Cloning and characterization of a myoblast cell surface antigen defined by 24.1D5 monoclonal antibody

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Summary

Monoclonal antibody 24.1D5 reacts specifically with an epitope expressed on the cell surface of mononucleate myoblasts in primary cultures of human skeletal muscle cells, but not with either multinucleate myotubes or fibroblasts. Polypeptides of 60 and 100 × 10^3 Mr were identified by immunoblotting with the McAb. Human muscle cDNAs encoding the 24.1D5 epitope were used to study further the structure and expression of 24.1D5 during skeletal muscle development. Two mRNA species of 3-0 and 2-5 kb were identified in primary cultures of human skeletal muscle and in mouse muscle cell lines. The levels of both transcripts decreased during myotube formation in vitro and were similarly decreased during myogenesis in the mouse embryo. 24.1D5 mRNAs were expressed by multipotent cells and myoblast derivatives of the mouse embryonic cell line C3H10T1, suggesting that 24.1D5 is expressed at an early stage during skeletal muscle development.

Key words: myoblast antigen, skeletal muscle development, monoclonal antibody.

Introduction

The formation of specialized tissues within a multicellular organism involves at the cellular level the appearance of distinct cell lineages and the migration and differentiation of lineage-specific stem cell populations in precise spatiotemporal patterns. The availability of increasingly specific groupings of cellular antigenic markers, in conjunction with defined tissue culture models, has led in recent years to remarkable progress in mapping stem and progenitor cell relationships within a number of diverse cell lineages, such as glia (Raff et al. 1983) and haemopoietic cells (Peault et al. 1983).

Myoblasts represent the first muscle lineage-specific stem cells observed during development of vertebrate skeletal muscle, which subsequently differentiate into multinucleate myotubes. Myogenic cells have been extensively studied in vitro and a number of lineage and developmentally regulated antigenic markers have been described (Miller & Stockdale, 1987; Stockdale & Miller, 1987; Wakshull et al. 1983; Kaufmann et al. 1985; Moore & Walsh, 1985). Recently, studies using monoclonal antibodies (McAb) that recognize specific myosin heavy chain isoforms have allowed three distinct myoblast lineages to be identified in avian muscle, namely fast, fast/slow and slow (Miller & Stockdale, 1987). However, it is unclear at present whether similar diversity arises during mammalian myogenesis.

During embryogenesis, the development of multipotent embryonic cells becomes restricted to specific lineages by a process of determination. Myogenic lineages arise in the somites and are thought to arise from a multipotent progenitor cell population in common with other mesodermal derivatives such as connective tissue, blood cells and fat cells (Balinsky, 1981). The molecular mechanisms underlying myogenic determination are little understood, although the mouse embryonic cell line C3H10T1 represents a multipotent mesodermal cell type that can be experimentally converted into one of three genetically stable determined cell lineages (myogenic, chondrogenic and adipogenic) and provides a useful model system for studying certain steps in this pathway (Taylor & Jones, 1979; Konieczny and Emerson, 1985; Lassar et al. 1986). Using this system, it has been possible to identify regulatory genes MyoD1 and myd which control the determination and differentiation of myogenic lineages (Davis et al. 1987; Pinney et al. 1988). Studies of myogenic determination have, however, been limited by the paucity of suitable antigenic markers that will allow the isolation and manipulation of cells. We report here studies on the expression of the 24.1D5 protein. Developmental control of 24.1D5 reactive epitopes has been shown to involve transcriptional activation by a positive trans-acting regulatory factor that was found in
mouse myoblasts, but was absent from differentiated myotubes (Blau et al. 1985). In the present study, using a combination of McAb and cDNA probes, we show that 24.1D5 is expressed at an early step of the myogenic programme of gene expression in skeletal muscle and that it is likely to be a useful marker for further analyses of muscle determination.

### Materials and methods

#### Cell cultures and tissue samples

Cultures of human skeletal myoblasts were produced from 16- to 20-week suction termination specimens as described previously (Moore & Walsh, 1985; Walsh & Ritter, 1981). Prefusion myoblasts were harvested at 80% confluence and early-, mid- and late-fusion cultures were obtained 2, 5 and 7 days, respectively, after induction of fusion (Moore & Walsh, 1985). Human skin fibroblasts were isolated and cultured as previously described (Moore & Walsh, 1985). Mouse myoblasts and myotubes of the G8-1 cell line were cultured as before (Moore et al. 1987). Cells of the mouse embryonic line C3H10T1/2 and their myoblast derivatives, obtained by treatment with 5-azacytidine, were a gift from Dr C. Emerson (Konnezny & Emerson, 1984). Multipotent precursor cells were cultured as described for skin fibroblasts. Cultures of 10T1/2 myoblasts and myotubes were produced as described for G8-1 cells.

