

Inhibition of Desmin Expression Blocks Myoblast Fusion and Interferes with the Myogenic Regulators MyoD and Myogenin

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Abstract. The muscle-specific intermediate filament protein, desmin, is one of the earliest myogenic markers whose functional role during myogenic commitment and differentiation is unknown. Sequence comparison of the presently isolated and fully characterized mouse desmin cDNA clones revealed a single domain of polypeptide similarity between desmin and the basic and helix-loop-helix region of members of the myoD family myogenic regulators. This further substantiated the need to search for the function of desmin. Constructs designed to express anti-sense desmin RNA were used to obtain stably transfected C₂C₁₂ myoblast cell lines. Several lines were obtained where expression of the anti-sense desmin RNA inhibited the expression of desmin RNA and

protein down to basal levels. As a consequence, the differentiation of these myoblasts was blocked; complete inhibition of myoblast fusion and myotube formation was observed. Rescue of the normal phenotype was achieved either by spontaneous revertants, or by overexpression of the desmin sense RNA in the defective cell lines. In several of the cell lines obtained, inhibition of desmin expression was followed by differential inhibition of the myogenic regulators myoD and/or myogenin, depending on the stage and extent of desmin inhibition in these cells. These data suggested that myogenesis is modulated by at least more than one pathway and desmin, which so far was believed to be merely an architectural protein, seems to play a key role in this process.

DESMIN is the muscle-specific member of the intermediate filament (IF)¹ protein multigene family (Lazarides and Hubbard, 1976). It is encoded by a single gene (Capetanaki et al., 1984a; Quax et al., 1985; Herrman et al., 1989; Li et al., 1989; Li and Capetanaki, 1993) which is expressed in all muscle types (for review see Lazarides, 1980; Holtzer et al., 1982; Lazarides et al., 1982; Osborn et al., 1982; Lazarides and Capetanaki, 1986; Franke, 1987; Steinert and Rop, 1988). During early development desmin seems to be one of the earliest myogenic markers (Bennet et al., 1979; Gard and Lazarides, 1980; Kaufman and Foster, 1988; Herrman et al., 1989; Schaart et al., 1989; Choi et al., 1990). It first appears at 8.25 d postcoitus (dpc) in the neuroectoderm where it is transiently expressed together with vimentin and keratin. Then it appears in the heart rudiment at 8.5 dpc where no other muscle-specific gene product has yet been found and at 9.0 dpc it appears in the myotome (Fürst et al., 1989; Schaart et al.,

1989). Desmin seems to be expressed at low levels in satellite cells (Allen et al., 1991) and replicating myoblasts (Dlugosz et al., 1984; Hill et al., 1986; Kaufman and Foster, 1988) which express high levels of the growth-regulated IF protein vimentin. Myogenesis in vitro is accompanied by down-regulation of vimentin and accumulation of desmin (Bennett et al., 1979; Gard and Lazarides, 1980; Holtzer et al., 1982; Capetanaki et al., 1984a,b; Fischman and Danto, 1985; Olson and Capetanaki, 1989). We have previously shown that C₂ myoblasts transfected with oncogenic ras genes express higher vimentin levels and fail to down-regulate vimentin and up-regulate desmin mRNAs (Olson and Capetanaki, 1989). Although these cells do not differentiate, they do express low levels of desmin suggesting that the regulation of the initial expression of desmin is independent of terminal differentiation.

Because of the lack of any functional studies, we do not know the role of desmin in myoblasts, early in development or in terminally differentiated muscle. The biological function of IF in general remains elusive (for review see Traub, 1985; Klymkowsky et al., 1989). It was initially suggested that in terminally differentiated myotubes, a potential role for desmin is to form a transcytoplasmic integrating matrix, linking individual myofibers laterally at their Z-disks and to the sarcolemma or longitudinally connecting successive Z-bands in elongating myofibrils (Granger and Lazarides,

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1. *Abbreviations used in this paper:* 3'UTRs, 3' untranslated regions; dpc, days postcoitus; GFAP, glial fibrillary acidic protein; HLH, helix-loop-helix; IF, intermediate filament; MARS, matrix-associated DNA regions; MCK, muscle creatine kinase.

1979; Tokuyasu et al., 1984; for review see Lazarides et al., 1982; Price and Sanger, 1983; Lazarides and Capetanaki, 1986). However, recent *in vitro* data have shown that disruption of desmin/vimentin filaments does not interfere with the assembly, lateral alignment, and maintenance of the striated contracting myofibrils (Schultheiss et al., 1991) suggesting that, at least *in vitro*, intact IFs are dispensable for these late processes of myogenesis. Additional data, accumulating the last few years have allowed speculation for alternative functions for these molecules. Vimentin and desmin seem to interconnect the nucleus to the plasma membrane (Woodcock, 1980; Granger and Lazarides, 1982; Fey et al., 1984; Goldman et al., 1985; Georgatos and Blobel, 1987a,b; Georgatos et al., 1987). This could be achieved through lamin B and ankyrin, respectively (Georgatos and Blobel, 1987a,b). In addition to lamin B these molecules also show high affinity for other nuclear constituents, like DNA and RNA (Traub, 1985; Cress and Kurath, 1988; Shoeman et al., 1988). Furthermore, it was recently demonstrated that lamin B specifically binds to nuclear matrix-associated DNA regions (MARS) (Ludérus et al., 1992). MARS are A-T-rich regions located in close vicinity to regulatory sequences or transcription unit boundaries (Mirkovitch et al., 1984; Gasser and Laemmli, 1986a,b; Jarman and Higgs, 1988; Phi-Van and Strätling, 1988) which by binding to nuclear matrix seem to activate gene expression (Stief et al., 1989; Phi Van et al., 1990; Grosveld et al., 1987). Considering all the above observations it could be envisioned that desmin and vimentin as well as other IF proteins could participate in signal transduction and transport processes between the cell surface and the nucleus and possibly modulate nuclear events. Indeed, it has been shown that mouse vimentin shares domains of significant similarity (Capetanaki et al., 1989b) with the DNA-binding domain and leucine zipper region of the transcription regulators *fos*, *jun* (Bohmann et al., 1987; Rauscher et al., 1988; Sassone-Corsi et al., 1988), and CREB, the cAMP-responsive DNA binding protein (Gonzalez et al., 1989; Hoefler et al., 1988). The significance of such similarities, if any, is completely unknown. In addition, over expression of vimentin has been linked to inhibition of differentiation of eye lens cells in transgenic mice (Capetanaki et al., 1989a) and in C₂ cells *in vitro* (Olson and Capetanaki, 1989).

All the above observations justified the need to search for the function of desmin during the initial and terminal stages of myogenic differentiation. We have isolated mouse desmin cDNAs and determined their complete primary amino acid structure. Computer sequence homology search has revealed significant homology between desmin and the basic and helix-loop-helix (HLH) region of members of the MyoD family which include MyoD (Davis et al., 1987), myogenin (Edmondson and Olson, 1989; Wright et al., 1989), MRF4 (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989), and Myf5 (Braun et al., 1989). All these myogenic regulators can convert fibroblasts to myoblasts through a mechanism requiring a basic region and a domain with homology to the myc family postulated to adopt a HLH conformation (Murre et al., 1989; for review see Emerson, 1990; Olson, 1990; Weintraub et al., 1991). All members of the myoD family are sequence-specific DNA-binding proteins requiring the basic and HLH region to bind to a consensus sequence CANNTG, known as an E box, and trigger

muscle-specific gene transcription (Braun et al., 1990; Brennan et al., 1991; Davis et al., 1990). To investigate further the biological significance of these homologies and study the role of desmin during myogenesis, we have inhibited its expression and demonstrated that this blocked the myogenic differentiation program by inhibiting myoblast fusion and interfering with the normal expression pattern of myoD and/or myogenin.

Materials and Methods

Isolation of Mouse Desmin cDNA

The mouse desmin cDNAs were isolated from a mouse myogenic BC3H-1 lambda-gt10 cDNA library (LaPolla et al., 1984), provided by Dr. N. Davidson (California Institute of Technology, Pasadena, CA), by screening with a chicken desmin cDNA probe (Capetanaki et al., 1984a) using conventional methods as previously described (Capetanaki et al., 1983).

Nucleotide Sequencing and Analysis of Protein Structure

Restriction fragments of the mouse desmin cDNA MD10₂ were subcloned in M13 and part of the nucleotide sequencing was performed by the dideoxy method (Sanger et al., 1980). In addition, the MD10₂ insert was subcloned in bluescript and deletion clones were similarly sequenced. Gaps were sequenced using synthetic desmin oligonucleotides as primers. The amino acid sequence was deduced from the nucleotide sequence by computer using the Eugene program. Sequence comparisons were performed using the Lawrence program (Lawrence and Goldman, 1988).

