Desmin/Vimentin Intermediate Filaments Are Dispensable for Many Aspects of Myogenesis

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Abstract. An expression vector was prepared containing a cDNA coding for a truncated version of the intermediate filament (IF) protein desmin. The encoded truncated desmin protein lacks a portion of the highly conserved α-helical rod region as well as the entire nonhelical carboxy-terminal domain. When transiently expressed in primary fibroblasts, or in differentiating postmitotic myoblasts and multinucleated myotubes, the truncated protein induces the complete dismantling of the preexisting vimentin or desmin/vimentin IF networks, respectively. Instead, in both cell types vimentin and desmin are packaged into hybrid spheroid bodies scattered throughout the cytoplasm.

Despite the complete lack of intact IFs, myoblasts and myotubes expressing truncated desmin assemble and laterally align normal striated myofibrils and contract spontaneously in a manner indistinguishable from that of control myogenic cells. In older cultures the spheroid bodies shift from a longitudinal to a predominantly transverse orientation and loosely align along the I-Z-I-regions of striated myofibrils (Bennett, G. S., S. Fellini, Y. Toyama, and H. Holtzer. 1979. *J. Cell Biol.* 82:577–584), analagous to the translocation of intact desmin/vimentin IFs in control muscle. These results suggest the need for a critical reexamination of currently held concepts regarding the functions of desmin IFs during myogenesis.

SINCE their description in maturing myotubes and chondroblasts over twenty years ago (38), intermediate filaments (IF)[1] have been the subject of considerable study. Much has been learned concerning the requirements for particular protein domains, as well as sites of phosphorylation, for IF assembly in cell-free systems (22, 36, 37), for incorporation of IF proteins into preexisting filament networks in vivo (1, 2, 24, 46, 57, 66, 69), as well as for de novo IF assembly within living cells (57). The sequence of several IF genes have been delineated, and the flanking regulatory regions of some have been determined as well (52, 56, 70; and see reference 61 for review). In contrast to this steady accumulation of molecular and biochemical data, and of information regarding the temporal and spatial expression of members of this multigene family during development (17, 31, 50, 51), virtually nothing is known about the biological function(s) of any class of IFs.

Several experimental approaches have been taken to attempt to uncover functional roles of IFs. Acrylamide has been shown to induce alterations in intermediate filament distribution within cells, however, it is cytotoxic when used for extended periods of time, and also causes alterations in general levels of protein synthesis (15). Several groups have microinjected antibodies to IF proteins into cultured cells. While the antibodies induce transient collapse of a small fraction of the IF network for a short period of time, no detectable abnormalities in cell division or motility have been detected (20, 40, 44, 64). Ectopic expression of IF proteins in transgenic mice thus far has produced little in the way of cytological perturbations, aside from defects in lens formation (7, 14, 48, 53). A recent publication reports that transgenic mice expressing a truncated cytokeratin molecule have disrupted cytokeratin filaments in the basal layers of the epidermis and exhibit a phenotype of easy blistering (67).

Desmin IFs are found predominantly in skeletal, cardiac, and smooth muscle cells (4, 19). In the skeletal myogenic lineage, the majority of normal replicating presumptive myoblasts are vimentin+/desmin−. Invariably these cycling myogenic cells are negative for all myofibrillar proteins. However, both in vivo and in tissue culture, a small subset of replicating presumptive myoblasts become vimentin+/desmin+ (12, 13, 29, 33, 34, 39). In contrast, all postmitotic mononucleated myoblasts and multinucleated myotubes are without exception vimentin+/desmin+ (8, 9, 29, 34, 35). For a period of 30–60 min after their birth, postmitotic myo-

1. *Abbreviation used in this paper:* IF, intermediate filaments.

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blasts may be desmin+/myofibrillar protein−, but are never desmin−/myofibrillar protein+. After fusion of myoblasts into multinucleated myotubes, the longitudinally oriented vimentin/desmin IFs gradually shift their orientation to form transverse bands loosely associated with the I-Z-I bands of neighboring striated myofibrils (compare references 4, 31, 32, 62, and 63 with 18, 19, 42, 43, and 55). Concurrent with this striking shift in orientation, vimentin is down regulated so that day 8 and older myotubes often fail to bind anti-vimentin, but continue to bind anti-desmin into transverse bands with an ~2.0 μm periodicity (4, 31, 32, 62).

Desmin is expressed both early and late in the myogenic program. That the synthesis of desmin marks a very early event in myogenesis is indicated by the behavior of nonmuscle cells in the process of being converted into the myogenic lineage by the forced expression of the gene MyoD1. Before MyoD1-converted fibroblasts, chondroblasts, smooth muscle, or retinal pigmented epithelial cells synthesize any myofibrillar protein and before fusing, they initiate the synthesis of desmin (9). It has been suggested that type III IFs in cells like replicating presumptive myoblasts may function (*a*) as guy wires maintaining the spatial distribution of nuclei in the sarcoplasm; (*b*) as a network along which nascent myofibrillar transcripts or proteins might be transported and distributed throughout the myoblasts and myotubes; or (*c*) may even play a role in regulating muscle-specific gene expression (25, 49, 54, 65; see reference 41 for review). Later in the myogenic program, desmin has been postulated to play a critical role in (*a*) forming the peripheral domain of expanding Z-bands and longitudinally connecting successive Z-bands in elongating myofibrils; (*b*) laterally linking Z-bands of adjacent myofibrils to one another and to the sarcolemma; and (*c*) "integrating" and orienting nascent myofibrils with other cell organelles (19, 42, 43, 55). However, previous experiments have already raised questions regarding some of these proposals. Myogenic cells raised continuously in Colcemide assemble normal, aligned, spontaneously contracting striated myofibrils despite the segregation of all desmin/vimentin IFs into meandering cables that greatly distort spatial relationships of IFs to all cell organelles (5, 10, 30, 33, 38).

To investigate these putative functions of desmin IFs, we monitored various phases of myogenesis in cells synthesizing a mutated desmin that induces the complete and specific disruption of desmin and vimentin IFs. Our strategy follows procedures introduced by Fuchs and co-workers (1, 2). A truncated desmin cDNA was constructed which, when expressed both in nonmuscle and muscle cells, blocks the assembly of desmin and vimentin IFs and also causes the disassembly of preexisting desmin and vimentin IFs. Myogenic cells transfected with this truncated desmin, in the complete absence of all desmin/vimentin IFs, assemble normally aligned, striated myofibrils that contract spontaneously. Myotubes devoid of all intact IFs also assemble and distribute microtubules, microfilaments, SR, and T-system elements in a fashion indistinguishable from that of control myogenic cells.

Materials and Methods

Construction of Expression Vectors

The complete hamster desmin cDNA was kindly provided by H. Bloemendal (University of Nijmegen, Nijmegen, The Netherlands). The full-length desmin cDNA and a truncated desmin cDNA cut at the unique CvnI site were subcloned using blunt-end ligation into the vector pRSV-β-globin (27; American Type Culture Collection, Rockville, MD) in place of the β-globin gene. Construct orientation was verified by restriction endonuclease mapping. The resulting constructs placed the full-length and truncated desmin cDNA's downstream of the RSV promoter and in front of human β-globin 3'-untranslated sequences. Large-scale plasmid preparations were purified using DNA-affinity columns (QIAGEN, Chatsworth, CA), and DNA was quantitated using UV-spectroscopy.

Cell Culture

All cells were cultured onto collagen-coated Aclar coverslips (Pro-Plastics, Linden, NJ) and placed into 35-mm tissue culture dishes. Primary chick dermal fibroblasts were cultured and maintained as previously described (9). They were passaged five times before transfection to remove all residual myoblasts. Skeletal myoblasts were cultured from day 11 chick embryonic pectoral muscle as previously described (29) and plated at an initial density of 300,000 cells per 35-mm culture dish. Colcemid (Sigma Chemical Co., St. Louis, MO) was added to some cultures at a concentration of 1 μM for a period of 24 h.

Transfections

Both fibroblast and muscle cultures were transfected with 2 μg of DNA using standard calcium phosphate precipitation methods (26). Cultures were incubated for 12 h in the presence of the calcium phosphate precipitate and then washed with serum-free medium, fed with full medium, and maintained up to 14 d. Fibroblasts were transfected ~24 h after passaging, when cultures were ~1/3 confluent. Muscle cultures were transfected ~24 h after culturing.