For analysis of 24.1D5 expression during development in vivo, limb buds or total limbs, respectively, were used from embryonic and newborn mice. Muscle tissue was dissected from the limbs of adult mice.

#### Indirect immunofluorescence and immunoblotting

To produce McAb 24.1D5, a plasma membrane fraction was prepared from a culture of human skeletal myoblasts and used to immunize Balb/c mice. The immunization schedule was as described previously (Walsh et al. 1981), using 0-1 mg protein per injection. Cell fusion and hybridoma growth were carried out as described (Walsh et al. 1981). Polyclonal antiserum HG-8 was produced as described below. Other antibodies used in the study were rabbit anti-mouse neural cell adhesion molecule (N-CAM) (Moore & Walsh, 1985) and rabbit anti-human Thy-1 (Walsh & Ritter, 1981). Double indirect immunofluorescence was carried out as described previously (Walsh et al. 1983). HG-8 and 24.1D5 McAb ascites fluid were used at 1:20 and 1:50 dilution, respectively. Anti-N-CAM and anti-Thy-1 were used at 1:50 and 1:100 dilution, respectively. Rabbit or mouse antibodies were detected using 1:500 dilution of Rhodamine-labelled anti-rabbit immunoglobulin (Ig) and a 1:100 dilution of fluorescein-labelled anti-mouse Ig (Sigma Chemical Co., St Louis, MO), respectively.

For electrophoresis, muscle cell homogenates scraped from tissue culture dishes, or bacterial pellets harvested by centrifugation, were treated with SDS-PAGE sample buffer. SDS-gel electrophoresis and immunoblotting were carried out as described previously (Moore et al. 1987), using 50 µg protein per lane. McAb 24.1D5 was used at 1:100 dilution in conjunction with horseradish peroxidase-conjugated sheep anti-mouse Ig (1:5000 dilution) and a colorogenic peroxidase substrate.

#### Isolation of 24.1D5 cDNA clones

A cDNA library (10⁶ recombinants) was produced in λgt11 using poly(A)+RNA isolated from midfusion human muscle cultures (Dickson et al. 1987). 24.1D5 cDNAs were detected by immunoscreening (Huynh et al. 1985) using 24.1D5 McAb (1:100 dilution) and horseradish peroxidase-conjugated sheep anti-mouse Ig as described above. Bacterial lysate (2 mg protein ml⁻¹), from a wild-type λgt11 lysogen, was included in the 24.1D5 McAb solution to absorb any anti-E-coli reactivity. Positive plaques were picked and purified by standard methods (Maniatis et al. 1982).

#### Preparation of antibody to 24.1D5 fusion protein

24.1D5-positive recombinant λgt11 clones were lysogenized in E. coli strain Y1089 and fusion proteins induced as described (Huynh et al. 1985). Cells were harvested by centrifugation, lysed directly in SDS–PAGE sample buffer and electrophoresed on 7-5% SDS–polyacrylamide gels. 2 mg fusion protein was added per gel and after electrophoresis a narrow strip of gel was removed from one side of the gel slab, transferred to nitrocellulose and immunostained with 24.1D5 McAb in order to localize fusion protein. The fusion protein band was excised from the remainder of each gel and crushed in liquid nitrogen. Crushed gel was suspended in PBS, mixed with complete Freund’s adjuvant (50% v/v) and injected subcutaneously into New Zealand white rabbits using approximately 100 µg fusion protein per injection. Booster injections were given at two-week intervals using 75 µg fusion protein in incomplete Freund’s adjuvant. Rabbits were bled one week after each booster injection.

#### Subcloning and labelling of cDNA probes

cDNA inserts from recombinant phage DNA were isolated and subcloned into the EcoRI sites of pUC13. Plasmid with inserted HG-8 cDNA was purified by equilibrium centrifugation and labelled with 32P by replacement synthesis (Dickson et al. 1986) for use as a probe in nucleic acid blot hybridizations. Sense and antisense single-stranded probes were prepared by transcription of M13 templates (Messing, 1983) prepared from M13mp18 recombinants containing the complete HG-8 cDNA. Probes HG-9A and HG-9B (see Fig. 4) were deletion clones produced in M13mp18 (see below) and were also labelled by transcription of single-stranded templates.