RNA Isolation and Analysis

C₂C₁₂ myoblasts were maintained and differentiated as previously described (Olson and Capetanaki, 1989). RNAs from different stages during myogenesis were isolated by a single step method using acid guanidinium thiocyanate-phenol chloroform extraction (Chomczynski and Sacchi, 1987). RNA blot analysis was performed as described previously (Capetanaki et al., 1983) and the quantitative levels were determined by densitometry and normalized for the loading differences, probe length, and specific activity.

Cultures and Transfections

The mouse skeletal muscle cell line C₂C₁₂ (Blau et al., 1983) was grown in DME containing 20% FCS as previously described (Olson et al., 1986; Olson and Capetanaki, 1989). To initiate differentiation, cultures at 80% confluency were transferred to DME containing 10% HS (or 2% FCS). Cultures of C₂C₁₂ cells in 100 mM culture dishes containing $\sim 0.5 \times 10^6$ cells were transfected by calcium phosphate precipitation (Graham and Van der Ed, 1973) with the pMAM_{neo} ± desmin constructs bearing the desmin cDNA in anti-sense and sense orientation, as shown in Fig. 4. The desmin in this construct was under transcriptional control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) aimed to confer glucocorticoid inducibility to the cDNA. All inserts in this construct are introduced in the SalI side of the polylinker of pMAM_{neo} vector obtained from Clontech (Palo Alto, CA), which also carries the neo gene for G418 selection. At 32 to 40 h after transfection, cells were transferred to three 100-mm dishes, and 60 h after transfection, 400 μg/ml G418 was added to the cultures of growth medium. Individual resistant colonies were isolated 14 d later and passaged into stable cell lines. Clonal cell lines were exposed to ~ 500 nM dexamethasone and were tested further. Rescue of the defective phenotype was achieved by the use of constructs expressing high levels of sense desmin from the very strong cytomegalovirus (CMV) enhancer/promoter. In this case the desmin cDNA was inserted into the pRc/CMV vector, a modification of pCMVβ (MacGregor and Caskey, 1989), obtained from Invitrogen (San Diego, CA) by directional cloning into the Hind-III/XbaI site of the polylinker. 20 μg of this vector were transfected into the fusion-suppressed antisense cell lines by calcium phosphate precipitation, followed after 15 h by glycerol shock, and the next day cells were switched to differentiation promoting media and analyzed after 4 d.

RNA PCR Analysis

cDNA synthesis of the desmin anti-sense RNA was performed with 10 µg total RNA using oligo(dT) as a primer. Part of the 3' half of the cDNA was amplified by PCR using Taq DNA polymerase as previously described (Saiki et al., 1985) to detect the expression levels of the anti-sense desmin RNA. Denaturation, annealing, and extension were performed for 1 min at 90°C, 2 min at 60°C, and 3 min at 72°C, respectively, in 40 cycles. PCR products were visualized by ethidium bromide staining.

Immunofluorescence and Western Blot Analysis

Immunofluorescence microscopy was performed using standard techniques. In the case of staining for desmin (D₃ antibody [Danto and Fishman, 1984] or D-8281 antibody; Sigma Chemical Co., St. Louis, MO) and myosin (MF20 antibody) (Bader et al., 1982), cells were fixed in ethanol at -20°C for 10 min and the antibody incubations were performed at 37°C for 30 min. For detection of MyoD and myogenin, the antibodies used were obtained from Dr. Weintraub's (Tapscott et al., 1988) and Dr. Wright's laboratories (Wright et al., 1991), respectively, and were incubated at room temperature for 1 h with cells fixed in 2% paraformaldehyde for 10 min and permeabilized with 0.15% Triton X-100 for 10 min. Western blot analysis was performed with the Sigma desmin antibodies as previously described (Capetanaki et al., 1989b).

Results

Isolation and Characterization of Mouse Desmin cDNAs

To isolate the mouse desmin cDNA, we screened a lambda-gt10 cDNA library from the mouse myogenic cell line BC3H-1, using the available chicken desmin-specific probe (Capetanaki et al., 1984a). Several clones were obtained. The longest one, pMD10-2, 1,497 bp, was subcloned into M13 and bluescript vectors and further characterized.

Genomic DNA blot analysis performed using pMD10₂ insert suggested that the desmin gene occurs once in the haploid mouse genome (data not shown).

The nucleotide sequence of the entire insert from the isolated desmin cDNA clone, pMD10₂, was established by sequence analysis of both strands. The obtained sequence (Fig. 1) comprises 1,497 bp which includes 83 bp of 5' untranslated region, 1,407 bp of coding sequence and only 7 bp of 3' untranslated region. The 1,407-bp coding region of this cDNA revealed the total primary structure of mouse desmin comprising 469 amino acids. Most of the 3' noncoding region is missing.

Analysis of the obtained mouse desmin protein sequence led to the assignment of the three structural domains established for all IF subunits studied to date (Geisler and Weber, 1982). These include a non- α -helical arginine rich NH₂-terminal head piece of 85 residues, a central rod-like α -helical domain of 330 residues consisting of three α -helices separated by two short non- α -helical spacers and a non- α -helical COOH-terminal tail piece of 45 residues (Fig. 1). The assigned α -helices contain the characteristic seven-residue repeat pattern with hydrophobic amino acids at the first (a) and fourth (d) position of the heptad (a to g), involved in formation of IF coiled-coil quaternary structure.

Amino acid sequence comparison between mouse desmin and the available sequences from other species revealed very high identity to hamster (98.7%), human (95.1%), and chicken (80.2%) (data not shown). Comparison of desmin with the other mouse IF proteins showed the highest homology to vimentin (61%).

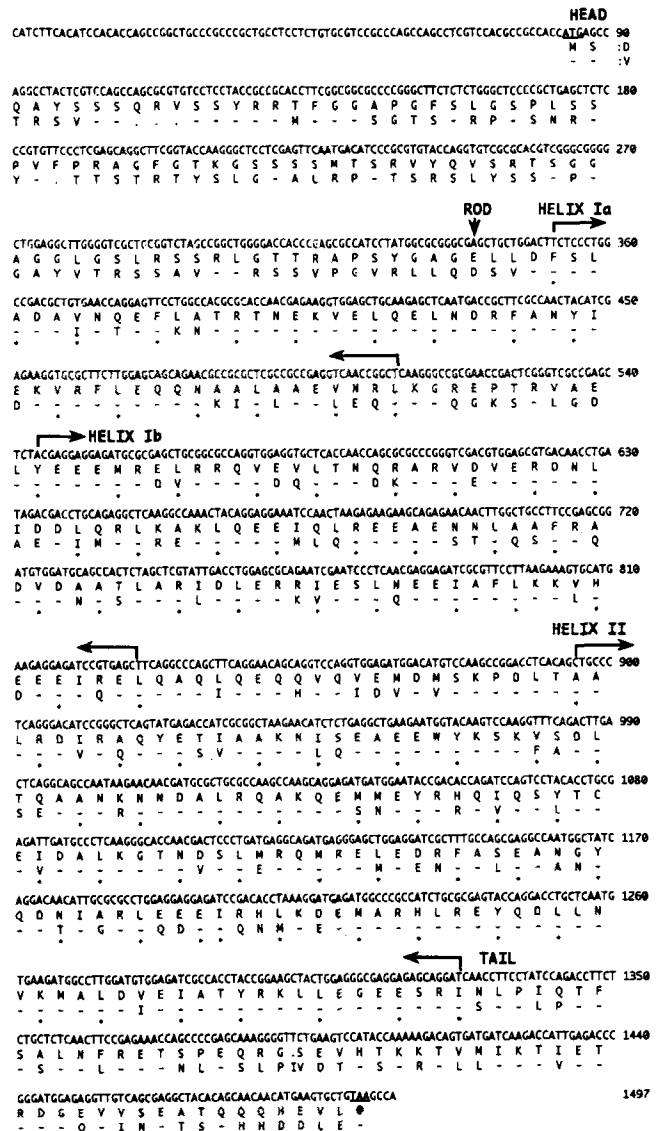


Figure 1. Nucleotide sequence and the predicted complete amino acid sequence of mouse desmin from BC3H-1 cells. Initiation and termination codons are underlined. Mouse vimentin (Capetanaki et al., 1990) is included for comparison. The non- α -helical head (NH₂-terminus) and tail (COOH-terminus) domains and the α -helical rod middle domain are indicated. Hydrophobic residues in positions a and d of the heptads are indicated by dots below vimentin sequence. This sequence data is available from EMBL/GenBank/DBJ under accession number L22550.