Antibodies

Monoclonal anti-vimentin clone Vim 3B4 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Polyclonal anti-desmin has been previously described (4, 32); it does not cross-react with vimentin. mAb 9A2B8, kindly provided by D. Fischman (Cornell University Medical College, New York, NY) recognizes α-actinin from skeletal and cardiac muscle; it does not react with α-actinin from smooth muscle or nonmuscle cells (45, 60). Monoclonal anti-myosin heavy chain specific for striated muscle (45, 60) was kindly provided by F. Pepe (University of Pennsylvania, Philadelphia, PA). Affinity-purified rhodamine-labeled goat-anti-mouse and fluorescein-labeled goat-anti-rabbit secondary antibodies were purchased from Jackson Immuno Research Laboratories Inc. (Avondale, PA).

Gel Electrophoresis

For one-dimensional gel electrophoresis, Triton-X-100-extracted cytoskeletons of muscle cultures were prepared in the presence of protease inhibitors (0.0001% PMSF, 50 μg/ml Leupeptin, 3 mM EDTA) and separated by 12.5% SDS-PAGE. Equal amounts of protein were loaded in each lane, as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). After electrophoresis, gels were stained with Coomassie brilliant blue.

Fluorescence Microassay

Processing of cells for immunofluorescence microscopy was carried out as described (45). All incubations were performed in a humidified chamber at 37°C for 60 min. Anti-vimentin was used at a concentration of 1:10, anti-desmin at 1:100, anti-MHC at 1:10 and anti-α-actinin at 1:200. Secondary antibodies were used at 1:80. Nuclei were visualized with 4',6-Diamidino-2-phenylindole (Sigma Chemical Co.). Coverslips were mounted using 60% glycerol in PBS with 2.5% 1,4-Diazabicyclo (2.2.2) octane (Sigma Chemical Co.) added to prevent bleaching. Preparations were examined using a epifluorescence photomicroscope (Zeiss, Oberkochen, Germany), using filters selective for rhodamine, fluorescein, or UV light. Photographs were taken using TMAX 400 film (Eastman-Kodak Co., Rochester, NY).

Electron Microscopy

For thin-section EM, chick embryo skeletal muscle cells were cultured on collagen-coated Aclar coverslips. 4 d cultures with or without transfection were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH

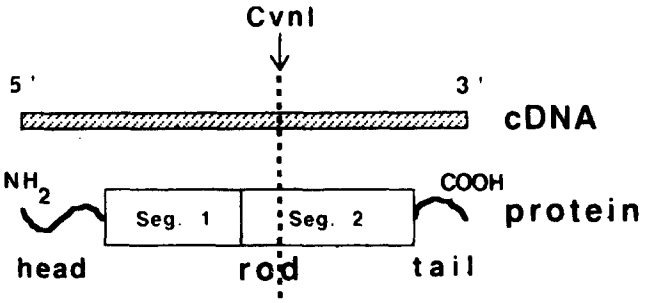


Figure 1. Outline of truncated desmin construct. To construct the truncated desmin plasmid, the desmin cDNA was cleaved at the unique internal CvnI site, and the NH_2-terminal portion was subcloned into the pRSV expression vector (see Materials and Methods). The encoded protein is truncated within segment 2 of the highly conserved α-helical rod domain and contains 272 *aa*, compared with 468 *aa* for the full-length desmin protein.

7.2, overnight at room temperature, rinsed with 10% sucrose in the same buffer and then postfixed for 1 h in cold OsO4 in the same buffer. After staining en bloc with 0.5% aqueous uranyl acetate for 2 h at room temperature, specimens were dehydrated in graded concentrations of ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (type H-800; Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 100 kV.

Results

Construction and Characterization of Plasmids

Epithelial cells are induced to collapse their preexisting cytokeratin IF networks when transfected with cDNAs encoding carboxyl-deleted cytokeratin molecules lacking portions of the central α-helical rod region (1). Based on this finding, we engineered a plasmid carrying a desmin cDNA truncated at amino acid 272 (Fig. 1). The truncated desmin lacks a portion of the central conserved α-helical rod and all of the carboxyl tail. Expression vectors driven by the Rous sarcoma virus promoter were created by subcloning either the truncated desmin cDNA or the full-length desmin cDNA into the plasmid pRSV-β-globin in place of the β-globin gene (see Materials and Methods). When transfected into muscle cells, the truncated desmin plasmid induces the appearance of a protein that on SDS-PAGE (Fig. 2) runs at a position consistent with the predicted molecular weight of the truncated desmin protein (30.5 kD). Preliminary examination of the gel also reveals the absence of several bands in the range of 130–150 kD in the cultures transfected with the truncated desmin plasmid. This finding is being pursued, as are additional details concerning the expression and stability of the truncated desmin protein and the relationships between the expression of truncated desmin protein and other myofibrillar and cytoskeletal proteins (T. Schultheiss and H. Holtzer, work in progress).

Expression of Full-length and Truncated Desmin in Primary Chicken Fibroblasts

Chick dermal fibroblasts contain vimentin IFs but no desmin or cytokeratin IFs. When these cells are transfected with the full-length desmin construct, ~2–5% of the cells exhibit desmin IFs, as determined by immunofluorescence staining with anti-desmin antibodies. At the light microscope level, these desmin IFs appear to colocalize precisely with the native vimentin IFs (Fig. 3, *A* and *B*). In these full-length desmin-transfected cultures, ~5–10% of the desmin-positive cells exhibit a desmin staining pattern that is not filamentous, but rather consists of large "pools" which occupy most of the cytoplasm (Fig. 3, *A* and *B*, *arrowhead*). Invariably, these pools costain with vimentin antibodies. In many cases, cells with pools of IF proteins contain deformed, fragmented, or even pyknotic nuclei. Currently, we cannot determine whether such cells are expressing excess desmin so that normal cellular function is impaired, leading to cell injury or death, or if the cells are moribund for some other reason connected with the transfection process.

The effects of transfecting dermal fibroblasts with truncated desmin cDNA differ considerably from the effects of introducing full-length desmin cDNA. In the fibroblast cultures transfected with truncated desmin, ~2–5% of the cells display round, smooth desmin+ spheroid bodies (Fig. 3, *C* and *D*). Without exception the desmin+ spheroids, which can range in diameter from 0.5 μm to several microns, costain with antibodies to vimentin (Fig. 3, *C* and *D*), but not with antibodies to tubulin or with phalloidin (data not shown). Most cells that contain desmin+/vimentin+ spheroid bodies fail to display long, intact IFs. Presumably the de novo synthesis of truncated desmin directly or indirectly interacts with the endogenous preexisting vimentin network inducing its subsequent disassembly. The transfected cells appear to be healthy; their nuclei are not pyknotic and they survive for at least seven days in culture. The expression of truncated desmin does not substantially alter the spatial dis-