#### DNA sequence analyses

HG-8 cDNA inserts were subcloned into M13mp18 and subclones in both orientations were identified by restriction site mapping. A series of overlapping deletion clones derived from subclones of both orientations were generated (Henikoff, 1984). Clones were sequenced by the dideoxy chain termination method (Sanger et al. 1977) and sequence data analysed as described previously (Dickson et al. 1987).

#### Nucleic acid blot analyses

Total and poly(A)+RNA was isolated, fractionated by glyoxal–agarose electrophoresis on 1% agarose gels and blotted to Genescreen membrane (New England Nuclear, Boston, MA) as described previously (Dickson et al. 1987).

Genomic DNA extracted from human fetal brain tissue was digested with restriction endonucleases, fractionated on 0.7% agarose gels and blotted to nitrocellulose filters (Dickson et al. 1987). Filters were hybridized with 32P-labelled probes at 42°C in the presence of 50% formamide, 10% dextran sulphate 1:1-sodium chloride, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 0.2% bovine serum albumin, 0.05 M-Tris–HCl (pH 7.5) and 100 µg ml⁻¹ salmon sperm DNA. Filters were washed first for 1 h at 65°C in 2 × SSC (0.3 M-NaCl, 0.03 M-sodium citrate) containing 1% SDS, followed by 1 h at room temperature in 0.1 × SSC. Labelled bands were detected by autoradiogra-
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Results

24.1D5 antigen is expressed by myoblasts in primary human muscle cultures

McAb 24.1D5 was produced from a cell fusion from mice immunized with a myoblast plasma membrane fraction and its reactivity at the cell surface examined by indirect immunofluorescence. In preliminary experiments, it was shown that 24.1D5 is expressed by myoblasts but not by myotubes, using double indirect immunofluorescence staining with 24.1D5 antibody and an antibody to the MM form of creatine kinase which is specifically expressed by myotubes (Walsh et al. 1985). In the present study, antibodies to well-characterized cell surface phenotypic markers, namely N-CAM (Moore & Walsh, 1985) and Thy-1 (Walsh & Ritter, 1981) were used with 24.1D5 McAb in immunofluorescence assays to further analyse the expression of this epitope in primary cell cultures derived from human skeletal muscle. N-CAM has previously been shown to be a specific marker for myoblasts and myotubes in such cultures (Moore & Walsh, 1985), whereas Thy-1 antibody reacts with both myoblasts and fibroblasts (Walsh & Ritter, 1981; Schweitzer et al. 1987).

In muscle cell cultures at the prefusion stage, two populations of Thy-1-positive mononucleate cells are discernable (Fig. 1A,B), which are either positive or negative for 24.1D5. Mononucleate cells that exhibit 24.1D5 immunoreactivity are also positive for N-CAM (Fig. 1C,D) suggesting that these cells represent myoblasts, whereas Thy-1-positive 24.1D5 negative cells are fibroblasts. These assignments were confirmed by analysing antigenic phenotypes in clonal cultures of human skeletal muscle in which there are no contaminating fibroblasts (data not shown). In addition, it was found that cultures of human skin fibroblasts that are Thy-1 positive do not exhibit reactivity towards 24.1D5 (data not shown).

Confirming earlier observations (Walsh et al. 1985), it was found that in differentiated muscle cultures (Fig. 1E,F), multinucleate myotubes that are positive for N-CAM are negative for 24.1D5. Myotube cultures also contain a small population of residual myoblasts which are stained by both N-CAM and 24.1D5 antibodies.

Immunoblotting experiments were carried out to determine the nature of the molecule reactive with 24.1D5 McAb. In myoblast cultures from human skeletal muscle two bands of immunoreactivity of 100 and 60 x 10^3 relative molecular mass are found (Fig. 2).

