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Replicating myoblasts express a muscle-specific phenotype

(desmin/muscle membrane glycoprotein H36/5-bromo-2′-deoxyuridine/regulation/skeletal muscle development)

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ABSTRACT **During the terminal stage of skeletal myogenesis, myoblasts stop replicating, fuse to form multinucleate fibers, and express the genes that encode the proteins that convey contractile capacity. Because of this dramatic shift in proliferative state, morphology, and gene expression, it has been possible to readily identify and quantitate terminally differentiating myoblasts. In contrast, it is not clear whether the proliferating cells that give rise to postmitotic myoblasts are equally distinct in their phenotype and in fact whether distinct stages in skeletal myogenesis precede the onset of terminal differentiation. To address these questions, monoclonal antibodies and immunofluorescence microscopy were used to determine that replicating myoblasts from newborn rats do express a muscle-specific phenotype. To identify replicating cells, incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) into DNA was assayed by using anti-BrdUrd antibody. The developmentally regulated, muscle-specific, integral membrane protein H36 and the intermediate-filament protein desmin were scored as markers of the myogenic phenotype. The percentage of BrdUrd$^+$ (i.e., proliferative) cells among H36$^+$ and desmin$^+$ myoblasts was equal to the percentage of BrdUrd$^+$ cells in the entire population, indicating that the expression of H36 and desmin is uniformly characteristic of replicating myoblasts. Inhibition of protein synthesis before and during growth in BrdUrd did not alter the frequency of desmin and H36 immunofluorescence in BrdUrd$^+$ cells. Thus, desmin and H36 were present in the replicating myoblasts prior to the onset of growth in BrdUrd. These results were confirmed using H36$^+$ cells selected by flow cytometry: these purified H36$^+$ myoblasts replicate, express desmin, and differentiate. Similar results were obtained with mouse myoblasts. Desmin expression in these mammalian cells differs from that in chicken embryo myoblasts: only a small proportion of replicating chicken embryo myoblasts express desmin. That replicating mammalian myoblasts have a muscle-specific phenotype serves to define a distinct stage in myogenic development and a specific cell in the myogenic lineage. Further, it implies that there is a regulatory event activated during myogenesis that precedes terminal differentiation and that is required for expression of those genes whose products distinguish the replicating myoblast.**

In what has come to be known as the terminal stage of skeletal muscle differentiation, myoblasts cease replicating and express a panoply of phenotypic characteristics intrinsic to this stage of development. During this same transition in myogenic development, skeletal myoblasts fuse to form multinucleate fibers in which assembly of myofibrillar proteins takes place. Once assembled in these fibers, these proteins provide the structural basis and enzymatic capacities that render the fibers functional contractile units, capable of responding to neuronal excitation. Expression of the genes that encode these proteins is largely regulated at the level of transcription (1–6); alternative splicing provides additional diversity and is often involved in the switching of isoforms (6, 7). Translation of muscle-specific transcripts may also be regulated (8–11).

The earliest expression of the terminally differentiated phenotype takes place in myoblasts that normally do not replicate further (12, 13). However, there appears to be a period in which these postmitotic, terminally differentiating myoblasts can be manipulated to replicate again (14, 15): when replication is reinitiated, expression of the differentiated phenotype stops. Analysis of developmental mutants and the use of tumor viruses and growth factors likewise have led to the conclusion that continued proliferation is incompatible with terminal myogenic development (16–23). Studies using heterokaryons suggest that soluble trans-acting factors may mediate or initiate terminal differentiation (24, 25). Transfection studies support this concept and indicate that the product of a single gene may mediate the onset of this stage of skeletal muscle development (26, 27).

By using antibodies and nucleic acid probes, and by measuring isozyme activities and myoblast fusion, it has been possible to identify and quantitate terminally differentiating myoblasts. In contrast, it is not clear whether the replicating cells that give rise to these postmitotic myoblasts have an equally distinct phenotype that distinguishes this particular stage of myogenic development. We believe that this is the case and demonstrate here that expression of the muscle-specific intermediate-filament protein desmin (28, 29) and the muscle-specific cell surface glycoprotein H36 (30, 31) are hallmarks of the population of replicating myoblasts that give rise to terminally differentiating myoblasts.