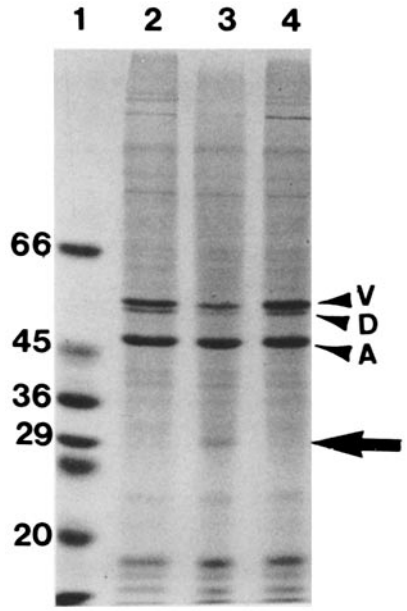
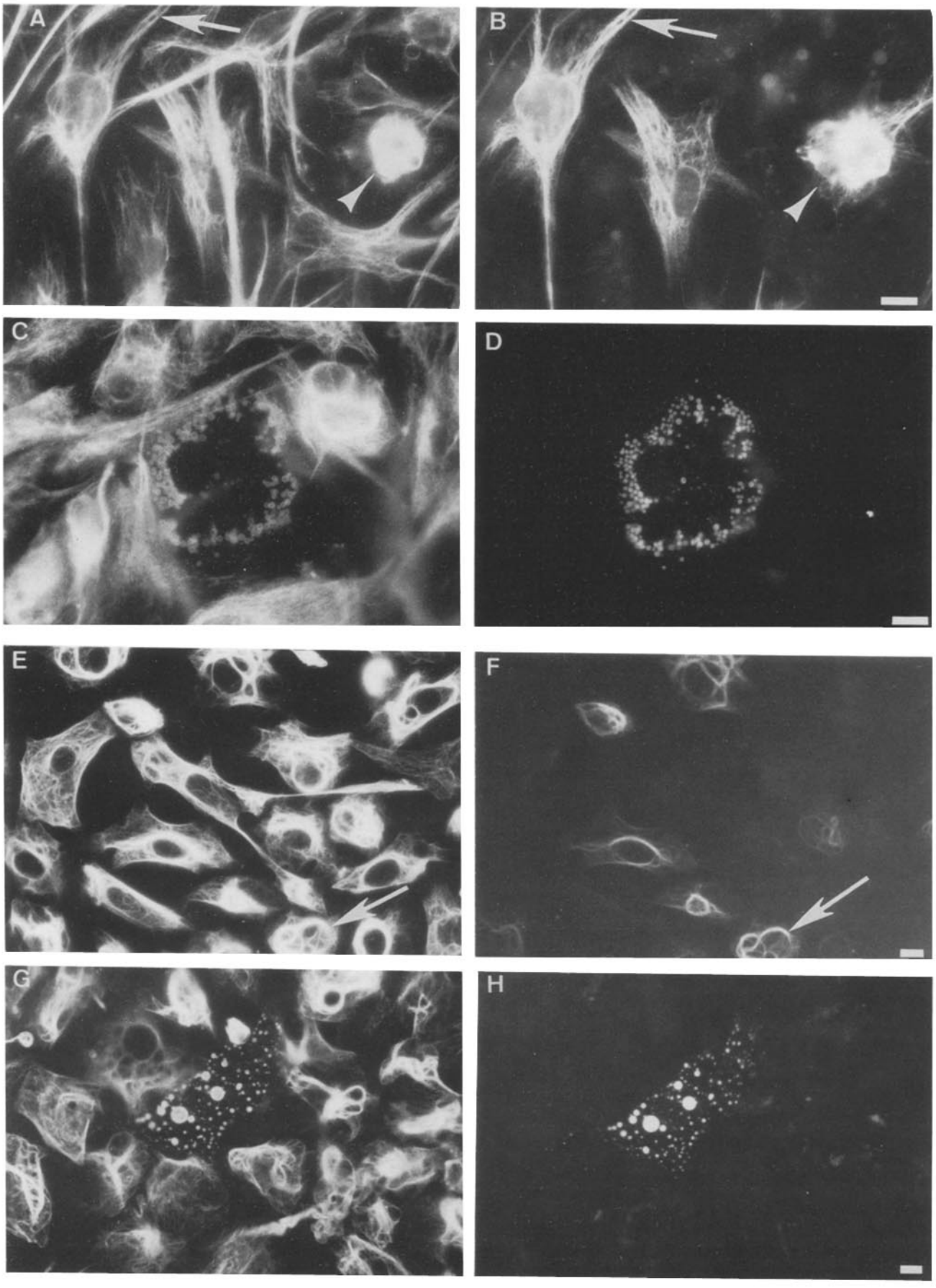


Figure 2. Coomassie blue–stained gel of transfected muscle cultures. Cytoskeletal extracts of skeletal muscle cultures transfected with the truncated desmin plasmid (lane *3*), the full-length desmin plasmid (lane *4*), or mock transfected with calcium phosphate and no DNA (lane *2*). Lane *1* contains protein molecular weight markers. Lane *3* contains a prominent band (*arrow*), not found in lanes *2* or *4*, at the predicted molecular weight for the truncated desmin protein (30.5 kD). *V*, *D*, and *A* refer to the positions of vimentin, desmin, and actin, respectively.


A
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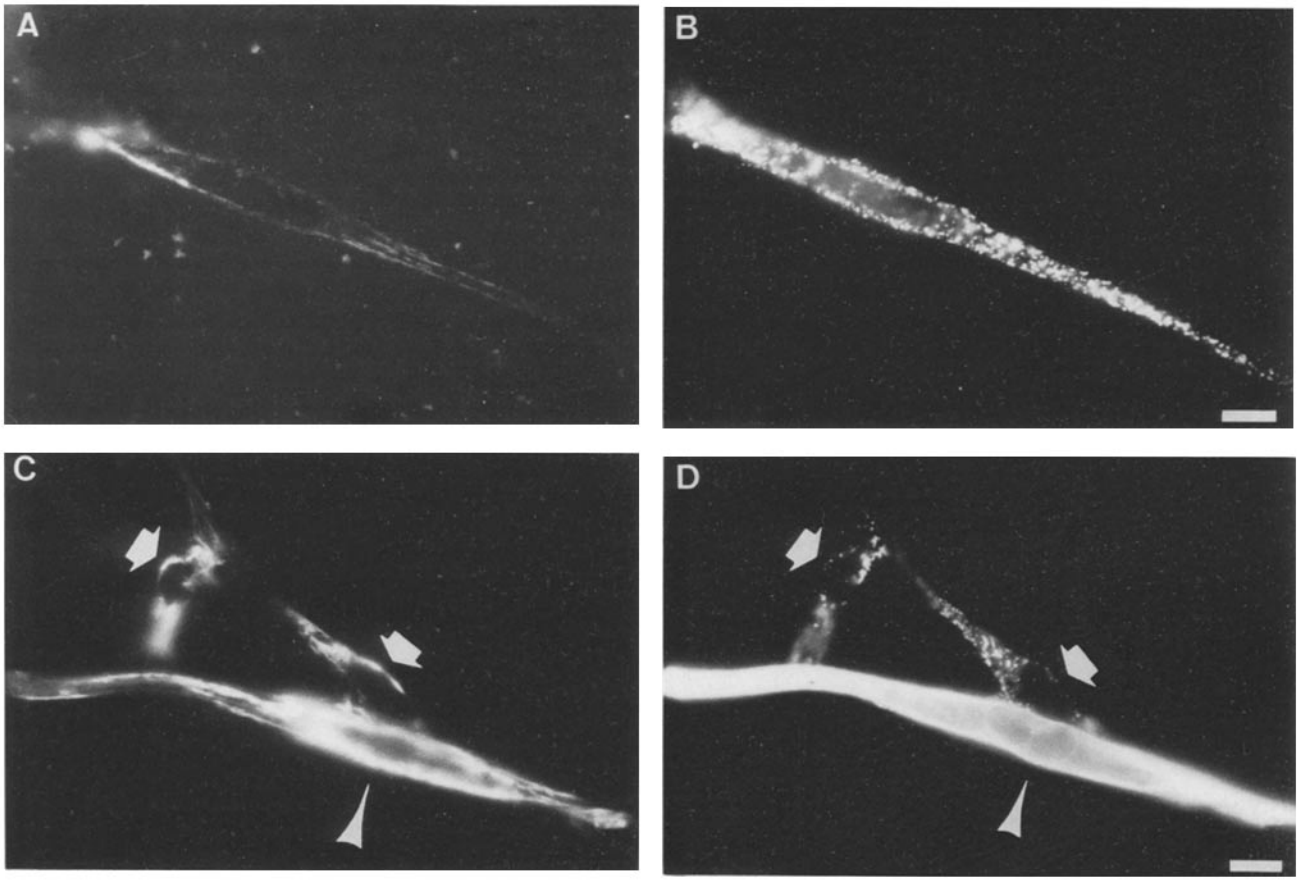


Figure 4. Muscle cultures transfected with truncated desmin construct: day 2.5. Muscle cultures were transfected with the truncated desmin construct 24 h after plating, fixed 36 h after transfection, and double stained with antibodies to sarcomeric myosin heavy chain (*A* and *C*, *rhodamine*) and desmin (*B* and *D*, *fluorescein*). *A* and *B* show a postmitotic myoblast that has begun the synthesis of sarcomeric MHC and in which the desmin IFs have already been completely replaced by spheroid bodies. In *C* and *D*, a binucleated myotube (*arrowheads*) is adjacent to two mononucleated postmitotic myoblasts (*arrows*). In the mononucleated postmitotic myoblasts, desmin IFs have been replaced with desmin+ spheroid bodies. In the binucleated myotube, the desmin IFs are intact. Owing to the low magnification, the individuality of the long desmin IFs is obscured. Small myotubes with intact desmin IFs are common in early cultures, but in later cultures (presumably owing to fusion of transfected with nontransfected cells) they are quite rare, constituting ~5% of all the myotubes in a culture. Bars, 10 μm.

tribution of stress fibers stained with rhodamine–phalloidin or microtubules as stained with anti-tubulin (data not shown). In brief, expression of the truncated desmin in dermal fibroblasts induces the formation of hybrid desmin and vimentin bodies, as well as inducing the complete and specific dismantling of the preexisting endogenous vimentin IF network. These desmin+/vimentin+ spheroids are similar to those found by Raats et al. (57) upon transfection of desmin mutants into various cell lines.