Isolation of 24.1D5 cDNA clones

In order to examine further the molecular structure of the 24.1D5 antigen and its mode of regulation, attempts were made to isolate 24.1D5 encoding cDNAs. A cDNA library was prepared in the bacterial expression vector Agtll using poly(A)⁺ RNA from human muscle cell cultures at midfusion stage of differentiation. cDNA clones encoding 24.1D5 antigen were detected by immunoscreening with McAb 24.1D5. From the entire library of 10⁶ recombinants, six positive clones were isolated which were subsequently prepared. β-galactosidase fusion proteins were induced in suspension cultures and culture lysates examined by Western immunoblotting with 24.1D5 McAb. All clones were found to encode fusion proteins expressing 24.1D5 antigenic determinants, but only two were strongly immunoreactive (HG-8 and HG-12, Fig. 3). HG-8 produced fusion protein of about 160 x 10^3 Mₚ with a minor band at 150 x 10^3 Mₚ. In the case of HG-12, bands of 130, 125 and 120 x 10^3 Mₚ were observed. The occurrence of multiple bands most likely reflects partial degradation often seen with bacterial expression systems.

Amino acid sequence of 24.1D5 cDNA clone HG-8

The 24.1D5 clone HG-8, which contained the largest insert, was examined in further detail. HG-8 was subcloned for restriction analysis (Fig. 4) and for sequencing. An overlapping series of deletion clones from both orientations was then prepared in M13mp18 (Henikoff, 1984; see Fig. 4). Fig. 5 shows the nucleotide sequence of the 2022 bp insert in this clone. The 5' to 3' orientation was determined by Northern analysis with complimentary single-stranded probes (data not shown). Translation of the HG-8 sequence revealed an open reading frame of 1188 base pairs (396 amino acids) ending with a COOH-terminal phe. Multiple stop codons downstream from this point clearly establish the end of the open reading frame and indicate the remainder of the clone to be 3' untranslated sequence. Secondary structure and hydropathicity analyses were performed to determine whether the protein encoded by HG-8 contains membrane-associated segments. Although the 24.1D5 antigen is clearly membrane-associated on the basis of immunofluorescence staining, there are no predicted hydrophobic domains that might represent typical transmembrane regions in the deduced amino acid sequence. Indeed, the COOH-terminal residues of 24.1D5 are predominantly basic. The sequence contains 1 Asn residue at position 66 that represents a potential site for N-linked glycosylation. Data bank searches of Genebank and NBRL data bases with the nucleotide and predicted amino acid sequences of HG-8 cDNA clone did not reveal any major similarities, although minor similarities between alpha-1 chains of types I, II and IV collagens and the deduced COOH-terminal region of HG-8 (residues 300–390) were detected. Residues 357 to 362 (gly-gln-gly-gly-ala) form the consensus sequence of ribonucleotide-binding proteins (Wierenga & Hol, 1983), which is expressed by many members of the tyrosine kinase family (Carpenter, 1987) and GTP-binding proteins (Gilman, 1987), but the significance of this is unclear.

Reactivity of HG-8 fusion protein antibodies

To further confirm that HG-8 cDNA encodes 24.1D5 antigen, the 160 x 10^3 Mₚ fusion protein from HG-8 was...
Fig. 1. Expression of 24.1D5 antigen compared with Thy-1 antigen N-CAM in primary cultures of human skeletal muscle. 
Indirect immunofluorescence double stains of prefusion myoblast (A–D) and differentiated myotube cultures (E,F), showing 
24.1D5 staining (A,C,E), Thy-1 staining (B, same field as A) and N-CAM staining (D,F, same field as C,E, respectively).
Scale bar, 100 μm.

isolated from polyacrylamide gels and used to immu-
nize rabbits for the preparation of polyclonal anti-
bodies. Antisera were tested by indirect immunofluor-
escence staining of primary human muscle cells in
culture, and compared to 24.1D5 McAb by double
staining. It was found that cells which are positive for
24.1D5 McAb are also uniquely reactive with HG-8
polyclonal antibody (Fig. 6A,B). These data indicate
that HG-8 polyclonal antibody has the same immuno-
chemical specificity as the 24.1D5 McAb.

Expression of 24.1D5 mRNA sequences in cells and
tissues
The HG-8 cDNA clone was used as a probe to study the
expression of 24.1D5 mRNAs by Northern blot analy-
sis. HG-8 hybridizes to two mRNA bands of 2.5 kb and
3.0 kb in human skeletal muscle myoblast cultures
(Fig. 7A), and levels of both mRNAs decrease during
myogenesis. Similar-sized mRNAs are found in the G8-
1 mouse muscle cell line (Fig. 7B). Higher levels of
24.1D5 mRNAs are present in this clonal cell line and
their decrease during myotube formation is much more
clearly observed in this highly myogenic culture model
than in heterogeneous primary cultures of human
skeletal muscle. Thus G8-1 cell myotube cultures
(Fig. 7B, lane 2) do not express appreciable amounts of
24.1D5 RNA transcripts.

