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Upstream regions of the hamster desmin and vimentin genes regulate expression during *in vitro* myogenesis

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Varying lengths of the hamster desmin and vimentin promoter regions were fused to the bacterial chloramphenicol acetyltransferase gene. These constructs were transfected into two different myogenic cell lines, T984 and C2C12. In both cell lines an increase in endogenous desmin expression takes place upon myogenesis. A region between −89 and +25 bp relative to the desmin transcription initiation site directs high-level tissue- and stage-specific expression upon *in vitro* myogenesis. At the myoblast stage, C2C12 cells appeared to express both desmin and vimentin, whereas in T984 myoblasts only vimentin expression was detected. Although vimentin is expressed during all stages of myogenesis, a strong decrease in vimentin expression occurs during differentiation of C2C12 cells. Vimentin−CAT constructs followed the endogenous expression pattern, showing that this down-regulation is mediated by 5′ flanking sequences. Vimentin promoter activity is modulated by at least two separate regions, both in myogenic and in non-myogenic cell lines.

Key words: intermediate filaments/intermediate filament genes/*in vitro* myogenesis/upstream DNA sequence

Introduction

Intermediate filaments (IFs) are unique cytoskeletal structures of 7−12 nm in diameter which occur in the cytoplasm of most eukaryotic cells. The members of this large multigene family of proteins can be divided into four major subfamilies on the basis of their biochemical and immunological properties (Lazarides, 1982; Steinert *et al.*, 1985; Traub, 1985): the acidic (type I) and basic (type II) cytokeratins, which are specific for epithelial cells, vimentin and desmin, glial fibrillary acidic protein (type III) and the three neurofilament polypeptides (type IV). All IF proteins are expressed in a developmentally regulated and tissue-specific manner. Recently it has been established that on the basis of substantial amino acid homology the lamins A and C may be included in the IF protein family (Fisher *et al.*, 1986).

For most classes of IFs, very little is known about the regulatory mechanisms underlying their expression. The evidence to date suggests that IF subunit expression is regulated mainly at the transcriptional- and/or post-transcriptional level, but not at the level of translation (e.g. Blikstad and Lazarides, 1983; Capetanaki *et al.*, 1983, 1984; Jorcano *et al.*, 1984; Ngai *et al.*, 1984; Nelson and Lazarides, 1985; Lilienbaum *et al.*, 1986).

Vimentin is expressed in cells of mesenchymal origin and in most cells in tissue culture, whereas in other cases vimentin is present in tissues during immature stages of development and ofter precedes the appearance of the cell type-specific IF subunit (for review see Traub, 1985). Desmin is expressed in adult cardiac, skeletal and smooth muscle cells (Lazarides, 1980, 1982; Schmid *et al.*, 1982). Regulation of desmin expression is stage- and tissue-specific, since it is induced during terminal development of, for example, skeletal muscle cell differentiation (Bennett *et al.*, 1979; Gard and Lazarides, 1980).

The detailed characterization of the hamster desmin and vimentin genes (Quax *et al.*, 1983, 1985) allows regulatory features governing muscle-specific versus non-muscle-specific IF expression at the molecular level to be investigated.

An important component of eukaryotic gene regulation is formed by *cis*-acting DNA sequences upstream from the transcription initiation site; these sequences are required for transcription and contain elements that control development- and/or tissue-specific expression (e.g. Breathnach and Chambon, 1981; Dynan and Tjian, 1985). We have identified DNA sequences within the upstream region of the desmin gene which are important in stage- and tissue-specific expression, as reflected in myogenic development. We fused the desmin promoter region to the bacterial chloramphenicol acetyltransferase (CAT) gene (Gorman *et al.*, 1982). The latter gene is used in transient transfection assays as a marker to demonstrate transcriptional activity of the upstream sequences. We performed experiments on two myogenic cell lines: T984 (Jakob *et al.*, 1978; Caravatti *et al.*, 1982) and C2C12 (Blau *et al.*, 1983). C2C12 cells have been used to study muscle-specific regulation of a number of genes due to their extensive and rapid differentiation into myotubes (Blau *et al.*, 1985; Hardeman *et al.*, 1986; Minty and Kedes, 1986; Minty *et al.*, 1986; Silberstein *et al.*, 1986). In order to gain more insight into the mechanisms that regulate desmin and vimentin co-expression during myogenesis, and to localize regulatory elements in the promoter region of the vimentin gene, we also transfected vimentin−CAT hybrids into these cells. Our results show that the desmin promoter region harbors a *cis*-acting regulatory element that determines tissue- and stage-specific expression. The flanking region of the vimentin gene contains at least two separate regulatory elements.

Results

Differentiation-related expression mediated by the upstream region of the desmin gene in T984 myogenic cells

The myogenic mouse teratocarcinoma skeletal muscle cell line T984 can be induced to differentiate by switching to a mitogen-deficient medium when the cells reach confluency. Within 7 days, multinucleated myotubes are formed which express the characteristic markers of skeletal muscle (Jakob *et al.*, 1978; Caravatti *et al.*, 1982).