Adjacent Regions of Desmin Show Similarity to the Basic and HLH Region of Members of the MyoD Family

We have recently shown that the growth-regulated IF protein vimentin shares domains of similarity with the DNA-binding/leucine zipper motif of the growth regulators fos, jun, and CREB (Capetanaki et al., 1990). We wanted to determine if a similar relationship exists between the muscle-specific IF protein desmin and the known myogenic regulatory proteins. A computer sequence search revealed a single domain of homology (Fig. 2). Interestingly, this domain is

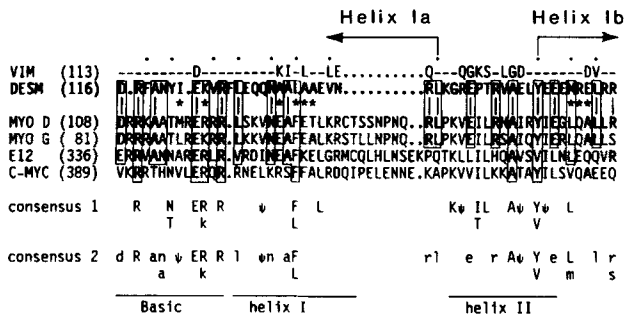


Figure 2. Similarity between desmin and myogenic members of the HLH motif containing family. Sequences were aligned to maximize similarity of desmin (*DESM*) to the shown consensus 1 sequence for the HLH region of all known members adapted from Murre et al., 1989. Consensus 2 is deduced by comparison of desmin, myoD (Davis et al., 1990), myogenin (myoG) (Edmondson and Olson, 1989), and E12 (Murre et al., 1989). The ψ symbol indicates hydrophobic residues. The capitals in consensus 2 indicate identity to consensus 1. Dots represent gaps in sequence introduced to allow the alignment. Dots above the vimentin sequence indicate the hydrophobic residues in positions a and d of the heptads of desmin and vimentin. Dashed lines above desmin show the amino acids of vimentin (*VIM*) identical to desmin. The end of helix Ia and beginning of helix Ib of vimentin and desmin are indicated by arrows above the sequences. The basic domain and the helix I and helix II of the HLH family are underlined below the sequences. Identity to desmin amino acids is indicated by a vertical line above the corresponding amino acids. Stars (*) show conserved amino acids.

the initially reported myc-like DNA-binding domain of myoD found to be sufficient for activation of the muscle differentiation program (Tapscott et al., 1988). It includes the proposed HLH motif present in all known members of the myoD family, necessary for obligatory heterodimer formation with the ubiquitous HLH proteins E12 and E47 (Murre et al., 1989), and overlaps the basic region responsible for DNA binding (Davis et al., 1990). The similarity to desmin covers the end of desmin helix Ia, the linker, and the beginning of helix Ib. Among all the known members of the HLH family, myoD, myogenin, and E12 show the highest similarity to desmin. Desmin closely follows the consensus proposed (Murre et al., 1989) for all the above HLH proteins (Fig. 2, *consensus 1*). When the group is restricted to desmin, myoD, myogenin, and E12, a more extensive consensus is revealed (Fig. 2, *consensus 2*). The observed similarities apply to other members of the IF family including vimentin which shows highest similarity at the basic and helix I region and the least in helix II. Lamin C, however, follows the consensus mainly in helix II and not in the other two domains (Murre et al., 1989). Further comparisons of mouse desmin also revealed domains of similarity with the transcription factors, fos, jun, and CREB, as predicted due to its high homology with vimentin (Capetanaki et al., 1990). An interesting aspect of all the above similarities is that they all are localized at the same overlapping or adjacent regions of desmin, that is the end of helix Ia and the beginning of the helix Ib (data not shown).

Expression of Desmin in Comparison to MyoD and Myogenin During Myogenesis In Vitro

To investigate the expression of desmin in relation to the

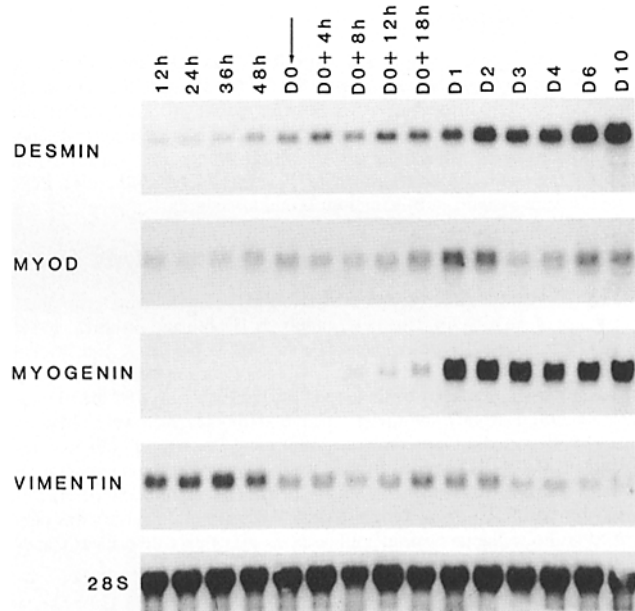


Figure 3. Comparison of the expression pattern of desmin relative to myoD, myogenin, and vimentin RNA during growth and differentiation of C_2C_{12} cells. 10 μ g total RNA from different times after plating (0 h, 12 h to 48 h) in growth medium (DME with 20% FCS; GM) or different times after transferring to differentiation/fusion promoting medium (DME with 10% HS;DM/FM) (Day 0, (D0), D0 + 4–18 h, D1–D10) of the myogenic C_2C_{12} cells were fractionated on 1.3% agarose/formaldehyde gels, transferred to nitrocellulose, and hybridized with the mouse desmin, vimentin, myoD (Davis et al., 1990), myogenin (Edmondson and Olson, 1989), and 28S rRNA 32 P-labeled cDNA inserts. The 28S ribosomal blot was used as internal control for RNA concentration. In lane D0 + 8 h, only 7.5 μ g RNA was used. On day 0 (68 h after plating), when the high serum was withdrawn, cultures had reached 80% confluency. X-ray exposure for desmin, vimentin, and myogenin was 18 h; for myoD 3.5 d and 28S 20 min.

transcription regulators, myoD, and myogenin, we examined the accumulation of their mRNA at very early stages and throughout myogenesis of C_2C_{12} cells (Fig. 3). Analysis of myoblasts growing in high serum concentrations any time after plating exhibit easily detectable levels of desmin mRNA, significantly lower levels of myoD mRNA (about 20% of that of desmin), and no detectable myogenin mRNA. There is no significant increase of desmin and myoD RNA by 48 h after plating. By 68 h (D0 in Fig. 3), however, an increase of around fivefold is observed in the abundance of desmin mRNA and threefold of myoD mRNA. It is not until 8 h after serum is withdrawn that myogenin mRNA is first detected, and then exhibits a fourfold induction by 18 h and a 24-fold increase by 24 h after switching to differentiation media. This data is consistent with previously reported observations (Edmondson and Olson, 1989; Wright et al., 1989). Interestingly, a 36–38% decrease in the abundance of myogenin mRNA is observed by day 4, followed by an increase which, by day 10, rises 23-fold above the basal level. After withdrawal of the high serum from the media, desmin mRNA shows an 18% increase in 4 h, while myoD mRNA does not seem to change before 12 h. By 24 h, however, myoD mRNA reaches its maximum level (about eight times the basal value), then drops to three times the basal level by the third

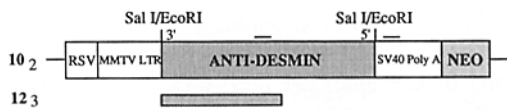


Figure 4. Constructs designed to express anti-sense desmin RNA under the dexamethasone MMTV inducible promoter. The entire cDNAs (10₂) or the truncated form 12₃ (nt 650–1,490) were inserted at the Sal I site of the multiple cloning site of pMAM_{neo} by blunt-ended ligations. The *E. coli* neo gene for selection is incorporated in the same vector. Small bars above the construct 10₂ indicate the position of PCR primers used in Fig. 6.

day and then in 3 d, increases again sixfold above the basal level and shows only slight decrease by day 10. Desmin mRNA, on the other hand, increases linearly to day 10 achieving a level 27 times basal concentration. Notably, an abrupt decrease by day 2 of both myoD and myogenin is observed when the accumulation of these RNAs is quantitated densitometrically and normalized by the 28S ribosomal signal (data not shown). The pattern of vimentin mRNA expression during C₂C₁₂ cell differentiation is also included in Fig. 3. It has already been shown that, contrary to desmin, the abundance of vimentin mRNA decreases during myogenesis (Capetanaki et al., 1983, 1984a,b; Olson and Capetanaki, 1989). By 68 h after plating, vimentin mRNA abundance decreases by 66%. 12 h after serum withdrawal from the media, vimentin mRNA decreases to 24% the starting level and interestingly, after six hours, shows a sharp increase (25% of basal level) and then decreases again. By day 10, vimentin mRNA decreases to only 9% of the basal level.