Previous work (32) demonstrated that detection of incorporated 5-bromo-2′-deoxyuridine (BrdUrd) by immunofluorescence, in conjunction with other antibodies, can be used to sequence the molecular events in single cells as they relate to the transition in DNA synthesis that accompanies myogenic differentiation. The selective prolongation by laminin of the proliferative phase of myogenic development was also demonstrated by using BrdUrd incorporation as an index of cell replication (33). We have adopted this same strategy in these experiments to show that replicating myoblasts from newborn rats express H36 and desmin and that replicating myoblasts from newborn mice likewise express desmin. This demonstration of a distinct muscle-specific phenotype in replicating myoblasts distinguishes these cells within the myogenic lineage and necessitates consideration of additional regulatory steps in the pathway of skeletal myogenesis.

In contrast with results found in mammalian cells, relatively few replicating myoblasts from the chicken embryo appear to express desmin, indicating that the regulation of desmin expression and the expression of a muscle-specific phenotype in replicating myoblasts may differ during avian and mammalian myogenesis.

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Abbreviations: mAb, monoclonal antibody; DPBS, Dulbecco's phosphate-buffered saline; BrdUrd, 5-bromo-2′-deoxyuridine.

METHODS AND MATERIALS

Cell Culture. Cultures from the hindlimbs of newborn Sprague–Dawley rats and newborn BALB/c mice (Holtzmann, Madison, WI) were prepared and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 10% fetal bovine serum, and antibiotics, as described (33). Chicken embryo hindlimb cultures were prepared from fertilized White Leghorn eggs (University of Illinois Poultry Farm) by dissociation of minced tissue by Vortex mixing (11- and 12-day embryos) (34); tissue from 18-day embryos was preincubated for 15 min at 37°C in 0.25% trypsin in calcium-free phosphate-buffered saline. The chicken cells were grown on gelatin-coated or laminin-coated (33) dishes in medium further supplemented with 2% chicken embryo extract. Expression of desmin in replicating chicken cells grown on laminin or gelatin was the same. Growth media and supplements were purchased from GIBCO.

Immunofluorescence Microscopy. The muscle-specific intermediate-filament protein desmin was detected with DE-R-11 monoclonal antibody (mAb), generously provided by M. Osborn (Max-Planck Institute, Goettingen, F.R.G.) (35). Anti-H36 mAb reacts with a developmentally regulated, muscle-specific, integral membrane protein on the surface of rat skeletal myoblasts and cardiac myocytes (30, 31) but not on smooth muscle. mAb 76.7, reactive with BrdUrd, was the generous gift of T. Ternynck (Institute Pasteur, Paris) (36). To render incorporated BrdUrd accessible to antibody, fixed cells were rinsed twice in Dulbecco's phosphate-buffered saline (DPBS), treated with 2 M HCl for 30 min at room temperature, neutralized by three 20-min washes in 50 mM NaCl/100 mM Tris·HCl, pH 7.4, and rinsed twice with DPBS. For simultaneous immunofluorescence labeling of desmin and BrdUrd, cells grown on coverslips were incubated for 90 min with 40 μM BrdUrd, fixed for 10 min with 95% ethanol, treated with 2 M HCl as cited above, and stained as previously described (32, 33). Cells labeled for H36 and BrdUrd were incubated with 40 μM BrdUrd for 90 min or with 4 μM BrdUrd for 15 hr, rinsed twice with cold DMEM, treated with fresh 1% formaldehyde for 10 min, and then incubated at 5°C with mAb (5 μg/ml) H36 followed by fluorescein isothiocyanate-conjugated rabbit antimouse IgG (FITC-RaMIg; Miles) diluted 1:50. The cells were washed, fixed with 95% ethanol, treated with 2 M HCl as above, and incubated with anti-BrdUrd mAb followed by FITC-RaMIg. All preparations were stained with 10 μM Hoechst 33342 to aid in enumeration of nuclei, mounted in glycerol/phosphate-buffered saline, 9:1 (vol/vol), pH 8.5, containing 0.01 M *p*-phenylenediamine (Eastman), sealed with FLO-TEXX (Fisher), and examined with a Zeiss Universal microscope equipped with epi-illumination optics and an HBO 100-W mercury lamp. Photomicrographs were made with Kodak Tri-X film and a Planapochromat ×63 objective. Handling of cultures grown in BrdUrd was done in gold light (General Electric F15T8-GO) prior to fixation.

