledge, this is the first description of the initiation of desmin expression in the vertebrate heart. Later stages of heart development have been described by Kuruc et al. (1988) in several species. Titin expression, however, has been described in the early stages of chick heart development by Tokuyasu et al. (1988), who also found a punctate staining pattern in the first stages. During further differentiation, this pattern changes into a fibrillar staining reaction.

In the underlying study, it became also evident that the first expression of mouse desmin could be noted in the neuroectoderm of 8.25 d.p.c. mouse embryos. At 8.5 d.p.c. and later stages, the neuroectodermal layer was negative for desmin.

Coexpression of different types of IFP is a common feature in developing embryonic tissues, and to some extent also in certain adult organs (reviewed by Traub, 1985; Viebahn et al. 1987, 1988). Recently, Van Muijen et al. (1987) and Gown et al. (1988) demonstrated a triple expression of keratins, vimentin and desmin in human fetal heart muscle cells. In mouse
Fig. 6. Immunofluorescence micrographs of frozen sections from 9.0 d.p.c. mouse embryos (Theiler stage 14), showing the neural tube (NT), thyroid anlage (TA), ventricular compartments (V), atrial compartments (A), and endocard (asterisks), incubated with (A) the polyclonal desmin antiserum (pDes); (B) the polyclonal titin antiserum (pTitin); (C) the monoclonal vimentin antiserum (BV1118); (D) the monoclonal keratin 18 antiserum (CK18-2). Bar indicates 100 µm.
embryos, vimentin and keratin 18 were coexpressed in the differentiating myocardium together with titin at first, and later also with titin and desmin. The endocardial cells were always stained by the vimentin antibodies only. Desmin and keratin coexpression was also found to be a transient feature in heart development by Kuruc et al. (1988). At 9.0 d.p.c., keratin reactivity in the myocardium was drastically decreased, resulting in a negative reaction in 9.5 d.p.c. mouse embryos. However, at this stage, vimentin and desmin are still coexpressed and even colocalized as concluded from their cross-striated immunofluorescence pattern.

Although in vitro studies of myogenesis show that desmin is a candidate for the first sign of myogenic differentiation, our studies clearly show that, in vivo, titin, and not desmin, is the first muscle-specific protein to be expressed in presumptive myogenic cells (Hill et al. 1986; Sassoon et al. 1988). The titin antiserum used in this study showed a specific and exclusive immunoreactivity in the heart anlage of the mouse embryo before desmin expression could be detected in this region, at 8.25 d.p.c. (Theiler stage 12). The typical punctate feature of this labeling pattern, which is comparable to that described by Tokuyasu and Mahler (1987) for early stages of cardiac myofibrillogenesis in chick embryos, supports the assumption that, at this stage, the antiserum does indeed react with titin.

Unfortunately, no immunochemical proof can be

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**Fig. 7.** Immunofluorescence micrographs of a double staining of embryonic mouse myocardium (Theiler stage 14, 9.0 d.p.c.), using (A) a monoclonal desmin antibody (DER11) and (B) the polyclonal titin antibody (pTitin). Note the cross-striation in the titin-staining reaction (arrow in B) and lack of obvious cross-striation at corresponding sites in the desmin staining (A). Bar indicates 6 μm.

**Fig. 8.** Immunofluorescence micrographs of frozen sections through the heart of a 9.5 d.p.c. mouse embryo (Theiler stage 14). Sections were incubated with (A) the polyclonal desmin antiserum (pDes) and (B) the polyclonal vimentin antiserum (pVim). Arrows show cross-striations in myocardial cells. Bar indicates 9 μm.
obtained for this observation, since the concentration of the antigen in 8.25 d.p.c. embryos is far too low to allow its detection in immunoblotting studies. At stage 8.5 d.p.c., titin is still present as spots in the developing myocard, while desmin can now clearly be detected and occurs in a filamentous fashion inside these premyocardial cells. Desmin and titin were coexpressed in 9.0 d.p.c. embryonic heart and myotome and showed similarly strong intensities in the immunofluorescence assays on frozen sections of this tissue. At this stage, however, a number of cells showed a striated titin staining pattern, while we could not observe such an organization for desmin. At 9.5 d.p.c., desmin, titin and vimentin were found to be colocalized in these crossstriations. Although it should be kept in mind that the studies so far have been performed in different systems, our observations that titin expression anticipates desmin synthesis seem in contrast with the in vitro findings of Hill et al. (1986). These authors showed desmin expression in presumptive replicating myoblasts present in embryonic chick skeletal muscle cultures. They could not detect titin until the postmitotic mononu-
cleaved myoblast stage and therefore suggested that this constituent is expressed later than desmin in the course of skeletal muscle myofibrillogenesis. Apparently, desmin and titin expression are differently coordinated in vivo and in vitro.

As far as the myotome is concerned, the present study shows almost simultaneous appearance of titin and desmin in nascent myotome cells. This establishes titin as an early myogenic marker in the myotome in addition to desmin which was hitherto thought to be the earliest marker of myotomal differentiation (Solursh and Meier, 1986; Kaehn et al., 1988; Viebahn, 1989). Furthermore, the characteristic developmental change of the intracellular distribution of titin from punctate to fibrillar is seen in the myotome as well as in the heart, suggesting similar sequences of early myogenic differentiation operating both organs.

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