T984 cells were first screened for desmin and vimentin expression in different stages of differentiation by immunofluorescence microscopy and Western blotting, using monoclonal and polyclonal antibodies respectively (Figure 1). It appeared that T984 myoblasts do not express desmin before they reach confluency. Upon differentiation into myotubes, desmin expression and assembly into intermediate filaments is observed (Figure 1A). After the cells have reached confluency, some mononucleated

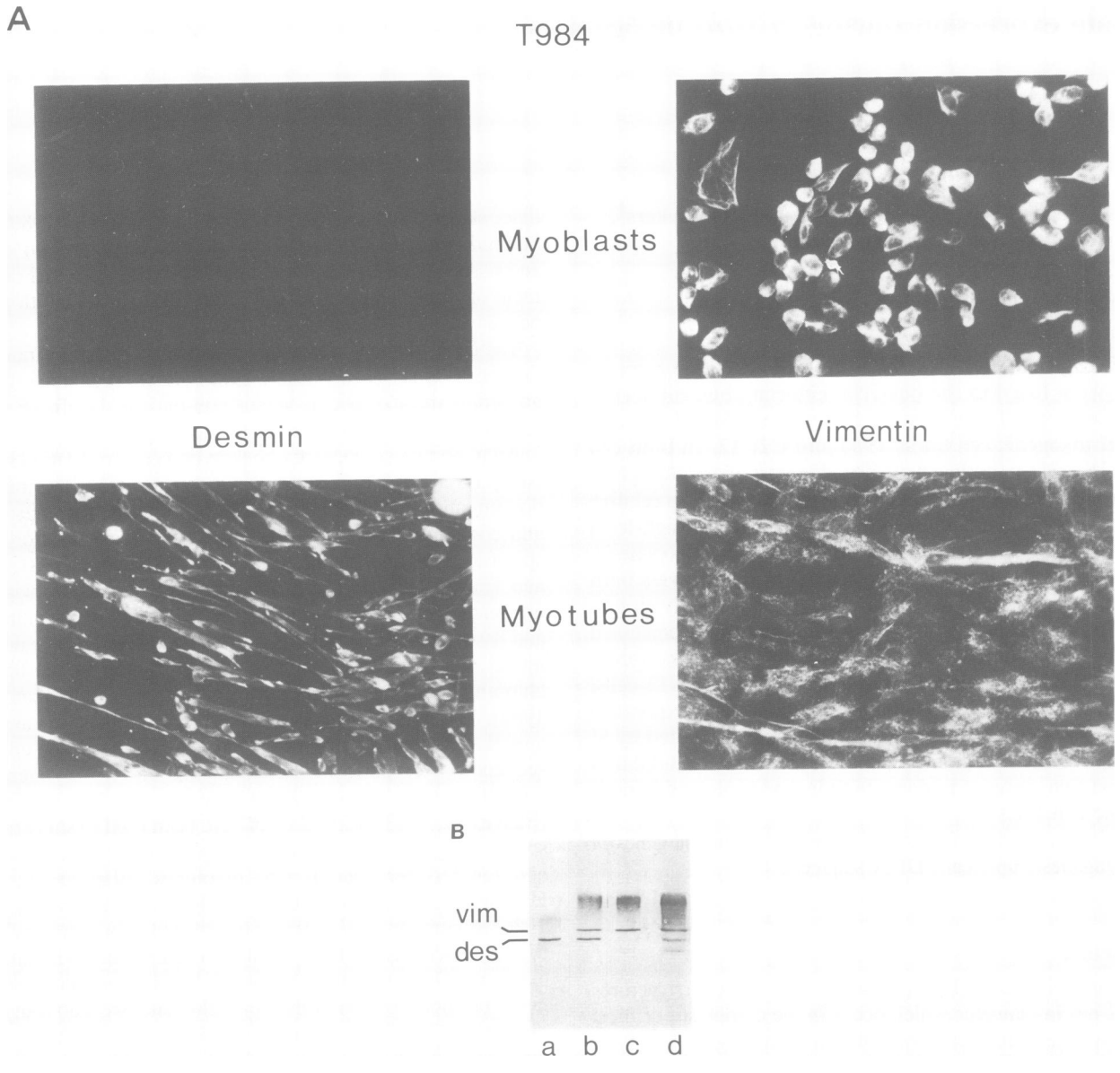

Fig. 1. Screening of T984 cells for desmin and vimentin expression during differentiation by immunofluorescence microscopy and Western blotting. (**A**) Immunofluorescent staining of T984 myoblasts (**top**) and myotubes (**bottom**). **Left**: cells incubated with monoclonal desmin antibodies; **right**: cells incubated with monoclonal vimentin antibodies. Incubation with polyclonal antibodies yielded identical results (not shown). (**B**) Western blotting of whole cell homogenates, prepared from subconfluent cultures of T984 myoblasts (**lane c**), and from T984 cultures which have been allowed to differentiate into myotubes for 7 days (**lane d**). Equal amounts of protein were loaded onto each lane. For comparison, a BHK cell extract was used (**lanes a** and **b**). **Lane a** was incubated with desmin antibodies only, **lanes b–d** were incubated with desmin antibodies and thereafter with vimentin antibodies. The faint bands in **lane c** were not observed after incubation with desmin antibodies and represent vimentin breakdown products.

myoblasts can also be seen to express desmin. On the other hand, vimentin is expressed during all stages of T984 differentiation. Western blotting of whole cell extracts with polyclonal antibodies against desmin and vimentin confirmed these observations: desmin expression could be detected in myotubes only, whereas vimentin is expressed throughout myogenesis (Figure 1B).

In order to identify DNA sequences within the 5′ flanking regions of the desmin and vimentin genes that are required for expression, we constructed a series of plamids in which decreasing lengths of 5′ flanking region were fused to the bacterial CAT marker gene (Gorman *et al.*, 1982). These desmin–CAT and vimentin–CAT hybrids (Figure 2) were transfected into proliferating T984 myoblasts and, in parallel, into multinucleated myotubes. CAT expression levels of the transfected cells were measured relative to the level of CAT activity present in cells transfected in parallel with pSV2CAT, a vector containing the SV40 early promoter.

When myoblasts were assayed for transient expression 48 h after transfection, no desmin–CAT expression could be detected (Figure 3A). Transfection of T984 myotubes yielded desmin–CAT expression levels that reached values of ~50% of that of pSV2CAT-transfected myotubes (Figure 3A). There were no significant differences between CAT expression levels of the individual desmin–CAT constructs, indicating that the region from −89 to +25 contains muscle-specific regulatory sequences which confer developmental control of desmin expression.