Flow Cytometry. Cells to be sorted by flow cytometry were grown for 24 hr in DMEM supplemented with 30% fetal bovine serum and 2% chicken embryo extract on laminin-coated dishes to maximize proliferation (33). The cultures, containing both myogenic cells and fibroblasts, were rinsed with calcium-free phosphate-buffered saline and dissociated with 0.05% trypsin. The cells were washed twice in DPBS containing glucose at 1 mg/ml and bovine serum albumin at 40 mg/ml (DPBS/BSA). The cells were resuspended to 10^7 per ml in DPBS/BSA containing H36 mAb or normal mouse serum IgG at 50 μg/ml and then were incubated at 5°C for 15 min, washed three times, incubated for 15 min in DPBS/BSA with FITC-RaMIg, washed, and resuspended to 10^6 cells per ml in DPBS/BSA for sorting. Myoblasts (i.e., $H36^+$ cells) were selected with a Coulter EPICS 751 cell sorter with an argon laser adjusted to emit 400 mW at 488 nm, using a 457- to 502-nm laser-blocking filter and a 515-nm long-pass filter. Cells in the H36-labeled sample were collected if brighter than 93% of the negative control cells.

RESULTS

To resolve whether replicating myoblasts express a distinct muscle-specific phenotype, we determined whether the muscle-specific proteins desmin and H36 were expressed *in vitro* in replicating myoblasts from newborn rat thigh muscle. At 24-hr intervals the cells were incubated for 90 min with BrdUrd at a final concentration of 40 μM. Incorporation of BrdUrd was detected by immunofluorescence using anti-BrdUrd antibody and scored in individual cells as an index of their proliferation. The specificity of the anti-BrdUrd mAb used is stringent and is restricted to the iodo and bromo derivatives of thymidine (36); this assay is equivalent in sensitivity to that achieved by [^{3}H]thymidine incorporation and autoradiography and it is by far more convenient (S.J.K. and M. Robert-Nicoud, unpublished data). Incubations with BrdUrd at the concentrations and times indicated had no effect on subsequent myogenic development.

Desmin and H36 were detected by using the respective mAbs DE-R-11 and anti-H36. The specificity of DE-R-11 for desmin has been established by immunoblotting with avian and mammalian desmin (35); the reactivity of anti-H36 is restricted to cardiac and skeletal muscle (30). Cultures prepared from fetal rat hindlimb contain both myoblasts and fibroblasts. Only the myoblasts stain with anti-desmin or anti-H36, whereas both fibroblasts and myoblasts react with anti-vimentin antibody. Immunofluorescence was used to define replicating cells that expressed H36 and desmin. That these muscle-specific proteins are expressed in replicating myoblasts is illustrated in Fig. 1. Quantitation and further documentation of this follows.