Effects of Colcemid on Fibroblasts Transfected with Desmin Plasmids

When myoblasts, chondroblasts, or fibroblasts are treated with the microtubule depolymerizing drug Colcemid, both

Figure 3. Chick dermal fibroblasts. Cultures in *A*, *B*, *E*, and *F* were transfected with the full-length desmin plasmid, while cultures in *C*, *D*, *G*, and *H* were transfected with the truncated desmin plasmid. Cultures were fixed 72 h after transfection. The cultures in *E–H* were placed in Colcemid for 24 h before fixation. Cultures were double stained for vimentin (*A*, *C*, *E*, and *G*; *rhodamine*) and desmin (*B*, *D*, *F*, and *H*; *fluorescein*). All cells stain with antibodies to vimentin, but only transfected cells stain with antibodies to desmin. In fibroblasts expressing full-length desmin, the desmin and vimentin IFs colocalize precisely (*arrows*, *A* and *B*). Occasionally, desmin is incorporated not into IFs but into amorphous pools (*arrowhead*, *B*); these pools also colocalize with vimentin antibodies (*arrowhead*, *A*). Fibroblasts expressing truncated desmin contain no intact IFs but instead contain numerous spheroid bodies that costain with desmin and vimentin (*C* and *D*). Note that cells containing desmin+/vimentin+ spheroids contain no intact IFs despite the fact that neighboring cells contain normal vimentin IFs. When full-length desmin–transfected fibroblasts are treated with Colcemid, typical cables are formed that costain with both desmin and vimentin antibodies (*E* and *F*). The desmin+/vimentin+ spheroid bodies in fibroblasts expressing truncated desmin show no rearrangement upon treatment with Colcemid (*G* and *H*). The size variation in the spheroid bodies in *G* and *H* is also seen in many truncated desmin-transfected cultures that have not been treated with Colcemid. Bars, 10 μm.

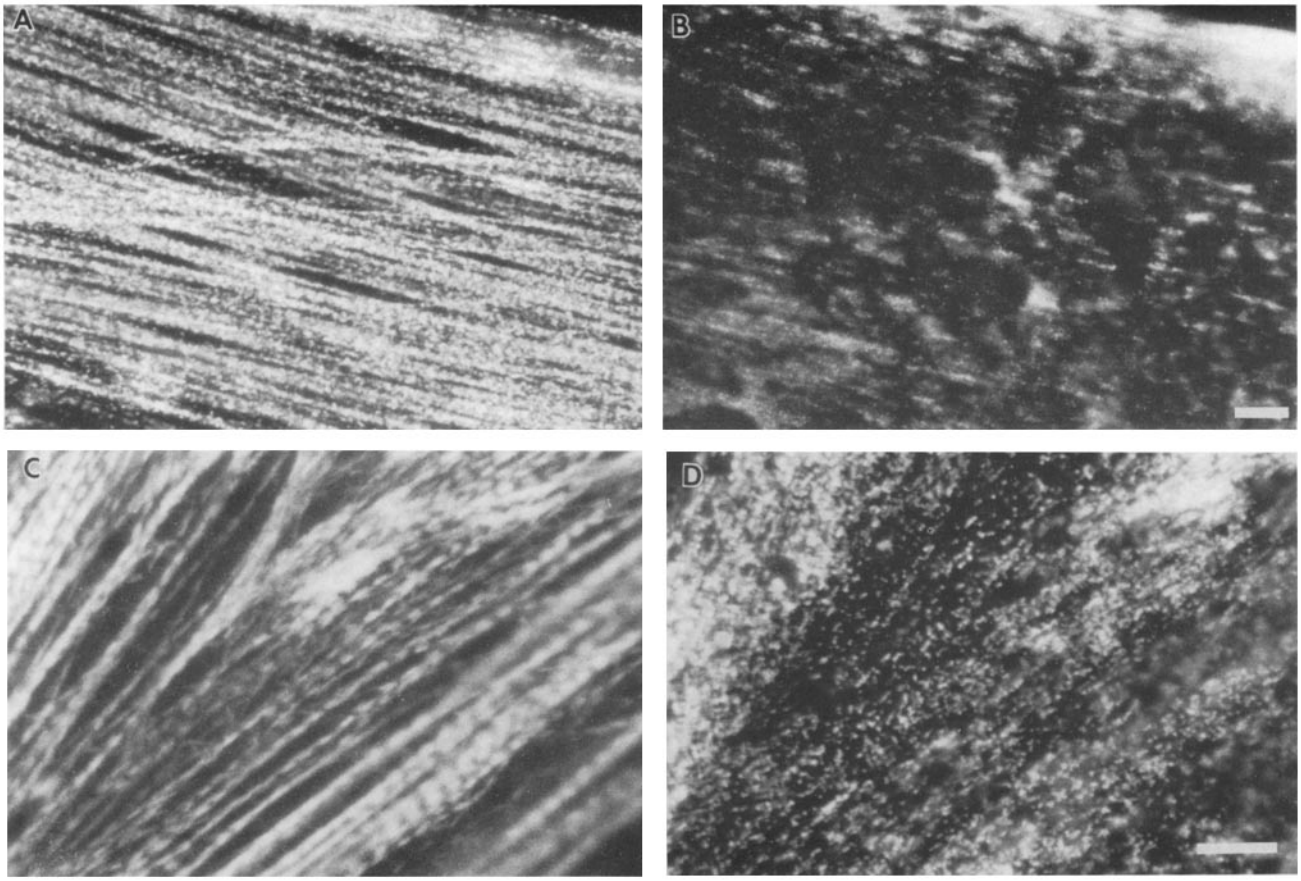


Figure 5. Muscle cultures transfected with truncated desmin construct: day 3.5. Muscle cultures were transfected with the truncated desmin plasmid 24 h after plating, fixed 60 h after transfection, and double stained with antibodies to sarcomeric α-actinin (*A*, *rhodamine*) or sarcomeric MHC (*C*, *rhodamine*), and desmin (*B* and *D*, *fluorescein*). By this stage, the cultures contain many multinucleated myotubes with 10–50 nuclei. In *A*, the myofibrils are entirely nonstriated, and the α-actinin is found in linearly aligned I-Z-I-like complexes. In *C*, striated myofibrils are forming, and the MHC is partially arranged into A-bands. Note that in both these multinucleated myotubes, the desmin IFs have already been completely replaced with desmin+spheroid bodies (*B* and *D*). Bars, 10 μm.

the desmin and vimentin IFs are induced to assemble into massive cables that exclude all cell organelles (10, 16, 30, 38). These massive IF cables are not confined to a perinuclear location but meander throughout the entire cell. When fibroblasts transfected with full-length desmin are treated with Colcemid, both the vimentin and the desmin IFs are integrated into the same meandering IF cables (Fig. 3, *E* and *F*). In contrast, when fibroblasts are transfected with truncated desmin and then treated with Colcemid, the distribution of the desmin+/vimentin+ spheroids is not affected (Fig. 3, *G* and *H*). Apparently, the mechanisms responsible for IF cabling, which are unknown (16), are not able to act on the desmin+/vimentin+ spheroid bodies induced in cells expressing the truncated desmin.

Myogenesis in Muscle Cultures Transfected with Desmin Constructs

The day after plating (day 1), replicating skeletal myogenic cells were either (*a*) transfected with the truncated desmin plasmid; (*b*) transfected with the full-length desmin plasmid; (*c*) sham transfected using calcium phosphate and no DNA; or (*d*) left untreated. The latter three treatments all gave similar results, and will therefore be discussed together as "controls." (The success of transfection with the full-length desmin construct into muscle cultures could not be determined using cytoimmunofluorescence since our antibodies could not distinguish between transfected hamster desmin and endogenous chicken desmin; however, the hamster and chicken desmin proteins have slightly different gel mobilities, and thus the presence of transfected full-length desmin could be verified by Western blot; data not shown.)