To date, studies using 24.1D5 McAb have been
restricted mostly to in vitro systems in the human
because of the species-specific nature of the antibody
and the lack of appropriate tissue. However, using HG-8 cDNA it was possible to extend the Northern blot analysis to a study of myogenesis in vivo in the mouse. A developmental analysis was carried out using various RNA samples harvested from embryonic or neonatal mice (Fig. 8). The two 24.1D5 mRNA bands of 2.5 and 3.0 kb are found at high levels in 12-day embryonic muscle. As the animal develops, however, the level of these two transcripts decreases such that by embryonic day 16 only very low levels are present. In newborn mice, 24.1D5 mRNAs are barely evident and adult mice do not express detectable 24.1D5 mRNAs (data not shown). These data show that expression of 24.1D5 mRNAs is decreased during myogenesis in vivo.

To attempt to further define the temporal and lineage specificity of 24.1D5 expression, use was made of the multipotential mesodermal cell line C3H10T1/2. Treatment with 5-azacytidine converts 10T1/2 cells into stably determined myoblasts which will fuse with high frequency to form myotubes (Taylor & Jones, 1979). mRNA was isolated from 10T1/2 stem cell cultures and also from 5-azacytidine-induced myogenic cells before and after fusion and this was analysed by Northern blot hybridization using HG-8 cDNA (Fig. 9). The two 24.1D5 mRNAs of 2.5 and 3.0 kb are clearly seen in parental 10T1/2 cells and also in 5-azacytidine-induced myoblasts (lanes 1 and 2). However, as these cells fuse to form myotubes the 24.1D5 mRNAs are correspondingly decreased (lane 3), indicating that 10T1/2-derived myogenic clones regulate 24.1D5 expression in a similar manner as the G8-1 muscle cell line. However, the 10T1/2 cells also express 24.1D5 mRNAs, suggesting that the 24.1D5 gene is activated at an earlier stage of development than is represented in the parental 10T1/2 cells.

The 24.1D5 gene locus
In order to relate the 24.1D5 cDNA sequence to genomic structure, human genomic DNA was digested with restriction enzymes that do not cleave the HG-8 clone and Southern blot analyses carried out with HG-8

Fig. 2. Analysis of polypeptides expressing 24.1D5 epitope in human muscle cultures. Western blot analysis of polypeptides immunoreactive with 24.1D5 antibodies in human skeletal muscle cultures. Size standards are indicated on the left.

Fig. 3. Analysis of bacterial fusion proteins with 24.1D5 antiserum. Bacterial lysates from lysogens of 24.1D5 positive clones HG-8 (lane 1) and HG-12 (lane 2) were fractionated by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue (A) or transferred to nitrocellulose and probed with 24.1D5 McAb (B). Size standards are indicated on the left.

Fig. 4. Restriction map of clone HG-8. Restriction endonuclease cleavage sites are shown above and the extent and direction of DNA sequence information gained from individual deletion clones are indicated by arrows below.
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728  Fig. 5. Nucleotide and deduced amino acid sequence of HG-8 cDNA. Nucleotides are numbered on the left and amino acids on the right. Restriction endonuclease sites are indicated above the sequence. The potential site for N-linked glycosylation is marked by an asterisk.

cDNA as the hybridizing probe. For most restriction enzyme digests, multiple HG-8 hybridizing bands are observed (Fig. 10), indicating that the HG-8 sequence may represent a series of exons that are interrupted by introns containing restriction sites. Alternatively, the human genome may contain multiple copies of the 24.1D5 gene. These possibilities were distinguished by performing Southern blot hybridizations, using smaller probes generated from the 5' end or the 3' end of clone HG-8 (probes HG-8A and HG-8B, respectively, from Fig. 4). It was found that for each digest, probes HG-8A and HG-8B hybridize to bands of different M_r (Fig. 10), indicating that the multiple bands observed with the complete HG-8 probe encode different regions of the HG-8 gene. These results show that the 24.1D5 gene exhibits a complex intron-exon structure.

Fig. 6. Cell surface immunoreactivity of HG-8 polyclonal antiserum in human muscle cultures. Double indirect immunofluorescence was used to compare the immunoreactivity of HG-8 antiserum (A) with that of 24.1D5 McAb (B) in prefusion cultures. Scale bar, 50 μm.