Expression of Desmin in Replicating Rat Myoblasts. The frequency of expression of desmin by replicating cells from

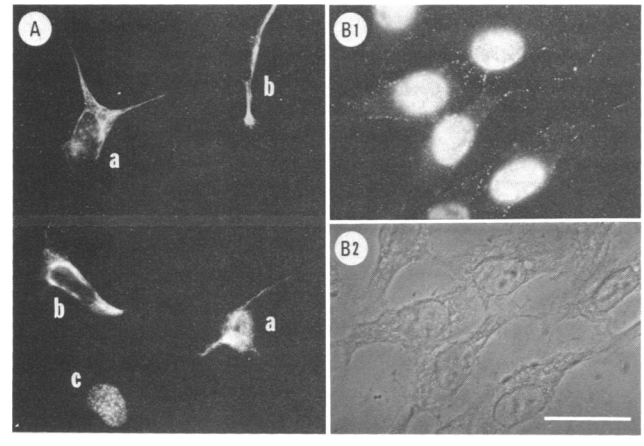

FIG. 1. Expression of desmin (*A*) and H36 (*B*) by proliferating cells from newborn rat hindlimb. (*A*) Cells were grown for 24 hr and incubated with 40 μM BrdUrd for 90 min, fixed with 95% ethanol, and stained with anti-BrdUrd and anti-desmin antibodies. Cells were phenotyped with respect to cytoplasmic desmin and BrdUrd incorporation into DNA. Examples are shown of $desmin^+$ $BrdUrd^+$ (a) and $desmin^+$ $BrdUrd^-$ (b) cells and of a $desmin^-$ $BrdUrd^+$ cell (c). For data see Table 1. (*B*) A culture grown for 24 hr was enriched for myoblasts (i.e., $H36^+$ cells) by flow cytometry, replated, and incubated with 4 μM BrdUrd for 15 hr, restained with anti-H36, and then fixed and stained with anti-BrdUrd antibody. Most cells (84%) incorporated BrdUrd and expressed H36 on their surface (*B1*), indicating that the myoblast population was replicating. *B2* shows a phase-contrast image of the cells in *B1*. For data see Table 2. (Planapochromat ×63 objective; bar = 20 μm.)

newborn rat hindlimb was determined. In 24-hr cultures, 32% of the mononucleate cells expressed desmin (experiment 1, Table 1). The frequency of S-phase cells in the desmin$^+$ population (i.e., myoblasts) was similar to that in the entire population (myoblasts and fibroblasts): 34% of the desmin$^+$ cells were in S phase during the 90-min incubation with BrdUrd (BrdUrd$^+$ cells), and BrdUrd incorporation was detected in 31% of the whole mononucleate population. This similar frequency of incorporation of BrdUrd into desmin$^+$ cells and cells in the whole population indicates that replication was characteristic of the desmin$^+$ population. Analogous results were obtained in experiment 2 of Table 1. Although the percent desmin$^+$ cells and the percent of myoblasts that are replicating vary from day to day, as expected since myoblasts withdraw from the cell cycle, we consistently found that a significant fraction (17–54%) of the cells expressing desmin were in S phase during the 90-min labeling period throughout the 72-hr duration of these experiments. Cells grown in the presence or absence of cycloheximide (5 μg/ml) 20 min prior to and during growth in BrdUrd exhibited the same frequencies of expression of desmin and incorporation of BrdUrd. These results rule out the possibility that the desmin detected in the BrdUrd$^+$ cells was synthesized subsequent to incorporation of BrdUrd (experiment 3, Table 1).

Expression of H36 on Replicating Rat Myoblasts. H36 was first shown to be expressed on the L8E63 and L6 lines of rat myogenic cells and on primary rat myoblasts (30). There is also a 99% coincidence in the expression of H36 and desmin in myoblasts from newborn rats (33). Therefore, as expected from the results with desmin, H36 was also found on replicating myoblasts from the newborn rat (Fig. 1). The expression of H36 on the surface of replicating myoblasts not only serves as a phenotypic marker for these cells but also permits the identification and isolation of these cells. Cells from 24-hr explants of newborn rat thigh muscle were enriched for myoblasts by using fluorescence flow cytometry to select for H36$^+$ cells. These cells were then (*i*) plated for 5 hr and then grown for 15 hr in 4 μM BrdUrd or (*ii*) plated for 18.5 hr and then incubated in 40 μM BrdUrd for 90 min. Eighty-four percent of these cells expressed both H36 and desmin and incorporated BrdUrd in the 15-hr labeling period (Table 2), confirming that expression of these proteins is characteristic of proliferating myoblasts.