Inhibition of Desmin Expression by Anti-sense RNA

In order to inhibit the expression of desmin during myogenesis in vitro, the constructs shown in Fig. 4 were prepared. The entire mouse desmin cDNA (10₂) or a truncated form of it (12₃) missing the 1/3 5' end of the molecule were inserted in both sense and anti-sense orientation in the pMEM_{neo} vector where the strong Rouse sarcoma virus-LTR (RSV-LTR) enhancer is linked to the dexamethasone inducible MMTV-LTR promoter for control of high level expression. In the present experiments, however, this promoter was leaking significantly so that constitutive expression was inevitable (see below). C₂C₁₂ cells were transfected with the above constructs and colonies resistant to G418 were isolated and screened by Southern blot analysis. One to five copies of the desmin constructs were incorporated in the stably transfected cell lines obtained (data not shown).

Several of these colonies were grown for 24 or 48 h at subconfluent densities in growth medium (DME with 20% FCS) in the presence of 0.5 μM dexamethasone. As mentioned above, under these conditions, C₂ myoblasts proliferate and express low levels of desmin and myoD (Fig. 3) but no myogenin or other myogenic regulatory proteins. When these cell lines reached 80% confluency, they were transferred to differentiation-promoting medium (DME with 2% FCS or 10% HS, also containing 0.5 μM dexamethasone). Normal C₂ myoblasts under such conditions start fusing to multinucleate myotubes and the onset of differentiation is marked by induction of myogenin mRNA and increased accumulation of desmin mRNA and the rest of the muscle-specific gene products.

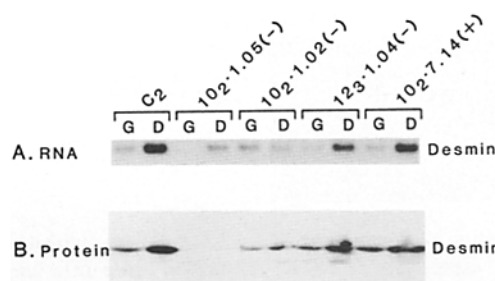


Figure 5. Inhibition of desmin expression by anti-sense RNA. (A) Northern blot analysis of total RNA from cell lines bearing the anti-sense (-) and sense (+) 10₂ and 12₃ constructs from Fig. 4, and cultured in growth medium (G) or, after achieving 80% confluency, switched to differentiation promoting medium (D) for 48 h. The blot with a 28S ribosomal probe used as an internal control for RNA concentration is shown in Fig. 8. (B) Western blot analysis with desmin antibodies was performed using total protein isolated from the above cell lines at the corresponding stages.

To determine the effect of the anti-sense RNA on the level of desmin expression in the transfected cell lines, we examined both RNA and protein. Total RNA isolated from cultures grown for 48 h in growth medium or switched and maintained for 48 h in differentiation-promoting medium was analyzed by Northern blots using the mouse desmin cDNA insert MD10₂. Fig. 5 A shows examples out of several anti-sense cell lines studied where a dramatic or moderate suppression of desmin mRNA accumulation had occurred. Densitometry of the obtained blots and normalization for RNA content against 28S ribosomal RNA (not shown) allowed approximate quantitation of the observed inhibition. In cell line 10₂.1.05 considerable desmin mRNA inhibition was displayed both in cultures maintained 48 h in growth (>99%) and differentiation (~85%)-promoting medium. On the other hand, clone 10₂.1.02 exhibited maximum desmin mRNA inhibition in cultures maintained for 48 h in differentiation-promoting medium (~93%), whereas during growth no detectable inhibition was observed. Clone 12₃.1.04 produced an inhibition pattern similar to that of 10₂.1.05 but to a lesser extent; under growth and differentiation conditions inhibition was ~22 and ~62%, respectively. No change was observed in those cell lines transfected with constructs of desmin in sense orientation (10₂.7.14).

When the desmin anti-sense RNA effect was examined at the protein level by Western blot analysis (Fig. 5 B), in most cell lines desmin protein was suppressed proportionally to the suppression of desmin mRNA. Clone 10₂.1.05 showed a 60% inhibition of desmin in growth and 85% inhibition under differentiation-promoting conditions. Similarly, in clone 12₃.1.04 there was a 27 and 70% inhibition of desmin under growth and differentiation conditions, respectively. There were cases however, like clone 10₂.1.02 where protein inhibition under growth conditions for instance, reached up to 50% of the normal level while no RNA inhibition was observed. Different mechanisms of anti-sense action could explain such observations (see Discussion). Under differentiation conditions the same clone demonstrated desmin inhibition at both protein (70%) and RNA level (93%). Similar results were obtained regardless of the presence or absence of dexamethasone, which initially suggested constitutive expression

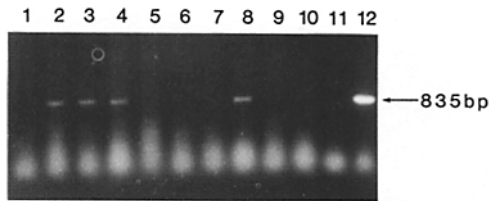


Figure 6. Detection of desmin anti-sense RNA in cells with inhibited desmin expression. Total RNA isolated from cell line 12₃.1.04 (described in Figs. 5 and 6) after 24 h (lanes 2 and 3) and 48 h (lane 4) in growth medium (GM) or 12 h (lane 5), 24 h (lane 6), 36 h (lane 7), and 48 h (lane 8) after switching to differentiation promoting medium (DM) was analyzed by reverse transcription-PCR using oligo (dT) and the primer shown in Fig. 4. Several controls are included: RNA from normal C₂C₁₂ cells (lane 1) cultured for 48 h in GM and from cell line 10₂.7.14 bearing the desmin construct in sense orientation and cultured for 48 h in GM (lane 9) or 48 h after switching to DM (lane 10). In lane 2, cells were cultured in the absence of dexamethasone in contrast to all the other cases. Lane 11 contains buffer only and lane 12 contains control plasmid. The expected 835-bp long PCR products were visualized by ethidium bromide staining.

of the anti-sense RNA from the constructs used and that is indeed documented in Fig. 6, lane 2.

Initial attempts to detect the desmin anti-sense RNA transcribed from the transfected constructs using RNA blot analysis were not successful. Given the fact that sense-antisense RNA duplex formation and rapid degradation is one of the accepted mechanisms of anti-sense action, the expected anti-sense RNA surviving this process could be of low abundance. To detect low levels of desmin anti-sense RNA from different cell lines, total RNA from cultures maintained in growth or differentiation-promoting medium in the presence or absence of dexamethasone was analyzed by reverse transcription followed by PCR (Fig. 6). Anti-sense RNA could be detected in only one (12₃.1.04) of the three transfected cell lines analyzed. Interestingly in those cell lines where the most dramatic endogenous desmin inhibition was observed, such as 10₂.1.05 and 10₂.1.02, no anti-sense RNA could be detected even as early as 6 h after plating. Similar observations of simultaneous reduction or disappearance of sense and anti-sense RNA have been reported in other systems (Sheehy et al., 1988; Smith et al., 1988; Van der Krol et al., 1988; Hamilton et al., 1990). The expression of the desmin anti-sense mRNA in the 12₃.1.04 cell line was further analyzed. As shown in lane 2 of Fig. 6 anti-sense RNA was expressed in the absence of dexamethasone at levels close to those attained in the presence of dexamethasone (lane 3). This confirmed the leakiness of the vectors and explained the above observations. The abundance of the anti-sense RNA did not change from 24 to 48 h in growth medium. However anti-sense RNA could not be detected during the initial 36 h after switching to differentiation-promoting medium but it reappeared at 48 h in differentiation medium at levels comparable to those attained in cultures maintained in growth medium.

Inhibition of Desmin Expression Blocks Myoblast Fusion and Myotube Formation

Morphological analysis of the desmin-defective cell lines

demonstrated the failure of these myoblasts to fuse and form multinucleated myotubes. Fig. 7, *bottom*, shows the morphology of one of the analyzed cell lines, 10₂.1.02, displaying inhibited desmin expression (Fig. 5), after 4 d in differentiation-promoting medium. In growth medium, these cells were initially morphologically indistinguishable from the nontransfected normal C₂C₁₂ myoblasts, or transfectant 10₂.7.14 with the desmin construct in sense orientation. When the cultures were transferred to differentiation-promoting medium, inhibition of fusion was observed. Even 4 d after the switch to this medium, no particular change in the behavior of these inhibited myoblasts was observed (Fig. 7, *bottom*). 70% of the 31 G418 resistant anti-sense cell lines analyzed demonstrated similar inhibition in myoblast fusion. In contrast, more than 90% of the 12 cell lines bearing desmin constructs in the sense orientation (Fig. 7, *middle*) fused and differentiated as well as normal C₂C₁₂ cultures (Fig. 7, *top*).