On day 2 (one day after transfection), all cultures contain both postmitotic mononucleated myoblasts and myotubes with small numbers of nuclei (typically 2–10). In the truncated desmin transfected cultures, some of these myoblasts and small myotubes contain intact IFs, while in others all of the IFs have been replaced by small desmin+/vimentin+ spheroid bodies (Fig. 4). By day 3, extensive fusion of myoblasts has occurred, and many myotubes containing 10–50 nuclei have formed. In the day 3 cultures transfected with truncated desmin, >95% of the myotubes are completely negative for both desmin and vimentin IFs and display in their stead enormous numbers of desmin+/vimentin+ spheroids (Fig. 5). Fewer than 5% of the myotubes assemble relatively intact desmin/vimentin IFs. Of this 5% of myotubes with intact IFs, the majority are small myotubes with fewer than 10 nuclei. Among the larger myotubes, which in general are filled with numerous desmin+/vimentin+ sphe-

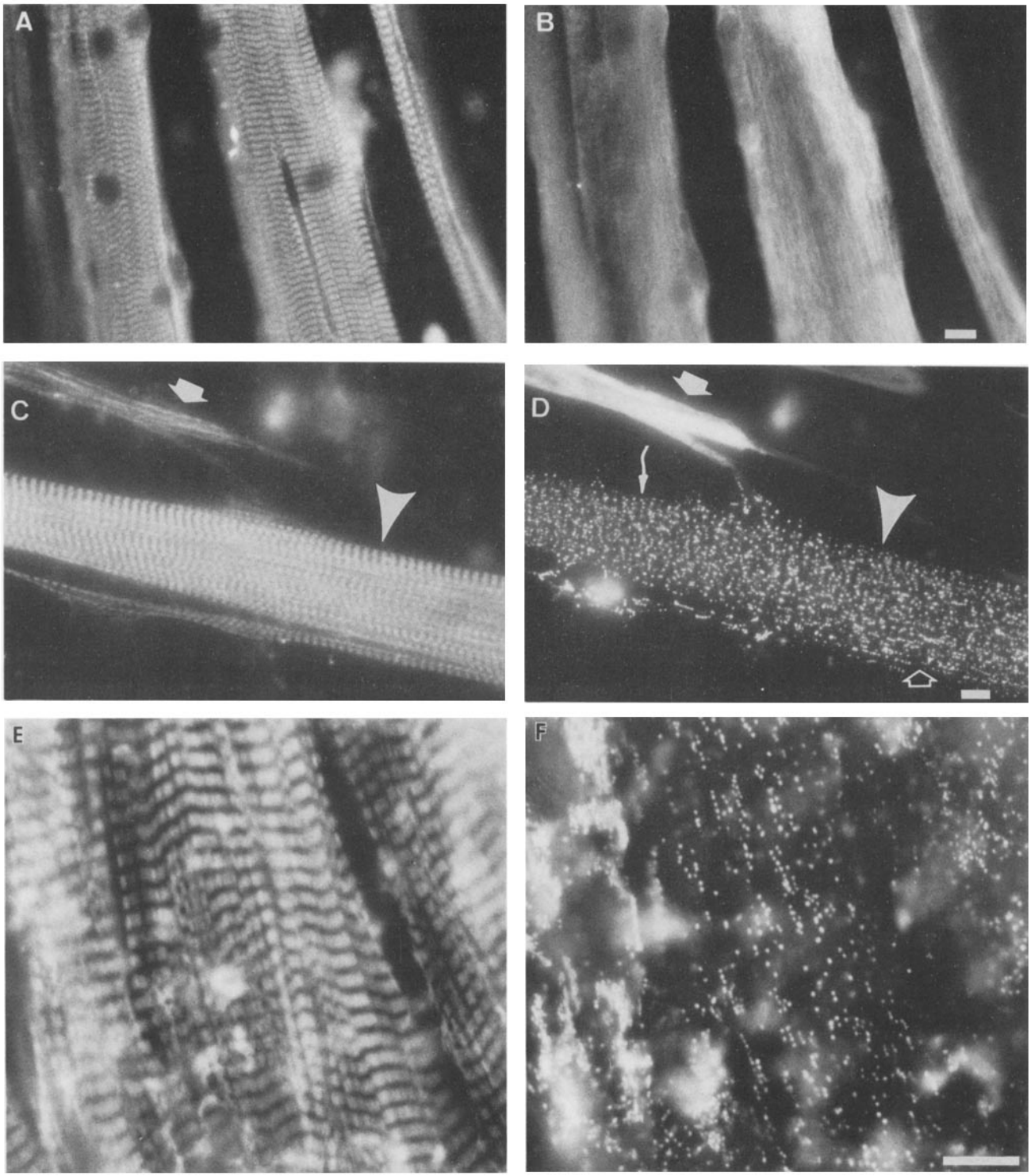


Figure 6. Muscle cultures: days 5–7. 24 h after plating, muscle cultures were either mock transfected using calcium phosphate and no DNA (*A* and *B*) or transfected with the truncated desmin construct (*C–F*). Cultures were fixed 4–6 d after transfection and double stained with antibodies to sarcomeric MHC (*A*, *C*, and *E*, *rhodamine*) and desmin (*B*, *D*, and *F*, *fluorescein*). Mock-transfected cultures show typical longitudinal IFs (*B*). In contrast, >95% of the myotubes in cultures transfected with the truncated desmin plasmid show complete absence of intact IFs and presence of desmin+ spheroid bodies (*arrowhead*, *C* and *D*; *E* and *F*). Despite the complete absence of IFs, myotubes have assembled striated myofibrils that are well aligned (*C* and *E*). At this stage, most of the spheroid bodies are oriented longitudinally within the myotubes (*open arrow*, *D*; center of *F*), although in some regions suggestions of spheroid bodies in a transverse alignment can be seen (*wavy arrow*, *D*). The upper arrows in *C* and *D* point to a small myotube that is among the small number of myotubes in these cultures with intact desmin filaments. Bars, 10 μm.

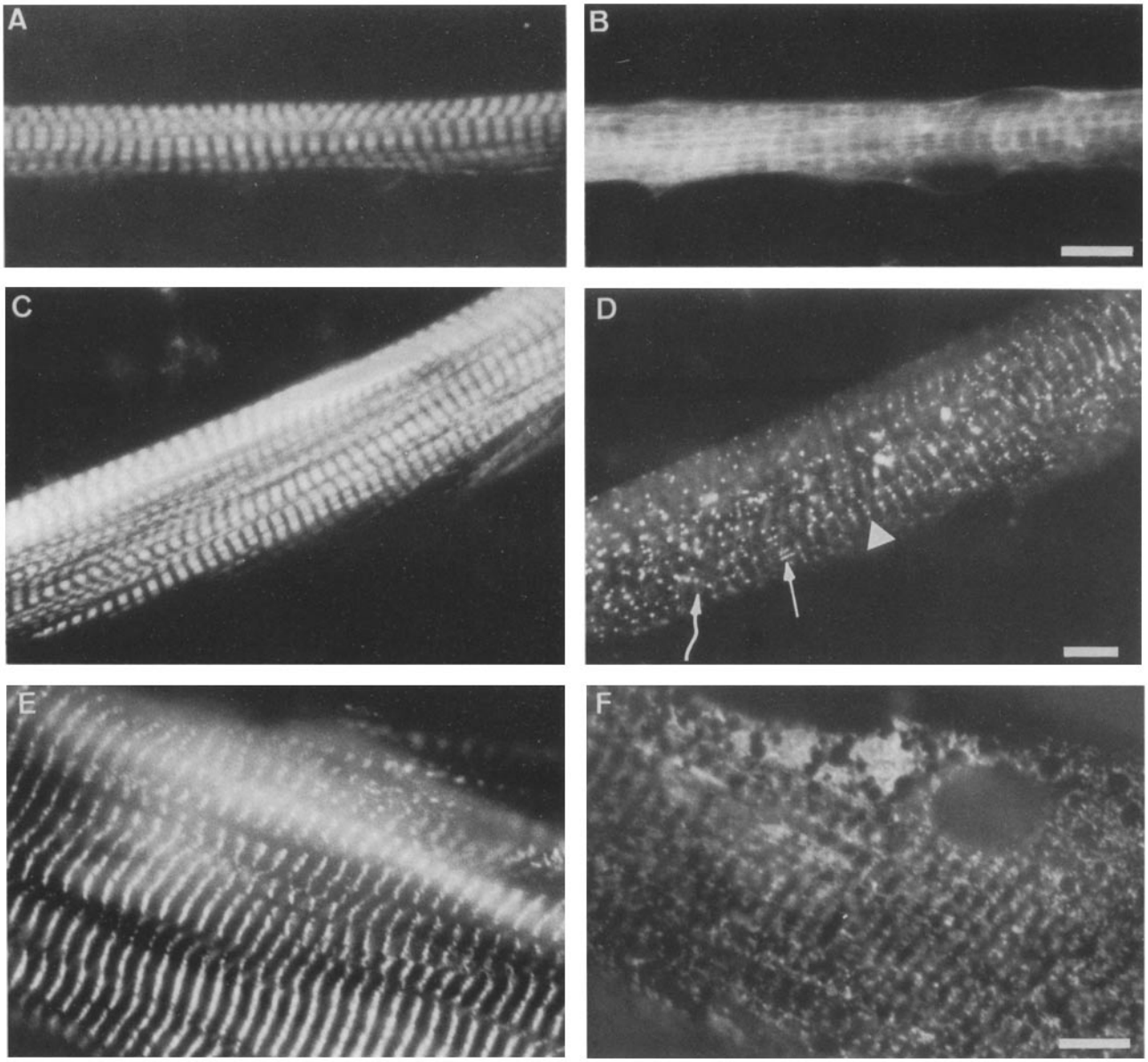


Figure 7. Muscle cultures: day 14. 24 h after plating, muscle cultures were either mock transfected using calcium phosphate and no DNA (*A* and *B*) or transfected with the truncated desmin construct (*C–F*). Cultures were fixed 13 d after transfection and double stained with antibodies to sarcomeric MHC (*A* and *C*, *rhodamine*) or sarcomeric α-actinin (*E*, *rhodamine*) and to desmin (*B*, *D* and *F*, *fluorescein*). In the mock-transfected control cultures, the desmin IFs have partially rearranged so that transverse as well as longitudinal desmin patterns are now apparent (*B*). The transverse IF banding corresponds to the I-Z-I-region of the sarcomere. In truncated desmin-transfected cultures, the spheroid bodies have also aligned to a large degree along the I-Z-I-band (*D*, *arrowhead*). Note that some spheroid bodies are still longitudinally oriented (*D*, *straight arrow*). Also, the myotube in *D* contains occasional faint wisps of linear desmin staining which may represent remnant IFs (*D*, *wavy arrow*). Bars, 10 μm.