Vimentin-CAT expression levels remained at a constant level during differentiation. Deletion of sequences upstream from

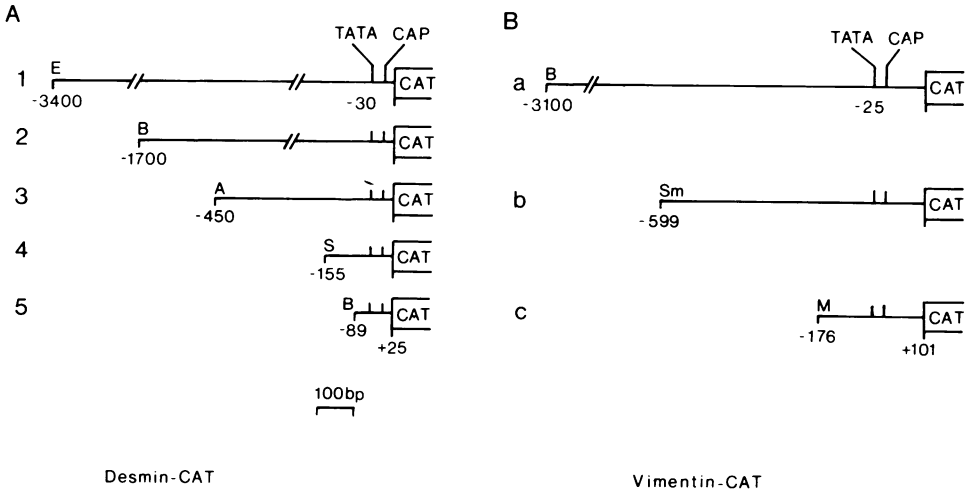

Fig. 2. Schematic representation of desmin and vimentin promoter−CAT hybrids. Details of construction are described in Materials and methods. Coordinates refer to base pairs of DNA sequence relative to the mRNA cap site (CAP) at position +1. Promoter−CAT fusion sites are located between cap site and initiation codon of the desmin and vimentin genes, at +25 and +101 respectively. Position of the TATA box homologies are indicated. Abbreviations used are: E, *Eco*RI; B, *Bam*HI; A, *Acc*I; S, *Stu*I; Sm, *Sma*I; M, *Mbo*I. (**A**) Desmin−CAT hybrids, 1 = p3.4DesCAT, 2 = p1.7DesCAT, 3 = p450DesCAT, 4= p155DesCAT and 5 = p89DesCAT. (**B**) Vimentin−CAT hybrids, a = 3.1VimCAT, b = p599VimCAT and c = p176VimCAT.

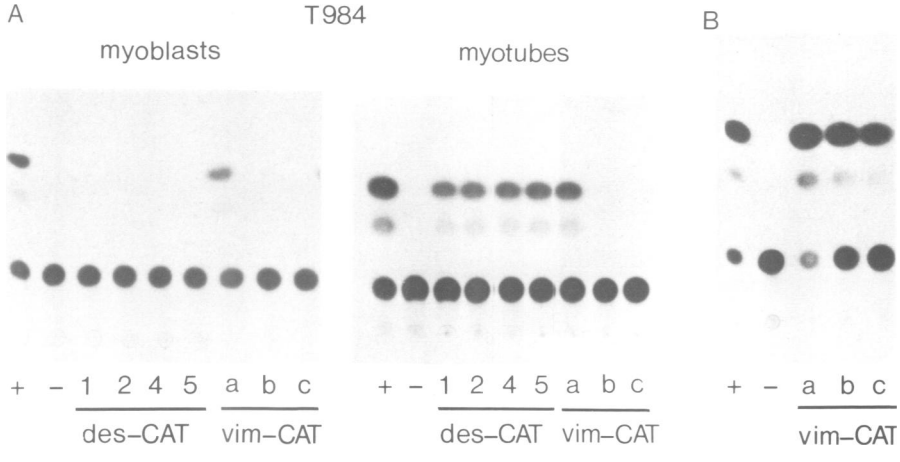

Fig. 3. Expression mediated by upstream regions of the desmin and vimentin genes in T984 myoblasts and T984 myotubes. (**A**) Plasmid DNAs were transfected into T984 myoblasts and myotubes. CAT activity was determined from equal volumes of cell extracts from myoblasts and myotubes, repespectively. (**B**) Transfections of vimentin−CAT hybrids into hamster lens cells. The assayed volumes of control extracts (+, −) were three times that of vimentin−CAT transfected cells. +, pSV2CAT; −, pSVOCAT; 1, p3.4DesCAT; 2, p1.7DesCAT; 4, p155DesCAT; 5, p89DesCAT; a, p3.1VimCAT; b, p599VimCAT; c, p179VimCAT.

−599 relative to the transcription initiation site almost abolished vimentin−CAT expression, both in transfected myoblasts and myotubes (Figure 3A). As a control, we transfected the vimentin−CAT hybrids into hamster lens cells, which express vimentin at high levels (Bloemendal *et al.*, 1980). All three constructs were expressed at high levels in these cells, with p3.1VimCAT expression 3-fold higher and p599VimCAT and p176VimCAT 1.5- to 2-fold higher than pSV2CAT expression (Figure 3B). From these results it can be concluded that at least two upstream regions are involved in vimentin expression, of which the most distal region is required for high-level expression in T984 cells.