Rescue of the Normal Myogenic Phenotype

To demonstrate that the inhibition of the myogenic differentiation observed above is indeed due to desmin inhibition and not a random defect of the isolated cell lines, rescue of the normal differentiation was achieved by two different ways. The first one was obtained due to spontaneous revertants when cells were cultured and passaged in the absence of G418 selection (Fig. 8). Up to about 5% of the cells seemed to fuse again and the majority of them formed single myotubes of relatively short length (Fig. 8 *b*). This does not happen when cells are cultured in the presence of G418 (Fig. 8 *a*). A very low percentage of these revertants form long myotubes, as shown in Fig. 8 *b*, and very rarely branched ones. 10₂.1.05 was the cell line with the highest percent of revertants. The second way the normal phenotype was rescued was when the desmin-defected cell lines were transiently transfected with the pRc/CMVdes construct which allows expression of high levels of desmin sense RNA from the strong CMV promoter described in methods (Figs. 8 *c* and 9). Transient transfections of the defected cell lines with this vector could rescue the myogenic phenotype, at least in 20% of the cell population, in 4 d (Figs. 8 *c*, and 9, *D* and *E*). Myoblast fusion and myotube formation looked very similar to that of normal C₂C₁₂ cells. In addition to the higher number of fusing cells in this case (Fig. 8 *c*), another prominent difference with the spontaneous revertants (Fig. 8 *b*) was the higher frequency of long and branched myotubes formed. These differences obviously must be due to the high number of cells expressing adequate levels of desmin from the transfected pRc/CMVdes vector. Indeed, immunofluorescence microscopy revealed that in contrast to the untransfected 10₂.1.05 cells (Fig. 9 *B*), those transfected with pRc/CMVdes vector showed high levels of desmin expression (Figs. 9, *C* and *D*). It is of interest to note that when cells with high levels of desmin expression, such as the one shown in *C* of Fig. 9, were surrounded by desmin negative cells, they often showed long extensions but did not fuse with neighboring cells, suggesting that fusion might take place only between desmin positive cells. Staining of the transfected cells with myosin heavy chain (MHC) antibodies demonstrated that in these desmin revertants the normal myogenic phenotype is accompanied by normal endogenous MHC expression (Fig.

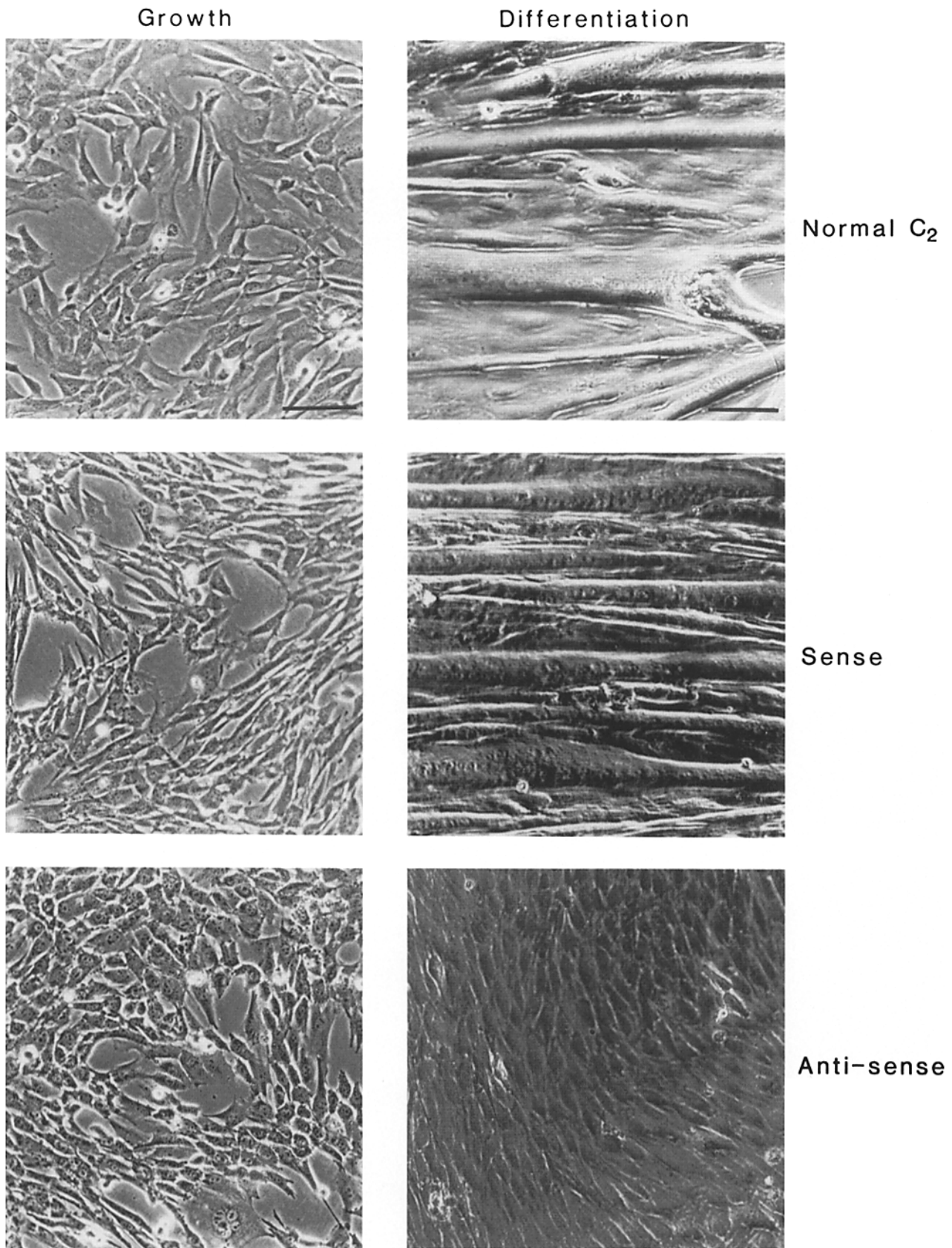


Figure 7. Inhibition of desmin blocks fusion and myotube formation of the C₂C₁₂ myogenic cells. Normal C₂C₁₂ cells (*top*) and cell lines bearing the desmin construct in sense orientation (*middle*) are compared with one of the anti-sense cell line (10₂.1.02) with inhibited desmin expression (*bottom*) due to anti-sense RNA. Cells were cultured as described in Fig. 5 in growth medium and after achieving 80% confluency, cultures were transferred to differentiation promoting medium for 4 d. Bars: (*Growth*) 20 μm; (*Differentiation*) 10 μm.