roid bodies, one may occasionally see small patches which contain modest numbers of intact IFs; however, the completeness of the disruption of the IFs is to be emphasized, and small patches of intact IFs within myotubes are the exception rather than the rule. Postmitotic, elongated myoblasts and multinucleated myotubes in day 3 cultures expressing truncated desmin display typical nonstriated myofibrils, which stain positively with antibodies to sarcomeric α-actinin and sarcomeric MHC (Fig. 5), as well titin, nebulin, and C-protein (data not shown). These nonstriated myofibrils are indistinguishable from those in control cultures (data not shown). Clearly, the absence of a normal complement of long desmin/vimentin IFs in early myogenic cells does not interfere with either the intracellular distribution of a cohort of myofibrillar proteins or with their arrangement into nonstriated myofibrils.

In day 4–5 truncated desmin-transfected cultures, the nonstriated myofibrils, in the absence of all IFs, rearrange into normal striated myofibrils (Fig. 6, *C–F*). There are no detectable differences in the events associated with this transition from nonstriated to striated myofibrils, including the number or spatial distribution of the emergent tandem sarcomeres, in truncated desmin-transfected versus control myotubes (compare Fig. 6, *A* and *B* with *C–F*). Nascent, typi-

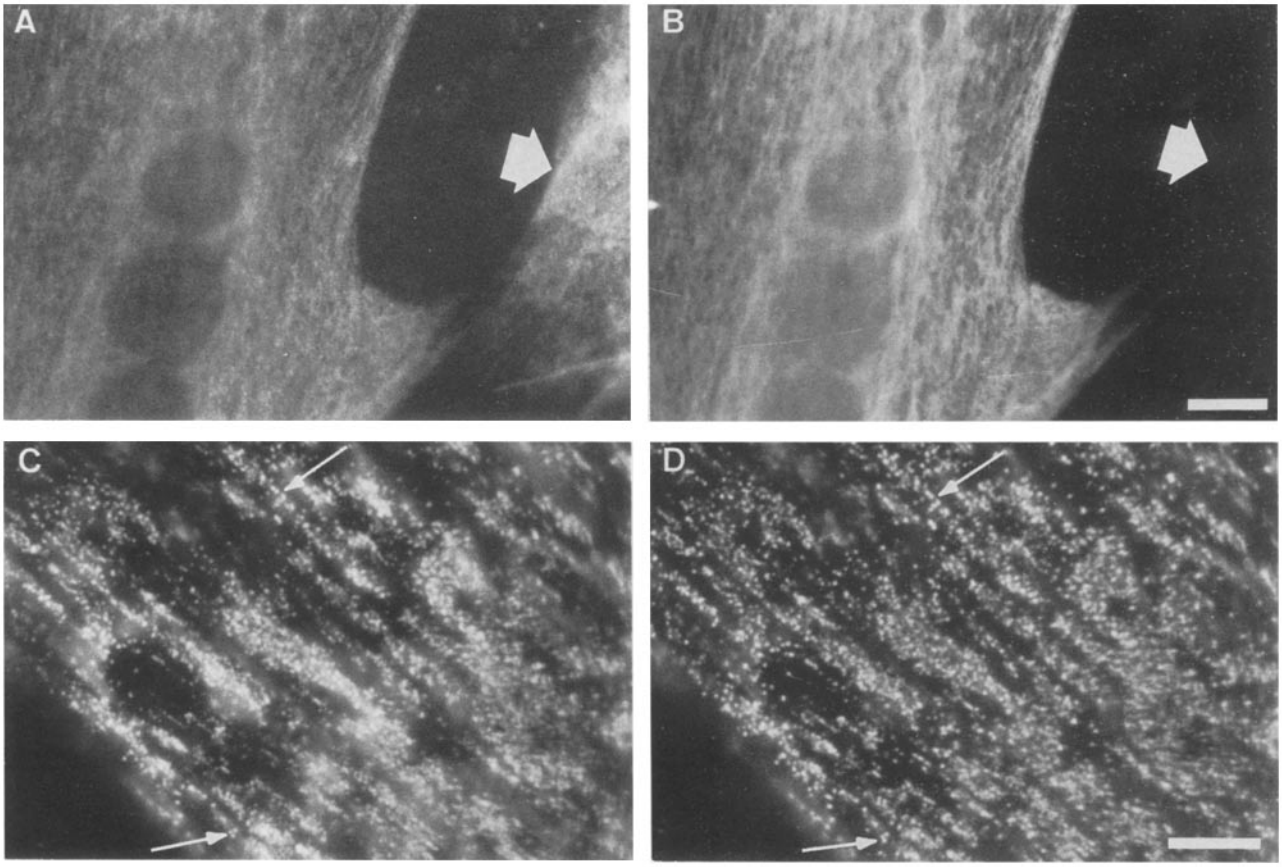


Figure 8. Muscle cultures: day 5. Cultures were transfected with truncated desmin plasmid (*C* and *D*) or mock transfected (*A* and *B*) 24 h after plating, fixed 4 d after transfection, and double stained with antibodies to vimentin (*A* and *C*) and desmin (*B* and *D*). In *A* and *B* the myotube on the left contains longitudinal IFs that costain with antibodies to vimentin and desmin, while the fibroblast at the right (*arrows*) contains only vimentin. Cultures transfected with the truncated desmin plasmid (*C* and *D*) contain numerous spheroid bodies that costain precisely with antibodies to vimentin and desmin (*arrows*). Bars, 10 μm.

cally striated myofibrils in turn align laterally in older myotubes (Fig. 6, *C–F*) and proceed to initiate spontaneous contractions. The concentration of spheroid bodies varies from myotube to myotube and even within different regions of a single myotube; however, there is no correlation between the presence or absence of desmin+/vimentin+ spheroids and the local density or lateral arrangement of adjacent striated myofibrils. No consistent differences in the arrangement of nuclei have been observed between myotubes with or without intact IFs. Truncated desmin-transfected myotubes are also indistinguishable from control myotubes in terms of the large increases in myotube size and myofibrillar content between days 3 and 5. In short, in addition to being dispensable for the intracellular distribution of myofibrillar proteins and the integrity of nonstriated myofibrils, long desmin/vimentin IFs are also dispensable for the formation of striated myofibrils, the lateral alignment of nascent myofibrils one to another, and for the initiation of spontaneous contractions.

In day 8 and older control cultures, there is a gradual shift of a portion of the desmin IFs from a longitudinal deployment to a transverse orientation in which the IFs loosely associate with the I-Z-I regions of the myofibrils (Fig. 7, *A* and *B*). In a portion of older cultures transfected with truncated desmin, the desmin+/vimentin+ spheroids likewise shift from a predominantly longitudinal distribution between myofibrils to one of lining up along the I-Z-I-regions (Fig. 7, *C–F*; for details on the redeployment of desmin IFs to the I-Z-I regions see references 4, 31, 32, 33, and 35).

In contrast to the observation that desmin+/vimentin+ spheroid bodies are capable of responding to signals in maturing myotubes to reorient along the I-Z-I-bands, the desmin+/vimentin+ spheroids have lost the property of cabling in the presence of microtubule depolymerizing drugs. As in fibroblasts, treating myotubes with Colcemid has no effect on the distribution of the desmin+/vimentin+ spheroids (data not shown).

An unexpected observation was that normal desmin or vimentin IFs did not return even in truncated desmin-transfected myotubes maintained in culture for 14 days. Transfected myotubes in these older cultures are still rich with desmin+/vimentin+ spheroid bodies and still totally lacking in intact IFs. In future experiments it will be important to follow the synthesis of the endogenous vs the truncated desmin and of the endogenous vimentin in transfected cells (see Discussion).