Since CAT expression levels of muscle-specific promoter regions do not necessarily reflect the expression levels of the endogenous gene (Seiler-Tuyns *et al.*, 1984; Minty and Kedes, 1986), T984 myoblasts and myotubes were assayed for the level of desmin and vimentin mRNA (Figure 4). No desmin mRNA was detected in T984 myoblasts, whereas T984 myotubes were shown to contain significant amounts of desmin transcripts. Vimentin mRNA was present in both myoblasts and myotubes,

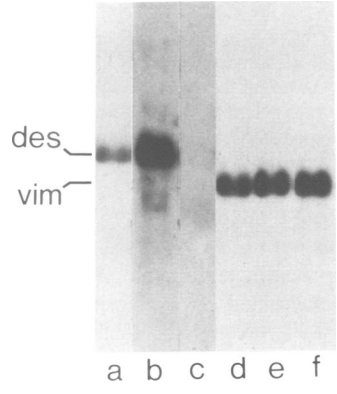

Fig. 4. Autoradiograms of Northern blots of RNA from T984 myoblasts and myotubes. Ten micrograms of total RNA were hybridized to a desmin probe (**lanes a−c**) and a vimentin-specific probe (**lanes d−f**). The same blot was used for both probes. The autoradiograms were exposed for 2 weeks or 6 h at −80°C respectively. RNA from vimentin- and desmin-expressing BHK-21 cells is shown as a marker. **Lanes a** and **f**, proliferating BHK-21 cells; **lanes b** and **d**, 8-day postfusion myotubes; **lanes c** and **e**, proliferating myoblasts.

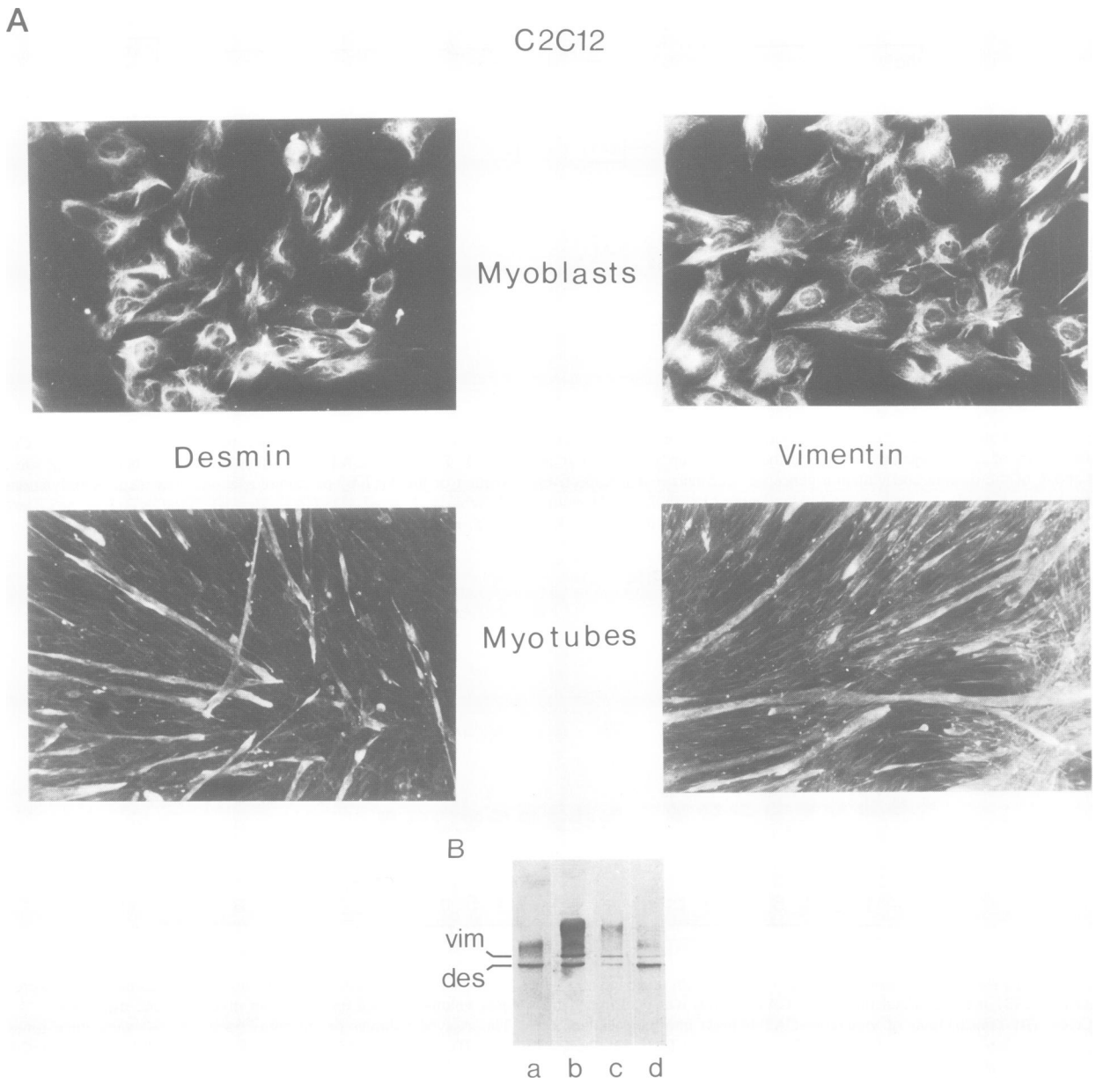

Fig. 5. Screening of C2C12 cells for desmin and vimentin expression during differentiation by the immunofluorescence microscopy assay and Western blotting. (**A**) Immunofluorescence staining of C2C12 myoblasts (**top**) and myotubes (**bottom**). The cells on the **left** were incubated with monoclonal desmin antibodies, and cells shown on the **right** with monoclonal vimentin antibodies. Incubations with polyclonal antibodies yielded identical results (not shown). (**B**) Western blotting of whole cell homogenates, prepared from subconfluent cultures of C2C12 myoblasts (**lane c**) and from C2C12 myotubes (**lane d**). Equal amounts of protein were loaded onto **lanes c** and **d**. For comparison, a BHK cell extract was used (**lanes a** and **b**). **Lane a** was incubated with the desmin antibody only, **lanes b–d** were incubated with desmin antibody and thereafter with vimentin antibodies.

although an ~2-fold reduction of transcripts was observed upon differentiation. Desmin–CAT expression apparently parallels the endogenous tissue-specific and developmentally regulated expression pattern as determined by Western and Northern blotting.