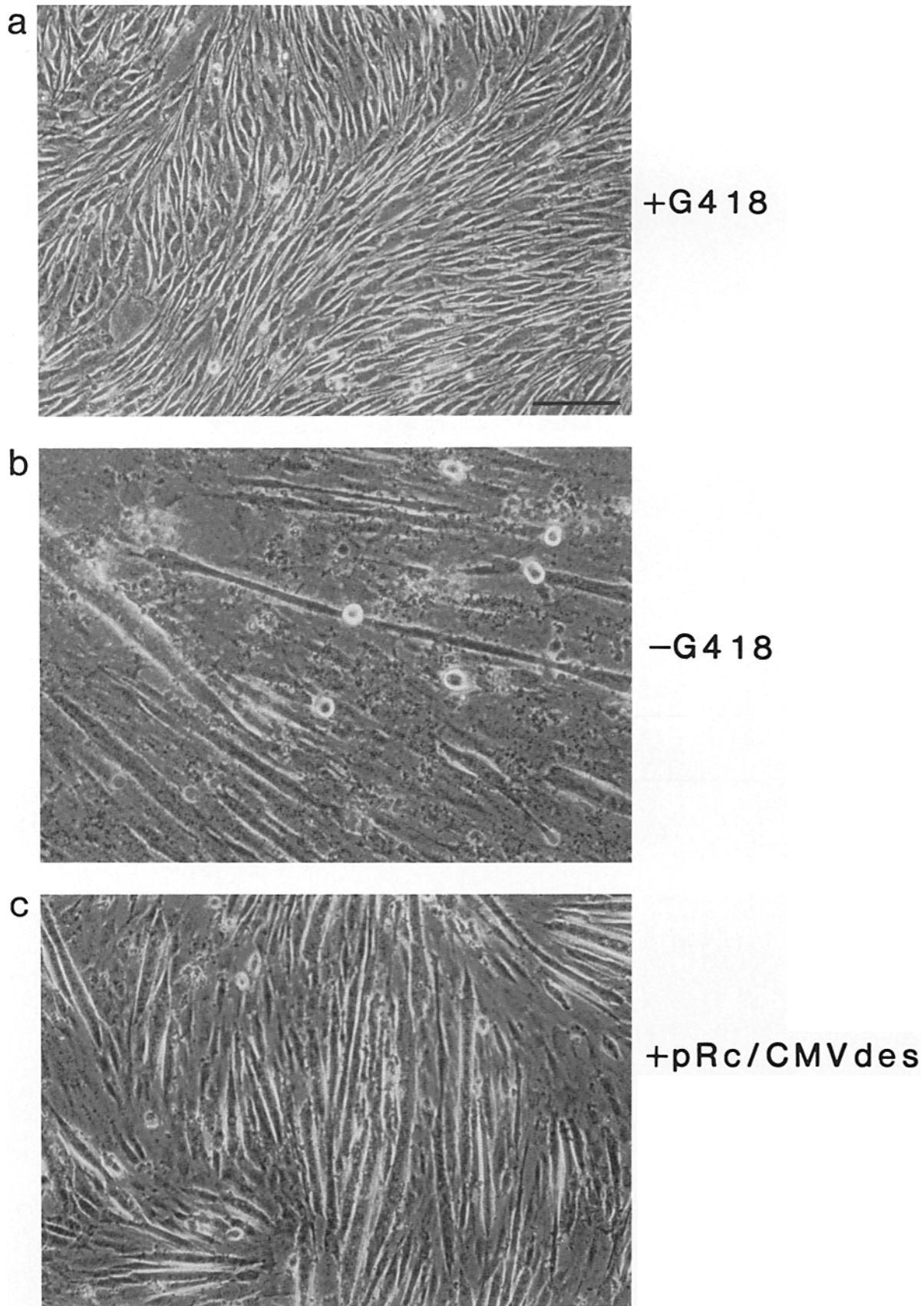


Figure 8. Rescue of the normal myogenic phenotype. Phase micrographs of the fusion suppressed anti-sense cell line 10₂.1.05, grown with (a) or without (b) G418 selection or transfected with the pRc/CMVdes construct overexpressing sense desmin (c). (a) Cells were cultured in the presence of G418 for 48 h in GM, and then switched to DM for 4 d. (b) Cells were grown and passaged for several days in the absence of G418 before the actual experiment. After reaching 80% confluency, they were switched as above to DM for 4 d. (c) 10₂.1.05 cells were grown in GM for 24 h before transfection with 20 μg pRc/CMVdes vector DNA. Cells were switched the next day to DM and maintained for 4 more days. Bar, 5 μm.

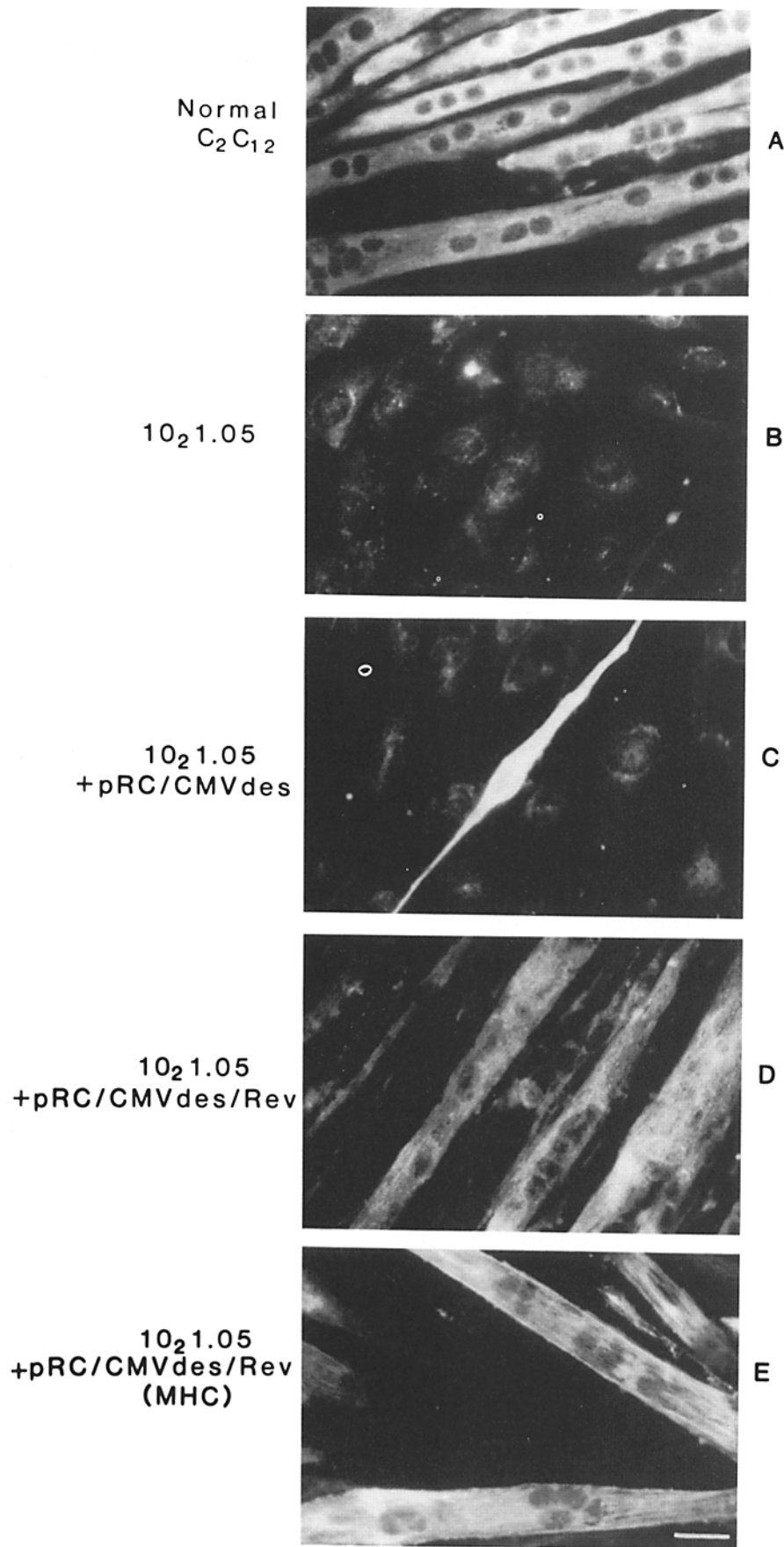


Figure 9. Immunofluorescence microscopy of desmin in normal C₂C₁₂ (A) cells and the fusion-suppressed 10₂1.05 anti-sense cell line before (B) and after (C and D) reversion (*Rev*) to the normal phenotype by pRC/CMVdes transfection and culture as described in Fig. 8. C shows an example of areas in the same culture dish as D with isolated cells expressing high desmin levels but unable to fuse with neighboring desmin negative cells. E shows MHC staining of cells from the same culture as D. Bar, 10 μ m.

9 E). The above rescue experiments demonstrated that the observed inhibition of fusion in the different anti-sense cell lines obtained was indeed due to the inhibition of desmin and not to a random defect of the isolated cell lines.

Inhibition of Desmin Interferes with the Expression of Myogenic Regulators and Other Muscle-specific Genes

The data had demonstrated that suppression by anti-sense RNA of desmin, a protein with some similarity to the myogenic regulators myoD and myogenin at the basic and HLH region caused inhibition of myoblast fusion and myotube formation. If desmin participates in the modulation of myogenesis, how does this correlate with the above two observations? To determine whether the inhibition or reduction of desmin has influenced the expression of other muscle-specific genes, RNA blots from one of the cell lines shown in Fig. 6, namely 10₂.102, were probed for myoD, myogenin, muscle creatine kinase (MCK), and actin. Interestingly, significant inhibition in the expression of both myoD and myogenin mRNA was evident in cultures maintained 48 h in differentiation medium as compared to normal C₂C₁₂ cells (Fig. 10 A). The extent of myoD and myogenin inhibition correlated, in most cases (70% of cell lines analyzed), directly to the extent of desmin inhibition. In this particular case it is clearly shown that when desmin is inhibited in cultures maintained up to 48 h in differentiation medium (Fig. 10 A, lane 4) both myoD and myogenin are inhibited. Earlier, however, when desmin has not yet been detectably decreased, no obvious change in myoD has occurred (Fig. 10 A, lane 3). Patterns of inhibition similar to those of myogenin mRNA were observed only for muscle-specific genes such as α -sarcomeric actin and MCK mRNA, but not for others like β , γ -actins, and vimentin (Fig. 10 A). This suggests that indeed, the effect is specific to the myogenic processes and not a random effect of the anti-sense RNA. Though the above described effect of desmin inhibition on the pattern of muscle gene expression is the most representative (70% of cases examined), there were cell lines like 10₂.105 where though the extent of myoD decrease directly reflected the level of desmin inhibition, myogenin did not (Fig. 10 B, lane 4). In this cell line, where the inhibition of desmin mRNA was dramatic, very strong inhibition of myoD mRNA was observed in both growth (94%) and differentiation (85%) conditions. Myogenin was not significantly inhibited, however (~13%) (Fig. 10 B, lane 4). In these cases α -sarcomeric actin and MCK expression looked very close to normal (data not shown). Fusion, however, was completely inhibited. On the other hand, there were cases, such as clone 12₃.104, where myoD mRNA was mainly inhibited during growth (81%) (Fig. 10 B, lane 5), with little inhibition under differentiation conditions (22%) (Fig. 10 B, lane 6), when myogenin displayed significant inhibition (90%) (Fig. 10 B, lane 6). In such cell lines, extensive inhibition of both MCK (67%) and α -actin (73%) mRNA was observed (data not shown). It should be noted here that the observed inhibition of myoD or myogenin cannot be due to the desmin anti-sense RNA since the similarity between desmin and these proteins shown in Fig. 2, is restricted only to the protein level. In addition, the construct 12₃.104 (nt 650-1,490) which shows similar results to construct 10₂ (Fig. 4) does not include this region (nt 348-498) which