Fig. 8 demonstrates that the spheroid bodies in muscle cells contain both desmin and vimentin in an identical distribution. The spheroid bodies in muscle cells are distinctly smaller than those found in fibroblasts, ranging from barely resolvable to up to 0.4 μm in diameter. These desmin+/

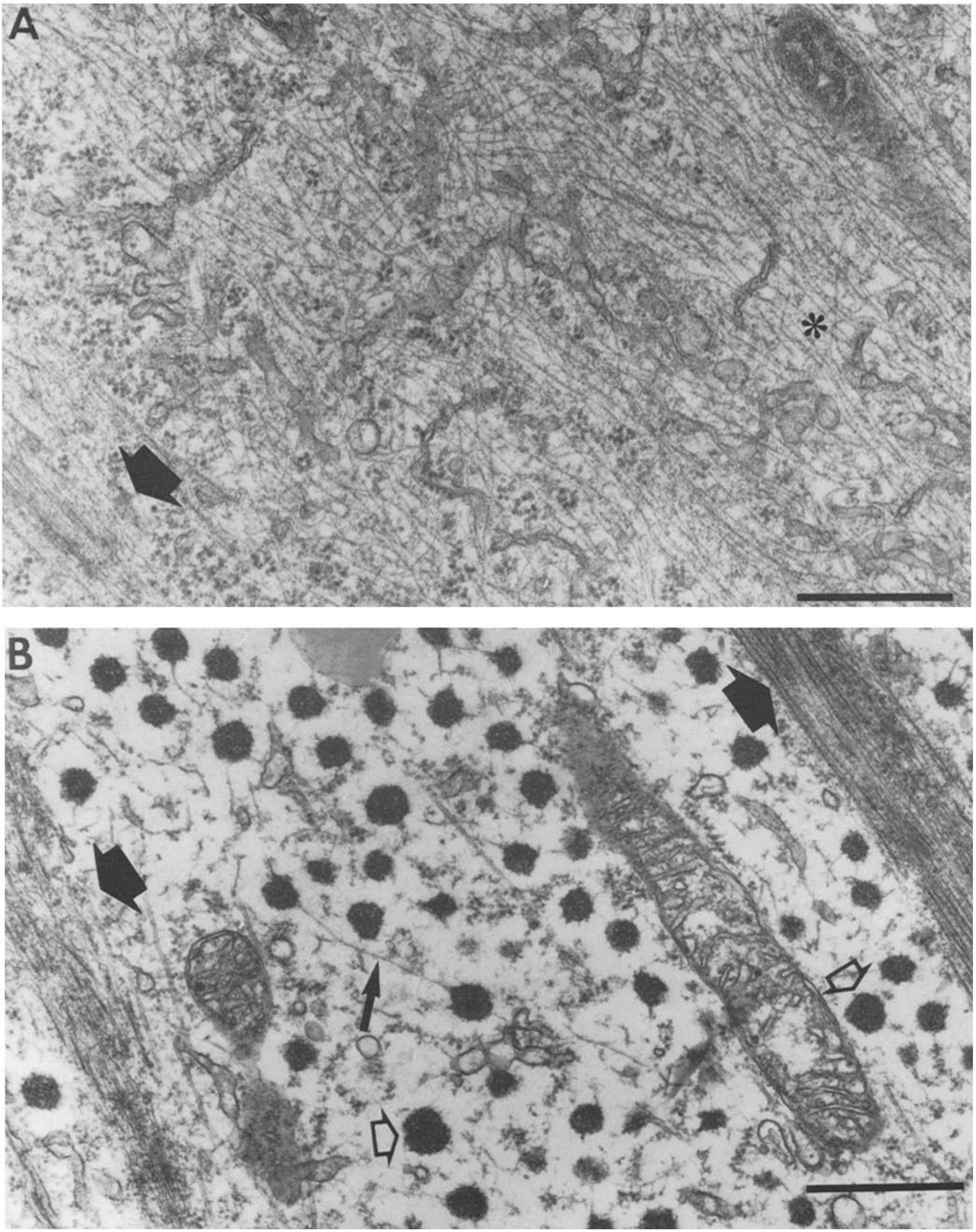
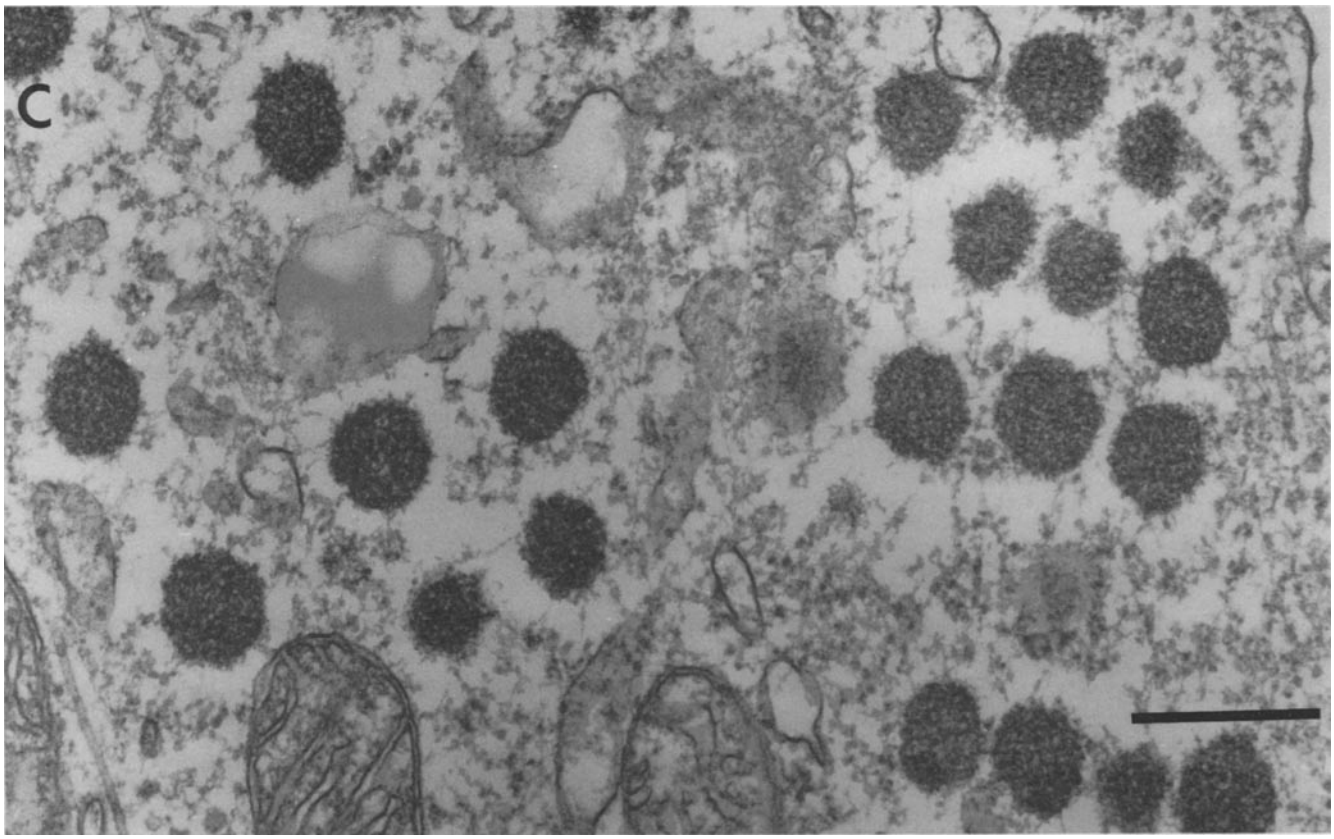


Figure 9. Electron micrographs: day 4. Muscle cultures were transfected with the truncated desmin plasmid (*B* and *C*) or mock transfected (*A*) and fixed 3 d after transfection. The control muscle cells (*A*) contain numerous longitudinally oriented, meandering IFs (*). An immature myofibril is seen at bottom left (*filled arrow*). In contrast, the myotube in *B* shows the virtually complete absence of IFs and instead the presence of numerous electron-dense spheroid bodies (*open arrows*). Occasionally filamentous strands can be seen connecting spheroid bodies (*small arrow*); however, in most cases the spheroid bodies are without filamentous projections. The large solid arrows indicate immature, forming myofibrils. *C* shows a truncated desmin-transfected muscle cell at higher magnification. The spheroid bodies are remarkably uniform in size. Bars: (*A* and *B*) 1 μm; (*C*) 0.5 μm.



vimentin+ spheroids are remarkably uniform in size within a given myotube, although there is some variation between myotubes. In contrast, the spheroids in fibroblasts, as described above, can vary greatly in size within a given cell.