Expression of Desmin in Replicating Mouse and Chicken Myoblasts. Because of the interest in myogenesis in other species, we determined whether replicating myoblasts from newborn mice and chicken embryos also express a muscle-specific phenotype. Due to the species specificity of the H36 antibody for the rat, these experiments were limited to examining desmin.

Cultures were prepared from newborn mouse hindlimb by the same procedure used for newborn rats. In a 24-hr culture, 40% of the cells were myogenic (i.e., they expressed desmin): 48–52% of these desmin$^+$ cells incorporated BrdUrd, while 50% of the entire population incorporated BrdUrd (Table 3). Thus in mouse, as in rat, the frequency of desmin$^+$ cells that are also in S phase parallels the state of proliferation of the whole population.

This incidence of desmin expression in replicating mouse and rat myoblasts differs from reports of desmin expression in chicken cultures, where desmin expression was noted as entirely (37–40) or largely (41, 42) confined to postreplicating cells. We therefore looked at expression of desmin in embryonic chicken myoblasts.

In six separate experiments, primary cultures were prepared from chicken embryos after 11, 12, or 18 days *in ovo*. These cultures were assayed for desmin in replicating myoblasts either 24 or 48 hr after seeding (Table 3). The incidence of BrdUrd incorporation into the desmin$^+$ population was very low compared with that in the whole mononucleate population, suggesting that either the desmin$^+$ population replicates more slowly or that most desmin$^+$ cells have withdrawn from the cell cycle. Because of the low incidence of expression of desmin in replicating chicken cells the data from six experiments have been pooled: a total of 9998 cells were scored in these experiments (Table 3). Of these, only 43 cells (0.45 ± 0.27%) both expressed desmin and incorporated BrdUrd. Because of this relatively low frequency, an additional 18,000 embryonic chicken thigh cells were scored (experiment 7 in Table 3). Of these, 18% expressed desmin but only 9 cells (<0.1%) were also BrdUrd$^+$. Furthermore, the relative immunofluorescence of desmin in the double-positive cells was considerably less than in desmin$^+$ BrdUrd$^-$ myoblasts.

DISCUSSION

The identity of replicating skeletal myoblasts has usually been established only after the fact: the cells that stop replicating and fuse, thereby giving rise to multinucleate fibers, are by definition replicating myoblasts. Although

Table 1. Expression of desmin in replicating rat myoblasts

	n	% BrdUrd$^+$	% desmin$^+$	% desmin$^+$ also BrdUrd$^+$	% BrdUrd$^+$ also desmin$^+$
Experiment 1					
24 hr	662	31.3	32.2	34.3	35.3
48 hr	834	43.6	20.3	45.0	20.9
72 hr	1908	25.4	15.7	38.3	27.4
Experiment 2					
24 hr	824	50.6	28.3	50.2	28.1
48 hr	2322	36.9	45.6	22.9	28.0
72 hr	2943	30.3	35.7	17.0	20.0
Experiment 3					
Control	1359	45.3	58.6	52.3	68.0
Cycloheximide	1254	51.6	58.3	54.3	61.3

Cells from the hindlimb of newborn rats were cultured for 24, 48, or 72 hr. These cultures contain myoblasts (desmin$^+$ cells) and fibroblasts (desmin$^-$ cells). The medium was supplemented with 40 μM BrdUrd during the last 90 min of growth. Coverslips were processed for immunofluorescence using anti-BrdUrd and anti-desmin mAbs. Cells were scored in 12 fields on each of three coverslips. *n*, Total number of mononucleate cells scored. The frequency of BrdUrd$^+$ nuclei among desmin$^+$ cells indicates that replicating myoblasts express desmin. In experiment 3, addition of cycloheximide (5 μg/ml) 20 min before and during BrdUrd incorporation in 24-hr cultures reduced [^{35}S]methionine incorporation by 95% but did not alter desmin expression in BrdUrd$^+$ cells.