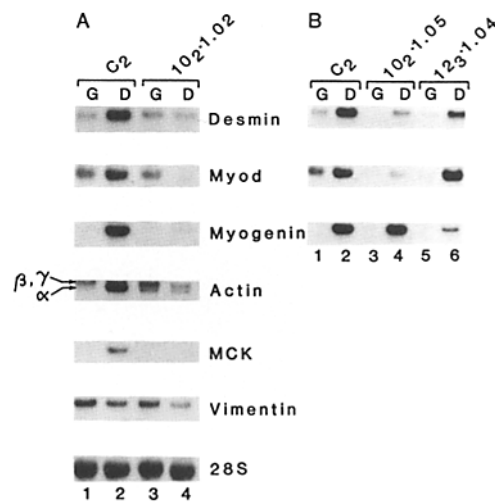


Figure 10. Inhibition of desmin interferes with the expression of myogenic regulators myoD and myogenin and their target genes. Northern blot analysis of total RNA from the cell line 10₂.102 (A) and 10₂.105 and 12₃.104 (B) with inhibited desmin expression described in Figs. 5, 6, and 7 cultured in growth (G)- or differentiation-promoting medium (D) for 48 h. Similar blots were hybridized, stripped, and rehybridized sequentially with the different probes as indicated.

shows the protein similarity between desmin and these proteins.

Finally, we examined the pattern of myoD and myogenin protein expression in these cell lines by immunofluorescence microscopy. As shown in Fig. 11 the nuclear staining patterns obtained are in direct agreement with the MyoD and myogenin RNA data shown in Fig. 10. For instance, in cultures maintained under differentiation condition, 10₂.102 cell line shows no nuclear staining for either myoD or myogenin, while 10₂.105 and 12₃.104 show staining only for one of these factors, myogenin or myoD, respectively. Furthermore, it is demonstrated that the degree of inhibition observed at the RNA level of these factors reflects mainly the number of cells expressing rather than levels of expression in single cells.

Discussion

Is Desmin Necessary for Myogenesis to Take Place and What Could Be Its Role during This Process?

Desmin is expressed both early and late in the myogenic program. It is one of the earliest myogenic markers both in vivo (Schaart et al., 1989) and in tissue culture, being expressed in replicating presumptive myoblasts (Dlugosz et al., 1984; Hill et al., 1986; Kaufman and Foster, 1988). That the appearance of desmin marks a very early event in myogenesis has been further supported by the fact that several nonmuscle cells, in the process of conversion into the myogenic lineage due to expression of exogenous myoD, start synthesizing desmin before the synthesis of any myofibrillar protein and before fusion (Choi et al., 1990). Similarly, during early development in mice, desmin has been shown to be one of the

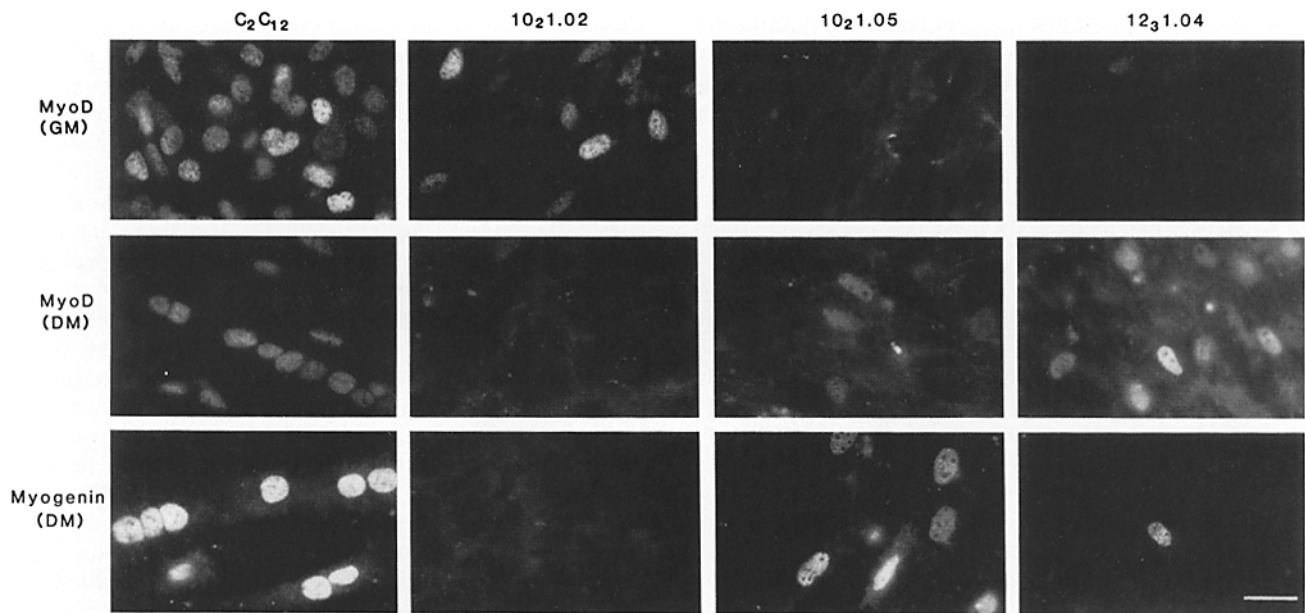


Figure 11. Immunofluorescence microscopy of MyoD and myogenin in normal C₂C₁₂ and different fusion-suppressed antisense cell lines. Cells cultured 2 d in growth (GM) and consequently switched for three more days in differentiation media (DM) were incubated with mouse myoD or myogenin antibodies. Bar, 10 μ m.

earliest expressed muscle-specific genes. The protein is initially detected in the neuroectoderm at 8.25 dpc, transiently expressed with vimentin and keratin while no other muscle-specific protein has yet been identified. It then appears in the heart rudiment at 8.5 dpc and in myotome at 9.0 dpc, well before myoD and as soon as the MRF4 mRNA is detected (Sassoon et al., 1989). Correlations with the appearance of Myf5 (Braun et al., 1990) and myogenin cannot be directly made since the time these proteins appear is unknown; their RNA is detected in the somites on 8.0 and 9.0 dpc, respectively. It would be interesting to determine what the role of desmin might be during these early events of myogenic determination and differentiation. Is desmin necessary for myogenesis to take place or for the maintenance of the myogenic phenotype?

Our present data have suggested that desmin might indeed be necessary for normal myogenesis to take place. Inhibition of desmin expression interfered with the normal induction of muscle-specific gene products in C₂C₁₂ cells and blocked myoblast fusion and myotube formation. These results suggested that the muscle-specific IF protein desmin, so far considered merely a component of the supporting cell architecture, might be a central molecule regulating myogenesis through direct or indirect modulation of myogenic regulators and myoblast fusion. In addition to vimentin (Capetanaki et al., 1989) and desmin (this article), another intermediate filament protein, glial fibrillary acidic protein (GFAP) has been linked to a differentiation-associated function (Weinstein et al., 1991). It is intriguing that all of these proteins are type III IF, showing the highest similarity to each other in comparison to the rest of the members of the IF superfamily. The present data cannot address possible direct involvement of desmin in myoblast fusion. This possibility, however, could be favored by the observation that ectopic ex-

pression of desmin in the lenses of transgenic mice causes, among other abnormalities, partial plasma membrane fusion (Krimpenfort et al., 1988; Dunia et al., 1990). On the other hand, from the present rescue studies it could be suggested that possibly only desmin positive myoblasts can fuse (Fig. 9 C). However, this is not proven from the present data, although it is consistent with recent observations on primary chick myoblasts (Lin et al., 1994) as well as in myoD-converted normal cells (Choi et al., 1990) which do not fuse before becoming desmin positive.