Electron Microscopy of Transfected Muscle Cultures

EM sections of muscle cells transfected with truncated desmin reveal the presence of huge numbers of round, electron-dense aggregates scattered throughout the sarcoplasm (Fig. 9, *B* and *C*). These are presumably the ultrastructural correlates of the desmin+/vimentin+ spheroids seen at the light microscopic level. They are not surrounded by a membrane, but there is a clearing of the intracellular ground substance immediately surrounding individual spheroids. Within a given myotube the size of the spheroids is relatively constant. Short filaments occasionally protrude from some electron-dense spheroids (Fig. 9 *B*). There are no intact IFs in most of these transfected myotubes, in contrast to control myotubes which are filled with large numbers of long, twisting 10-nm filaments (Fig. 9 *A*). Myotubes lacking IFs appear normal with respect to their deployment of such organelles as mitochondria, microtubules, giant polysomes, SR, and T-system elements. Further ultrastructural studies of the relationships of spheroid bodies to myofibrils, the nuclear and plasma membranes, and other organelles are in progress.

Discussion

The truncated desmin construct provides us with a highly specific tool to investigate one by one the several biological roles that IFs have been postulated to play in the differentiation of muscle cells. In the complete absence of intact IFs, the following aspects of myogenesis proceeded normally: (*a*) the transition of nonstriated into striated myofibrils; (*b*) the normal extension of the peripheral domain of each Z-band; (*c*) the alignment laterally of nascent striated myofibrils; and (*d*) the initiation of spontaneous contractions. It is also likely that disruption of IFs does not greatly affect fusion of myoblasts (see below).

Because myoblasts or myotubes lacking IFs are not deflected in a specific way from expressing their normal differentiation program, our results do not pinpoint a specific function for IFs. They do, however, prompt a more critical view of a number of seemingly plausible and commonly accepted notions concerning their roles during myogenesis. For example, our findings are not readily compatible with the suggestions that desmin/vimentin IFs (*a*) constitute an essential network that connects the nucleus to the cell membrane and provides a pathway for distributing or transporting myofibrillar and other mRNAs or proteins throughout the cell (25, 65); (*b*) are responsible for the lateral alignment and organization of nascent myofibrils by cross-linking neighboring Z-disks to each other and to the sarcolemma (19, 42, 43); or (*c*) are responsible for integrating myofibrils with other cell organelles (19, 42, 43, 55), at least to the extent necessary for forming striated myofibrils or initiating contractions.

Because of the timing of the disruption of IFs relative to the events of myogenesis in our cultures, we are somewhat limited in the conclusions that we can draw regarding the importance of IFs for very early events in myogenesis, including the fusion of myoblasts. Complete IF disruption is evident in a significant proportion (>5%) of myoblasts on day 2, when fusion is just beginning. By day 3, substantial fusion has occurred, and the IFs in >95% of the cultures have been

completely disrupted (Fig. 5). If IF disruption interferes with fusion, one would have to postulate that a significant number of cells that carried the truncated desmin plasmid failed to express it until after fusion; this would require significant heterogeneity among cells in terms of when they start expressing the truncated protein. In addition, the size of the myotubes in later truncated desmin-transfected cultures is not affected despite the facts that (*a*) significant numbers of mononucleated and oligonucleated muscle cells in day 2–3 cultures have disrupted IFs; and (*b*) fusion of myoblasts to myotubes continues after day 3 in control cultures (6). Thus, it is highly unlikely that disruption of IFs significantly inhibits fusion. This conclusion would be strengthened by future experiments in which IF disassembly is induced before all fusion.

Nothing is known of the signals that in older myotubes initiate, or the mechanisms that effect, the translocation of longitudinally deployed desmin+/vimentin+ IFs to their final transverse association along the I-Z-I-bands (4, 32, 33). In the current experiments virtually all IF proteins were confined to small spheroid bodies. Nevertheless, at the appropriate stage of maturation these spheroids were redeployed so as to loosely associate with the I-Z-I-bands. Clearly, this striking spatial shift must involve properties of the desmin and vimentin molecules that are independent of their assembly into long IFs.

Recent studies on the vectorial growth and trajectory of IFs have suggested that nucleation occurs by interaction of their carboxy ends with lamin B at the nuclear envelope and that IFs terminate by interaction of their amino termini with ankyrin-like molecules at the sarcolemma (23). While such interactions might account for the distribution of intact IFs in nonmuscle cells, it is difficult to see how they could account for either the longitudinal distribution of the desmin+/vimentin+ spheroids in early myotubes or of their subsequent shift to a transverse, sarcomeric distribution in older myotubes. Ultrastructural studies of the relationship between spheroid bodies, the nuclear membrane, and the I-Z-I-regions of myofibrils are in progress and will be reported elsewhere.

One striking finding in these experiments is that >95% of the myotubes in cultures transfected with the truncated desmin plasmid exhibited the mutant phenotype of spheroid bodies with dismantled IFs. Using the same culture conditions, the transfection efficiency in chick dermal fibroblasts was 5%, and others have reported a similar transfection efficiency for skeletal myoblasts (47). The most likely explanation for the high rate of expression of the truncated phenotype in myotubes is that myoblasts were transfected before fusion, and that a few transfected mononucleated cells subsequently contributed truncated desmin to an entire myotube. This interpretation is supported by the observation that the small number of myotubes in the older cultures that contain intact IFs are those that have only a few nuclei (Figs. 4 and 6, *C* and *D*). It is currently unknown whether fusing cells carry cDNA only in their nuclei, and thus are the only source of truncated mRNA for the entire myotube, or whether the fusing cells also carry cDNA in their cytoplasm which upon fusion is capable of being taken up by other nuclei in the myotube and subsequently expressed. Also possibly contributing to the high rate of expression of the mutant phenotype is the high rate of expression of the mutant protein, as suggested by Coomassie blue–stained gels (Fig. 2). In addition, truncated NF-M molecules expressed in fibroblasts at a level of only 1% of the endogenous full-length protein have resulted in dismantling of endogenous IF networks (69).

Another unexpected finding was that there was no suggestion of recovery of normal IFs, even after 14 d in culture. The desmin+/vimentin+ spheroids remained, essentially unchanged in size, morphology, or in density per unit sarcoplasm. This result was not anticipated, given the findings of Albers and Fuchs (1) that cytokeratin filaments were initially disassembled in PtK2 cells, but that intact IFs reappear ~5 d after transfection, with full recovery of normal IFs several days later. The DNA, the RNA or the protein may be more stable in nonreplicating myotubes than in various types of replicating cultured cells. Wolff et al. (68) have reported the long-term expression of exogenous genes in mouse muscle in vivo. It is also possible that the accumulation of truncated desmin leads to a down regulation of endogenous desmin and/or vimentin expression by way of still unknown feedback loops.

What, then, are possible roles for desmin during myogenesis? It may be that the consequences of the lack of intact desmin IFs cannot be detected in our in vitro system, which never attains the maturity of in vivo muscle. The truncated desmin-transfected myotubes may be defective in some unknown biomechanical or biochemical properties that will be manifested only if the cells are stressed in a physiologically relevant manner. By analogy, the muscle of children with Duchenne muscular dystrophy, despite the complete absence of dystrophin, initially appear normal, and degenerate only with further maturation and usage. Future studies with transgenic animals expressing truncated desmin proteins that disrupt the endogenous IFs should shed light on this aspect of IF function.

Our experiments also cannot rule out a possible role for desmin IFs (or even desmin molecules independent of their assembly into filaments) in very early myogenic events, including the transition from replicating presumptive myoblast to postmitotic definitive myoblast. In this context it is worth stressing that the issue of when desmin is first expressed in the myogenic lineage still requires further study. Although desmin has been shown in a variety of systems to be expressed before most myofibrillar proteins (8, 9, 12, 29, 34), direct information is lacking with regard to the appearance of desmin relative to, on the one hand, the earliest expressed structural muscle proteins (e.g., cardiac α-actin and smooth α-actin; 58) and, on the other hand, the muscle-regulatory proteins of the MyoD family (11).

The current experiments provide evidence against many commonly accepted notions of the functions of IFs in developing muscle cells. They also establish a new model system for the study of IF function during various stages of myogenesis. The elimination of background cytological "noise" that results from the intermingling of long IFs and nascent myofibrils should render this system ideal for following in detail the assembly of thick and thin filaments, SR, and T-system both in living cells and in EM sections. Further investigation of the properties of IF-deficient myogenic cells, in tissue culture and especially in transgenic animals, should provide insight into the still wholly unknown function(s) of desmin and vimentin IFs.

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