Desmin and vimentin expression in C2C12 myogenic cells

Like T984 cells, myoblasts of the mouse muscle cell line C2C12 can be induced to fuse and form multinucleated myotubes. Using monoclonal and polyclonal antibodies against desmin and vimentin, we screened these cells for intermediate filament expression by immunofluorescence and Western blotting during different stages of differentiation (Figure 5). Contrary to T984 cells, C2C12 cells were found to express desmin and vimentin during all stages of myogenesis. From immunofluorescence studies using desmin antibodies combined with Hoechst staining, it was clear that even in sparse cell cultures each individual C2C12 myoblast expresses desmin. Therefore, the observed desmin expression is not due to premature differentiation of some of the myoblasts. Western blotting with polyclonal desmin and vimentin antibodies confirmed that C2C12 myoblasts express both desmin and vimentin, as do C2C12 myotubes (Figure 5B).

Transfection of desmin–CAT hybrids into proliferating C2C12 myoblasts resulted in CAT expression levels of 25% of the level of pSV2CAT-transfected myoblasts (Figure 6A). In myotubes, however, desmin–CAT expression levels were similar to the pSV2CAT expression level, indicating a 4-fold increase in ex-

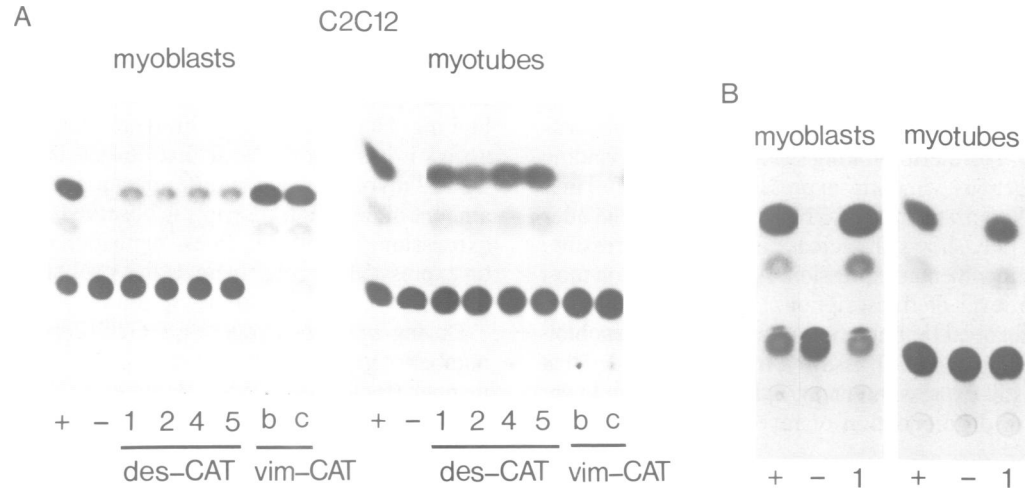

Fig. 6. Expression mediated by upstream regions of the desmin and vimentin gene in proliferating C2C12 myoblasts and in C2C12 myotubes. Plasmid DNAs were transfected into C2C12 myoblasts and myotubes. CAT activity was determined in equal volumes of cell extract from myoblasts and myotubes, respectively. +, pSV2CAT; −, pSVOCAT; 1, p3.4DesCAT; 2, p1.7DesCAT; 4, p155DesCAT; 5, p89DesCAT; b, p599VimCAT; c, p176VimCAT. (**A**) CAT activity in transfected myoblasts and myotubes, assayed 40 h after transfection. (**B**) CAT activity in myoblasts, which were allowed to differentiate for 7 days after transfection before CAT assays were performed, and in transfected myotubes, which were assayed for CAT activity after 40 h.

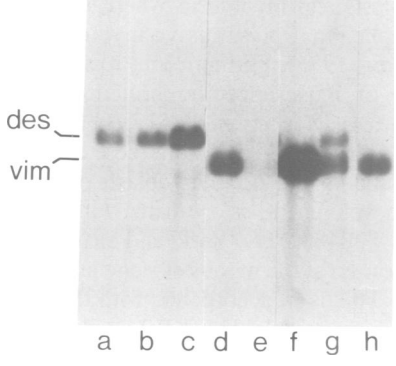

Fig. 7. Autoradiograms of Northern blots of RNA from C2C12 myoblasts and myotubes. Ten micrograms of total RNA were first hybridized to a desmin-specific (**lanes a−c**) and then to a vimentin-specific probe (**lanes d−h**). The same blot was used for both probes and was not dehybridized after desmin probing. Autoradiograms were exposed for 4 days (desmin probe), 8 h (vimentin probe, **lanes d** and **e**) or 24 h (vimentin probe, **lanes f** and **g**). BHK-21 is shown as a marker. **Lanes a** and **h**, proliferating BHK-21 cells; **lanes b, d** and **f**, proliferating myoblasts; **lanes c, e** and **g**, 5-day postfusion myotubes.