In the present study the different cell lines obtained showed differential inhibition of myoD and myogenin in response to the inhibition of desmin. The extent and stage of myoD inhibition directly correlated with the extent of desmin inhibition and the stage of the C₂C₁₂ cells. However this was not the case for myogenin. Only 60% of these lines showed substantial inhibition of myogenin following desmin inhibition. It is not yet clear how this differential effect takes place. It could be explained, however, by the already suggested multiple pathway process for myogenic differentiation in vivo (Sassoon et al., 1989; Cusella-De-Angelis et al., 1992). How the absence or decrease of desmin could influence the accumulation of these myogenic factors is difficult to predict. At the present time we do not really know if this effect is the first consequence of desmin inhibition or if it follows after myogenesis has been inhibited by an independent mechanism. Furthermore, we do not know if the observed mRNA decrease takes place at the transcriptional or posttranscriptional level. Sequence comparison revealed (Fig. 2) that the region of desmin (116–207) which covers part of helix Ia, the linker, and part of helix Ib has significant sequence similarity to members of the HLH family, particularly the myogenic regulator myoD, myogenin, and the KE2 (immunoglobulin enhancer)-binding protein E12 (Murre et

al., 1989). Overlapping and adjacent regions of desmin also show similarity to the basic and leucine zipper domains of the transcription factors jun, fos, and CREB. It is interesting that two adjacent regions of desmin demonstrate similarity to the very important functional regions of members of two different transcription factor protein families acquiring either the HLH or the leucine zipper motifs. It should be further noted here that members of these families seem to function antagonistically, possibly by using these important domains (Bengal et al., 1992; Li et al., 1992). Nuclear lamins, the nuclear counterpart members of the IF family, also show some similarity to the HLH family members but not to the same extent as does desmin with the myoD family (Murre et al., 1989). The biological significance of these similarities is completely unknown. The effect of desmin inhibition on the expression of MyoD and myogenin could allow some speculations that have to be proven experimentally. One could assume that the above discussed similarities may reflect possible association and modulating interactions between the homologous α -helical domains of these proteins at some stage during muscle differentiation, as we have previously suggested for the observed corresponding similarities between the growth-regulated IF protein vimentin and the growth regulators fos, jun, and CREB during cell proliferation (Capetanaki et al., 1990). This can be a mechanism by which desmin filaments could be involved in signal transduction and transport of these myogenic factors to the nucleus or in the modulation of muscle chromatin conformation. Neither desmin nor vimentin has been found in the nucleus. However, they both seem to interlink the nucleus to the plasma membrane and the possibility of penetrating the nucleus and interacting with nuclear constituents cannot be excluded; on the contrary, it is favored by the already demonstrated interactions of desmin and/or vimentin with different nuclear constituents including lamin B, RNA, DNA, and histones (Traub, 1985; Georgatos and Blobel, 1987*a,b*; Cress and Kurath, 1988; Shoeman et al., 1988). As mentioned above, recent data have demonstrated that lamin B specifically binds to MARS, which when bound to nuclear matrix activate gene expression; however, the functional role of the lamin B binding to MARS is unknown. Considering the above data, one could envision that the connection of the cytoplasmic IF with the nuclear lamins could somehow influence nuclear events that might be modulated through lamin B-MARS binding. Recent data on mechanotransduction across the cell surface and through the cytoskeleton, including the IF system (Wang et al., 1993; for review see Ingber, 1993), complements all the above considerations. Alternatively, the desmin filaments could just facilitate the transport of myoD and myogenin into the nucleus or of myoD, myogenin and the other muscle-specific RNAs out in the cytoplasm. It has been recently reported that 3' untranslated regions (3'UTRs) of muscle-specific genes seem to be responsible for the maintenance of the muscle-differentiated state (Rastinejad and Blau, 1993). If desmin filaments associate with muscle mRNAs as proposed for keratins and maternal Vgl mRNA in *Xenopus* oocytes (Pondel and King, 1988; for review see Klymkowsky et al., 1989) their lack might inhibit myogenesis by blocking the function of the 3'UTRs of the muscle RNAs. On the other hand, given the above described importance of the 3'UTR region of some muscle-specific mRNAs on muscle differentiation one

should seriously consider that the effects of the desmin anti-sense RNA on muscle differentiation might not be just due to the decrease of the desmin protein, but the decrease of its mRNA and thus its 3'UTRs. It should be pointed out that the obtained rescue of the normal phenotype here by the overexpression of sense desmin RNA is probably not achieved by the exogenous RNA using the same mechanism since the construct used lacks the desmin 3'UTR. It could be, however, due to the endogenous desmin mRNA and 3'UTR which was able to accumulate in such rescue experiments.

Of interest is the correlation of the presently obtained cell lines with the known fusion-defective BC3H1 cell line. It has been shown that the fusion defect of these myoD-negative, myogenin-positive cells can be reversed by expressing myoD in these cells (Brennan et al., 1991). Desmin is expressed in this cell line but at lower levels which would correlate with the expression pattern in the 10₂.105 cell line. The data from the BC3H1 study and that of the present study suggest: (a) MyoD by itself is not sufficient for normal myogenesis to take place. (b) Normal myogenin levels are required for normal myogenesis and muscle-specific gene expression consistent with the recent data on the disruption of the myogenin gene by homologous recombination (Hasty et al., 1993). (c) Inhibition of desmin leads to negative regulation of myoD which is always linked to inhibition of fusion. (d) Inhibition of desmin is not necessarily followed by myogenin inhibition. (e) More than one pathway seem to be responsible for the control of myogenesis.

Does desmin play different or additional roles at the later stages of muscle differentiation? After fusion of myoblasts into multinucleated myotubes, desmin IF gradually rearrange from a radial and random distribution to form transverse bands surrounding the Z-disks and connecting neighboring myofibrils (Bennett et al., 1979; Gard and Lazarides, 1980; Holtzer et al., 1982; Fuseler et al., 1983; Tokuyasu et al., 1984). Thus it has been postulated that later in myogenesis desmin might play a critical role in the lateral alignment of the myofibrils, their connection to each other through their Z-disks, to other organelles, and the whole system to the sarcolemma (Lazarides, 1980; Lazarides et al., 1982). Recent *in vitro* data, however, have shown that disruption of desmin/vimentin filaments by expressing exogenous truncated desmin protein did not interfere with the assembly, lateral alignment, and the maintenance of the striated contracting myofibrils (Schultheiss et al., 1991). This data indirectly suggest reconsideration of those older hypotheses of desmin function and by extension, the function of IF proteins in general. Nevertheless, possible structural roles of at least keratins and neurofilaments in the organization and integrity of cells and tissues have been indirectly suggested from work in transgenic mice and genetic diseases (Coulombe et al., 1991; Vassar et al., 1991; Cheng et al., 1992; Chipev et al., 1992; Fuchs et al., 1992; Rothnagel et al., 1992; Xu et al., 1993; for reviews see also Liem, 1993; Coulombe, 1993).

Mechanism of the Inhibition of Desmin by Anti-sense RNA

The mechanism of the presently reported inhibition of desmin by the anti-sense RNA is not completely clear. A major portion of the inhibition was obviously due to a decrease in

the steady state of the endogenous RNA level. The process by which this decrease is effected can only be speculated based upon previous observations (Kim and Wold, 1985; Stout and Casky, 1990). The anti-sense RNA might interfere with RNA processing by hybridizing with the sense desmin RNA in the nucleus. The unprocessed or the double stranded RNA would then be poorly or not transported to the cytoplasm and would be quickly degraded. This mechanism explains the presently obtained, intriguing mutual decrease or disappearance of both sense and anti-sense desmin RNA in those cases where the highest endogenous desmin RNA inhibition was observed. This is consistent with previously reported data in other systems including the plant hormone ethylene (Hamilton et al., 1990), chalcone synthase (Van-Bevern et al., 1983), and polygalacturonase (Sheehy et al., 1988; Smith et al., 1988). In such cases it can be argued that the possibility of indirect nonspecific contribution of the anti-sense RNA on the observed phenotype is considerably decreased since this RNA is quickly degraded and never reaches the cytoplasm. Finally, an additional inhibition mechanism could directly involve inhibition of desmin translation by the anti-sense RNA that reaches the cytoplasm. The extent to which the latter mechanism may be contributing to the observed decrease of desmin in the obtained cell lines is unknown. In the case of the 10₂.1.02 cell line under growth conditions the desmin mRNA seems normal but the protein is reduced ~50%. Obviously, just inhibition of translation seems to be the predominant mechanism of the antisense action here.

Similar anti-sense RNA approaches have been used to show that inhibition of another IF protein, GFAP, blocked the formation of stable astrocytic processes (Weinstein et al., 1991), suggesting that active involvement in the regulation of cell differentiation may be a function of all tissue-specific IF proteins.

This work is dedicated to the memory of Ilmar R. Kuisk.

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