Fig. 7. Expression of 24.1D5 mRNAs during myogenesis in vitro. Northern blot analysis using HG-8 to probe. (A) Total cellular mRNAs (7 μg per lane) from human prefusion myoblasts (lane 1), and from early fusion, midfusion and late fusion human myotube cultures (lanes 2, 3, 4 respectively). (B) Poly(A)^+ RNA (1 μg per lane) from G8-1 mouse muscle myoblasts (lane 1) or myotubes (lane 2). Positions of 28S and 18S rRNA standards are shown on the left and the sizes of 24.1D5 mRNAs are indicated on the right in kb.
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Fig. 8. Expression of 24.1D5 mRNAs during muscle development in vivo. Northern blot analysis using HG-8 to probe total cellular mRNAs (7μg per lane) from 12-day (lane 1), 14-day (lane 2) or 16-day (lane 3) embryonic and newborn (lane 4) mouse muscle. Positions of 28S and 18S rRNA standards are shown on the left and the size of 24.1D5 mRNAs are indicated on the right in kb.

Fig. 9. Expression of 24.1D5 mRNAs in multipotential 10Ti cells and their myogenic derivatives. Northern blot analysis using clone HG-8 to probe total cellular mRNAs (10μg per lane) from multipotential 10Ti cells (lane 1) and 5-azacytidine-induced prefusion myoblasts (lane 2) or postfusion myotubes (lane 3). Positions of 28S and 18S mRNA standards are shown on the left and the size of 24.1D5 mRNAs are indicated on the right in kb.

Discussion

A number of McAb have now been isolated that define antigens whose expression is decreased following myoblast fusion in skeletal muscle. These include the following antigens: Thy-1, 30-2A8, transferrin receptor and 24.1D5 (Walsh et al. 1984). 24.1D5 reactivity is distinct from the others as it is the only member of this family that is not generally expressed on the surface of fibroblast cells (Walsh et al. 1984). Furthermore, in relation to other muscle cell surface antigens, it can be distinguished in terms of M, from C3-1 (Wakshull et al. 1983), H36 (Kaufmann et al. 1985), N-CAM (Covault et al. 1986; Moore et al. 1987), CSAT (Neff et al. 1982) and Thy-1 (Williams & Gagnon, 1982) and in terms of cell expression and primary amino acid sequence from the transferrin receptor (Schneider et al. 1984).

The species-specific reactivity of 24.1D5 McAb has previously been of immense value for studies of human gene activation in heterokaryons and somatic cell hybrids (Blau et al. 1985), but it has not allowed a full characterization of this antigen during myogenesis in vivo mainly because of the difficulties in obtaining human fetal material. It was therefore clearly desirable to obtain more versatile reagents specific for 24.1D5 in order to study the structure and expression of this protein and for this reason cDNA clones encoding 24.1D5 were isolated. Evidence that clone HG-8 represents an authentic 24.1D5 cDNA was provided by indirect immunofluorescence experiments in which an antibody prepared against fusion protein produced by HG-8 exhibited the same immunochemical specificity as 24.1D5 McAb.

The mode of attachment of 24.1D5 to the external surface of the plasma membrane is still uncertain. Although the HG-8 clone does not have an initiation site for translation and does not therefore encode the full amino acid sequence of 24.1D5, it is clear, however, that this antigen does not have a predicted transmembrane domain near its COOH-terminal. It is possible that 24.1D5 has a membrane-associated region in its NH2-terminal domain similar to the 4F2 antigen (Teixeira et al. 1987) although further sequence analysis is required to establish this point. Other experiments have been carried out to determine whether 24.1D5 associates with the plasma membrane via nonhydrophobic interactions. However, 24.1D5 immunoreactivity is not released from the plasma membrane by treatment with collagenase or phospholipase C or by extraction with salt (data not shown). This is in contrast to nontransmembrane N-CAM isofoms which can be released from myotubes by treatment with phospholipase C and which are associated with the plasma membrane via a glycosylphosphatidylinositol anchor (Moore et al. 1987). The nucleotide and deduced amino acid sequence of HG-8 cDNA showed no major homology with any known sequences in Genebank and NBRL data bases. The observed matches between the predicted COOH-terminal sequence of HG-8 and regions of collagen alpha-1 chains are unlikely to be significant, but are thought to arise due to the relatively high proportion of glycine residues in both sequences. The 24.1D5 predicted amino acid sequence also contains the consensus sequence that identifies ribonucleotide-binding proteins (Weirenga & Hol, 1983; Carpenter, 1987). Since HG-8 cDNA does not encode a hydrophobic domain, the possibility exists that 24.1D5 is a protein ectokinase.