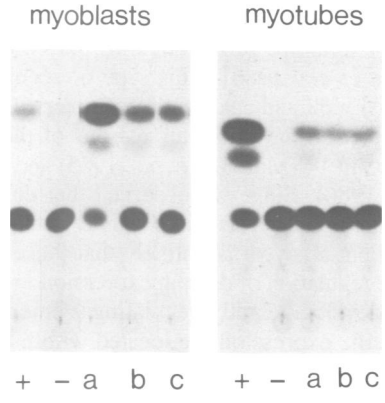

Fig. 8. Transcriptional activity of upstream regions of the vimentin gene in proliferating C2C12 myoblasts and in myobtubes. Vimentin−CAT hybrids were transfected into C2C12 myoblasts or myotubes. Equal amounts of cell extract from transfected myotubes and transfected myoblasts were assayed respectively. +, pSV2CAT; −, pSVOCAT; a, p3.1VimCAT; b, p599VimCAT; c,p176VimCAT.

pression levels during C2C12 myogenesis (Figure 6A). The observed increase in desmin−CAT activity corresponds to the increase in desmin concentration, as determined by Western blotting (Figure 5B). Northern blotting of RNA from C2C12 myoblasts and myotubes clearly shows that the endogenous levels of desmin transcription follow the same expression pattern as the desmin−CAT constructs (Figure 7, lanes a−c). This indicates that the developmentally regulated expression of desmin synthesis is not controlled at the level of translation, but takes place at the transcriptional level, although an enhanced stability of desmin transcripts and desmin−CAT transcripts upon differentiation cannot completely be excluded.

When C2C12 myoblasts were transfected at low cell density and then allowed to differentiate into myotubes, the same increase in desmin−CAT activity was observed (Figure 6B). Since transfection of myotubes did not result in lower expression levels, the increase must be induced by a muscle-specific regulatory process which is fully active in myotubes.

Contrary to expression levels in T984 cells, p599VimCAT and p176VimCAT expression amount to ~150% of pSV2CAT expression upon transfection into C2C12 myoblasts (Figure 6A). Obviously, sequences upstream from −599 in the vimentin flanking sequence are not necessary for high level expression in C2C12 myoblasts. Conversely, transfection of p599VimCAT and p176VimCAT into C2C12 myotubes resulted in low CAT expression levels, hardly exceeding pSV0CAT expression (Figure 6).

In order to compare p3.1VimCAT expression with p599VimCAT and p176VimCAT activity, the constructs were introduced into proliferating C2C12 myoblasts and into myotubes. In myoblasts p3.1VimCAT activity exceeded p599VimCAT and p176VimCAT expression 2- to 3-fold, reaching values of 300−400% of pSV2CAT expression (Figure 8). After transfection of these constructs into myotubes, vimentin−CAT expression was very low, indicating that sequences upstream from −176 are not responsible for the observed drop in vimentin transcriptional activity. Since it is very unlikely that a dramatic increase in pSV2CAT activity takes place upon differentiation (Grichnik *et*

al., 1986; Minty *et al.*, 1986), a strong decrease in vimentin−CAT expression levels must take place. Vimentin−CAT expression in C2C12 myotubes was 30-fold lower than in transfected myoblasts.

An analysis of vimentin mRNA levels in C2C12 myoblasts and myotubes by Northern blotting shows a corresponding decrease of endogenous vimentin expression (Figure 7, lanes d−h). Likewise, Western blotting of C2C12 cells before and after differentiation not only shows an increase in desmin expression, but also a decrease in vimentin expression. Again, regulation most probably is at the level of transcription.

From the data obtained by immunofluorescence, immunoblotting, Northern blotting and CAT assays it may be concluded that C2C12 myotubes still express vimentin, although at much lower levels than prior to differentiation of myoblasts.

Discussion

Transfection of desmin−CAT hybrid constructs into the mouse myogenic muscle cell lines T984 and C2C12, before and after differentiation, demonstrated that a region from −89 to +25 bp relative to the desmin transcription initiation site contains regulatory sequences that are involved in muscle-specific induction of desmin expression. Immediately upstream from the region between −89 and +25 bp at least one (at −130) and perhaps two (−110) potential Sp1 protein binding sites are located (CG-box; Kadonaga *et al.*, 1986). This type of regulatory element occurs in several viral and eukaryotic promoters. CG-boxes have also been detected in the promoter regions of the IF genes for human cytokeratin 14 (Marchuk *et al.*, 1985) and cytokeratin 19 (Bader *et al.*, 1986). Since we observed that deletion of these sequences did not influence desmin promoter activity in myogenic cells in a transient assay, it is unlikely that these GC boxes are involved in the regulation of desmin expression in these systems.

Cis-acting, developmentally regulating elements which control tissue-specific expression are located within the 5′ flanking regions of a number of muscle-specific genes. The genes for skeletal α-actin (Melloul *et al.*, 1984; Nudel *et al.*, 1985; Bergsma *et al.*, 1986; Shani, 1986), cardiac α-actin (Minty and Kedes, 1986; Minty *et al.*, 1986; Mohun *et al.*, 1986), muscle creatine kinase (Jaynes *et al.*, 1986) and troponine I (Konieczny and Emerson, 1985) contain such muscle-specific transcriptional control elements. By comparing the regulatory sequences of these and other genes, α-actin-specific (Minty and Kedes, 1986) or even muscle-specific consensus sequences have been proposed (Jaynes *et al.*, 1986). We compared the desmin promoter sequence from −89 to +25 bp with the 5′ flanking sequence of published muscle-specific genes. An 11-bp sequence from −82 to −72 (CAGCTGTCAGG) has a perfect match at −440 in the mouse and human cardiac α-actin flanking region. However, sequences homologous to the α-actin or muscle-specific consensus sequences (Jaynes *et al.*, 1986; Minty and Kedes, 1986) could not be detected. Deletion of a region from −443 to −395 of the human cardiac α-actin gene, including the 11-bp sequence, decreased transcriptional activity of the cardiac actin promoter 2-fold, but it was not essential for high-level, tissue-specific expression. We are currently investigating whether this 11-bp or other sequences in the desmin promoter region are responsible for tissue- and stage-specific expression.

Other conserved, muscle-specific sequences could not be detected in the 1.7-kbp 5′ upstream region sequence of the desmin gene, when compared with the EMBL data base (version 9.0, EMBL, Heidelberg).

In the mouse myogenic cell line T984, desmin expression was detected only after the cells had reached confluency. This corresponds with the induction of desmin−CAT expression that occurred upon myogenesis. From the results obtained by Western blotting, Northern blotting, immunofluorescence tests and CAT assays it has become clear that desmin is already expressed in C2C12 myoblasts. Upon differentiation, a 4-fold increase in the amount of desmin transcripts is observed, resulting in increased expression. In both cell lines, regulation of desmin and vimentin expression is probably not at the level of translation, but, most likely, at the level of transcription.

During *in vitro* myogenesis, C2C12 cell cultures express a number of genes in a muscle-specific, developmentally regulated manner (Blau *et al.*, 1983; Bains *et al.*, 1984; Seiler-Tuyns *et al.*, 1984; Minty and Kedes, 1986; Silberstein *et al.*, 1986). This involves induction as well as down-regulation of gene expression. We show here that in C2C12 cells desmin expression does not follow the temporal expression pattern that is characteristic for other muscle-specific genes; its expression is initiated at an earlier stage. In fact, desmin is the earliest known marker for cells in the myogenic lineage (Hill *et al.*, 1986). Gene activation during myogenic development is a multistep process, characterized by an independent regulation of different classes of muscle-specific proteins. Most myofibrillar proteins are expressed in post-mitotic myoblasts and myotubes, whereas desmin synthesis is initiated in replicating, presumptive myoblasts (Dlugosz *et al.*, 1983; Hill *et al.*, 1986). Furthermore, it is possible to block the accumulation of muscle-specific proteins without repressing desmin synthesis (Dlugosz *et al.*, 1983; Holtzer *et al.*, 1985; Nelson and Lazarides, 1985). From these data and our results it may be concluded that C2C12 myoblasts represent a later stage in myogenic development, as compared with other myogenic cell lines, such as T984 and L8 (Yaffe and Saxel, 1977a), which do not express desmin at the myoblast stage and differentiate much more slowly. This is in accordance with the findings of Minty *et al.*(1986) who reported that C2C12 myoblasts contain muscle-specific, transcription-modulating factors. It is possible that these factors are sufficient for desmin expression. The increase in transcriptional activity of the desmin promoter upon differentiation may be caused by a higher concentration or different type of *trans*-acting regulatory factor(s) in myotubes (Blau *et al.*, 1985).

It is a matter of debate whether desmin and vimentin are co-expressed in adult myotubes. Bennett *et al.* (1979), Osborn *et al.* (1982) and Tokuyasu *et al.* (1984) reported that vimentin gradually disappears as myotubes mature, whereas others observed a continued co-expression (Granger and Lazarides, 1979; Gard and Lazarides, 1980; Hill *et al.*, 1986). Zehner and Paterson (1985) observed an eightfold decrease in vimentin mRNA levels during *in vivo* myogenesis, whereas Ngai *et al.* (1985) reported that during *in vitro* myogenesis vimentin mRNA levels did not change. We observed only a slight decrease in vimentin expression in differentiating T984 cells. In differentiating C2C12 cells, however, vimentin expression decreased ~30-fold, although Western blotting and immunofluorescence tests show that C2C12 myotubes still contain vimentin. At present we do not know whether the difference in regulation of vimentin expression between T984 and C2C12 cells is caused by insufficient maturation of the T984 myotubes (Blau *et al.*, 1985; Silberstein *et al.*, 1986), or if different control mechanisms exist in these cell types. It was recently shown in studies on the expression of sequentially expressed myosin heavy chain (MHC) genes that in T984 myotubes the embryonic MHC gene is predominant and

only a small amount of adult MHC is expressed (Weydert *et al.*, 1987). Therefore, insufficient maturation of T984 myotubes seems the most likely explanation.

Vimentin expression in T984, in C2C12 and in hamster lens cells is regulated by at least two separate regions. One is located between −3100 and −599, the other between −176 and +101. This second region contains a putative CAAT box at −90 bp, which is flanked by two potential Sp1 binding boxes at −120 and −55 (Kadonaga *et al.*, 1986; Jones *et al.*, 1986), and a TATA box at −25 bp (Quax *et al.*, 1983). The first region increased transcriptional activity in transient transfection assays; only low-level expression remained in T984 cells when this region was deleted. In C2C12 myoblasts and hamster lens cells sequences upstream from −176 were neither required for high-level expression, nor were they needed for the down-regulation of transcriptional activity in C2C12 cells. We are presently investigating the enhancing capacity of these sequences. Preliminary results from experiments with further deleted vimentin promoter regions show that deletion of the CAAT box greatly diminishes vimentin−CAT expression levels, both in hamster lens cells and in C2C12 cells. Deletion of the potential Sp1 binding box at −120 bp has no effect on CAT expression (data not shown).

It remains to be established whether desmin induction and vimentin repression are co-ordinated at the molecular level. The data on the regulation of desmin and vimentin co-expression strongly suggest that their expression is not completely interdependent.

Materials and methods

Cells and cell culture

SV40-transformed hamster lens cells (Bloemendal *et al.*, 1980) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The teratocarcinoma mouse muscle cell line T984 was originally isolated by Jakob *et al.* (1978). T984 myoblasts were maintained in DMEM supplemented with 15% fetal calf serum. For differentiation into multinucleated myotubes, confluent cultures were switched to nutrient-deficient medium (DMEM supplemented with 2% fetal calf serum). The C2C12 cell line (Blau *et al.*, 1983) was a subclone of the C2 line isolated by Yaffe and Saxel (1977b), and cultured as described by Blau *et al.* (1983). To induce myotube formation. confluent cultures were switched to low mitogen fusion medium (DMEM supplemented with 2% horse serum). C2C12 cells started to fuse after 24−36 h (Bains *et al.*, 1984), whereas T984 myoblasts started to fuse after 48 h.