A number of issues remain to be resolved regarding the structure and expression of the 24.1D5 protein. In particular, the relationship between the two polypeptide chains on Western blots of human myoblast cultures remains to be elucidated. It is possible that the
Fig. 10. Southern blot analysis of 24.1D5 genomic structure. Human genomic DNA (20 μg per lane) was digested with EcoRI (lane 1), PstI (lane 2), PvuII (lane 3) and ApaI (lane 4) and analysed by Southern blot hybridization using probes. (A) Whole HG-8 insert; (B) 5′ subfragment HG-8A; (C) 3′ subfragment HG-8B. Size markers indicated in kb were from HindIII digests of λ phage DNA.

60 × 10^3 M_r band is a degradation product of the 100 × 10^3 M_r band. Alternatively, the two subunits may be generated from two 24.1D5 mRNAs. Indeed, Northern blot analyses show the presence of two 24.1D5 mRNAs in human myoblast cells which are presumably generated by alternative processing of a single primary transcript. However, it is not known whether these mRNAs differ in their coding or noncoding sequences.

The cross-species reactivity of HG-8 cDNA has allowed a more detailed study of 24.1D5 mRNA expression. The results indicate that 24.1D5 is a marker of one of the earliest programmes of gene activation during myogenesis, both in culture and in developing skeletal muscle tissue. Both 24.1D5 mRNAs are reproducibly decreased in amount as myoblasts fuse to form myotubes. The decreased level of 24.1D5 mRNAs was most obvious in the G8-1 cell line in which myotubes did not express either mRNA transcript. G8-1 myoblasts, however, expressed high levels of both mRNAs. A similar developmental transition was found during skeletal muscle development in the mouse. Embryonic day-12 skeletal muscle expressed both mRNA bands but at later stages of development the level of 24.1D5 mRNAs was decreased. Newborn and also adult mouse muscle did not express appreciable amounts of the 24.1D5 mRNAs.

As 24.1D5 is expressed at an early stage of skeletal muscle development, it is possible that this antigen may be a marker of skeletal muscle determination although a more detailed lineage analysis should be carried out. To further analyse this, experiments were carried out utilizing the 10T^f^ cell line. This cell line has been found to be useful for the study of muscle determination as multipotential 10T^f^ cells can be induced to form stable myoblasts after 5-azacytidine treatment (Konieczny & Emerson, 1984; Lassar et al. 1986; Davis et al. 1987; Pinney et al. 1988). In the present study, it was found that 24.1D5 is expressed by multipotential 10T^f^ cells and determined myoblasts, but not by myotubes. Thus the activation of 24.1D5 expression at the level of transcription must occur at an earlier stage of development than is represented by the multipotential 10T^f^ cells and before absolute commitment to myogenesis. N-CAM mRNAs are also expressed at low levels in 10T^f^ precursor cells (data not shown), perhaps indicating that these cells may be at a stage closer to commitment to myogenesis than to other mesodermal lineages. Indeed, it has already been observed that multipotential 10T^f^ cells preferentially convert into myocytes (Konieczny & Emerson, 1984), leading to the suggestion that myogenic determination requires the alteration of fewer gene loci than, for example, commitment to chondrogenesis (Lassar et al. 1986). It is possible that continued expression of 24.1D5 is necessary for skeletal muscle determination whereas expression of this antigen is down-regulated when multipotential mesodermal 10T^f^ cells become committed to alternative developmental pathways. Experiments are currently underway to test this hypothesis by analysing patterns of 24.1D5 expression in clonally stable lineages of adipogenic and chondrogenic cells that can also be produced from multipotential 10T^f^ cells by treatment with 5-azacytidine.

Further investigation regarding the expression of 24.1D5 at the protein and mRNA levels and a detailed analysis of its expression in different cell lineages will be
of considerable interest. It should now be possible to analyse further the expression of 24.1D5 during embryogenesis by in situ hybridization and immunocytochemistry. Availability of cDNA probes to 24.1D5 should also allow isolation of the 24.1D5 gene and may ultimately lead to the characterization of the trans-acting factor (Blau et al. 1985), which may control the expression of a family of early genes during skeletal muscle myogenesis.

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