Construction of plasmids and preparation of plasmid DNA

In general, the procedures suggested by Maniatis *et al.* (1982) were followed for construction of plasmids and preparation of plasmid DNA. Five different desmin promoter−CAT hybrids were constructed (Figure 1A). A 114-bp *Bam*HI−*Hpa*II fragment, containing sequences 89 bp upstream and 25 bp downstream from the CAP site including the TATA box of the desmin gene (Quax *et al.*, 1985), was subcloned into the *Bam*HI−*Acc*I site of pSP65. From this plasmid, p89DesCAT was constructed by isolation and subcloning of the *Bam*HI−*Hin*dIII desmin fragment into superCAT, a derivative of pSVOCAT (Gorman *et al.*, 1982). The 5′ upstream 1.6-kbp *Bam*HI fragment was ligated in front of the 114-bp *Bam*HI−*Hpa*II fragment in pSP65. From this plasmid, a 180-bp *Stu*I−*Hin*dIII fragment, a 475-bp *Acc*I−*Hin*dIII fragment and a 1.7-kbp *Sac*I−*Hin*dIII fragment were isolated and subcloned in superCAT, resulting in p155DesCAT, p450DesCAT and p1.7DesCAT respectively. The plasmid p89DesCAT was partially digested with *Eco*RI, cutting only the *Eco*RI site in the polylinker, and completely with *Bam*HI. The 5′ upstream 1.7-kbp *Eco*RI−*Bam*HI desmin fragment was ligated into this plasmid. This plasmid was subsequently linearized with *Bam*HI and ligated to the 1.6-kbp *Bam*HI upstream fragment resulting in p3.4DesCAT. Three vimentin−CAT hybrids were constructed (Figure 1B). A 1.2-kbp *Eco*RI−*Pst*I fragment, containing sequences from −1100 to +101 relative to the CAP site, was isolated from the hamster vimentin gene (Quax *et al.*, 1983). This fragment was *Mbo*I digested and the 277-bp *Mbo*I−*Pst*I fragment was ligated into superCAT, resulting in p176VimCAT. The 1.2-kbp *Eco*RI−*Pst*I fragment was also digested with *Sma*I, and the resulting 700-bp *Sma*I−*Pst*I fragment was subcloned in superCAT, giving p599VimCAT. p3.1VimCAT was constructed by ligating the 1.9-kbp 5′ upstream *Bam*HI−*Eco*RI fragment and the 1.2-kbp *Eco*RI−*Pst*I fragment into superCAT.

All DNA preparations to be used in transfection experiments were purified on two successive CsCl gradients.

Cell transfection and CAT assays

Cells were transfected by the calcium phosphate precipitation method, essentially as described by Wigler *et al.* (1979). For hamster lens cells, T984 myoblasts and C2C12 myoblasts respectively, cells were plated at equal density in 100-mm^2 culture dishes the day before transfection. Transfections were carried out on cell cultures which had reached ~20% confluency. To each culture dish 10 μg of plasmid DNA was added as a calcium phosphate precipitate, and 20 min later 5 ml medium was added. After 5 h of incubation, the cells were glycerol-shocked for 2.5 min, and incubated in normal growth medium for ~40 h. Thereafter, CAT assays were performed or, in certain experiments with T984 and C2C12 myoblasts, the cells were incubated in fusion medium for 7 days and afterwards used in CAT assays. Special care was taken to ensure that 40 h after transfection of myoblasts, when the cells were harvested for use in CAT assays, the cultures had not yet reached confluency. This was done to minimize possible effects of differentiation and/or increasing cell density on desmin and vimentin expression. For transfection of T984 or C2C12 myotubes, myoblasts were allowed to differentiate for 7 days. Transfections were carried out on the resulting confluent myotube cultures as described; CAT assays were performed 40 h afterwards. Cell extracts were prepared and used in CAT assays as described by Gorman *et al.* (1982). Transfections, performed in parallel with pSVCAT (containing the SV40 early promoter) and with pSVOCAT (without promoter) were used to compare expression levels. Quantitation of CAT assays was performed by scintillation counting of the appropriate areas of the chromatogram. Experiments were carried out at least four times, using two different preparations of plasmid DNA.

Immunofluorescent staining and Western blotting

In this study polyclonal rabbit antisera to chicken gizzard desmin (Ramaekers *et al.*, 1983a, 1987) and to bovine lens vimentin (Ramaekers *et al.*, 1983b) were used, both for Western blotting and immunofluorescent staining assays. Monoclonal antibodies to desmin (RD301) and vimentin (RV202) (Broers *et al.*, 1986; Ramaekers *et al.*, 1987) were used in immunofluorescence assays only, since they reacted very weakly in immunoblotting assays. The indirect immunofluorescence technique was performed as described previously (Ramaekers *et al.*, 1983a). Gel electrophoresis and immunoblotting were carried out essentially as described by Broers *et al.* (1986).

RNA isolation and Northern blotting

RNA from proliferating BHK-21 cells, T984 and C2C12 myoblasts and 5-day post-fusion (C2C12) or 8-day post-fusion (T984) myotubes was isolated by the LiCl−urea method (Auffray and Rougeon, 1980). Total RNA samples (10 μg) were glyoxylated, fractionated on 1% agarose gels and transferred to Highbond-N (Amersham). As a desmin probe we used a 350-bp hamster desmin *Sau*3A fragment in M13, covering a region from 25 bp of exon 8 to 120 bp into the 3′ non-coding region (Quax *et al.*, 1985). The vimentin probe was a 520-bp hamster vimentin *Sau*3A fragment in M13, ranging from −150 to +340 bp relative to the transcription initiation site (Quax *et al.*, 1983). Hybridization of Northern blots was performed as described by Church and Gilbert (1984).

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