Table 2. H36 and desmin expression in sorted rat myoblasts

Time with BrdUrd, hr	n	% BrdUrd$^+$	% H36$^+$	% desmin$^+$
15	988	84.2	ND	ND
0	610	ND	84.1	84.8
1½	1043	43.6	ND	ND

Cells were selected by fluorescence flow cytometry on the basis of H36 expression: myoblasts express H36 and desmin, fibroblasts do not. The enriched H36$^+$ population was subsequently grown in 4 μM BrdUrd for 15 hr or in 40 μM BrdUrd for the last 90 min of incubation. The cells were then stained for immunofluorescence with anti-H36, anti-BrdUrd, and anti-desmin antibodies. The frequency of each phenotype was determined by scoring at least 30 fields on each of two coverslips. n, Number of cells scored; ND, not determined.

expression of many muscle-specific myofibrillar proteins and isozymes and the acetylcholine receptor have been used as hallmarks of the postproliferative phase of myogenesis, no distinct phenotype has been associated with replicating myogenic cells.

Since terminal differentiation can be readily identified by the capacity of postmitotic myoblasts to fuse, conditions that promote the cessation of proliferation and terminal differentiation have most often been used in studies of myoblasts *in vitro*. Therefore the relative lack of focus on the replicative phase of myogenic development *in vitro* is not surprising. The replicating cells in the developing limb that are not directly myogenic are predominantly fibroblasts. Differences in morphology and developmental capacity between myoblasts and fibroblasts suggest that replicating myoblasts should also have distinguishing muscle-specific biochemical markers. However, in the absence of known functions specifically attributable to these cells, such biochemical markers have largely gone undetected. Furthermore, the absence of this information has fostered the notion that such markers and functions may not exist and that the preeminent, if not sole, mechanism underlying the differentiation of skeletal muscle is associated with the terminal phase of development and cessation of replication. The expression of two muscle-specific proteins, desmin and H36, establishes that replicating mammalian myoblasts do have a muscle-specific phenotype and establishes criteria for enumerating another cell in the myogenic lineage. This expression of muscle-specific genes prior to the onset of terminal differentiation suggests that there are additional regulatory events expressed at earlier stages of development.

Expression of desmin was previously reported to be restricted to postmitotic differentiated myoblasts (37–40) and to rhabdomyosarcoma cells (43). Desmin$^+$ cells have been reported in day-3 to -5 embryonic chicken limb bud (44), but the proliferative state of these cells was not determined. More recently, desmin expression was found in embryonic chicken myoblasts that were denoted as replicating. Duglosz *et al.* (42) reported that expression of desmin in chicken myoblasts *in vitro* generally follows what they suggest is the terminal DNA synthesis in the myoblasts. They did find that about 1% of cells grown with [^{3}H]thymidine for 45 min both express desmin and incorporate thymidine. This frequency could be increased by growth of cells in the presence of the tumor promoter phorbol 12-myristate 13-acetate. However, unlike the untreated controls, these cells also expressed myosin heavy chain and thus were more analogous to rhabdomyosarcoma cells, which also exhibit aberrant growth control and express desmin as well as other characteristics of the differentiated myogenic phenotype. More recently, Hill *et al.* (45) stated that 7% of fibroblastic cells in 4- to 8-day primary cultures of 12-day chicken embryo breast muscle express desmin (but not myosin or titin) and concluded that these cells are most probably replicating presumptive myoblasts. In that experiment no attempt was made to demonstrate that these cells were proliferating; however, it was stated that 8% of metaphase-arrested cells in 2-day cultures treated with Colcemid for 4 hr did express desmin. This suggested a population of desmin$^+$ replicating cells. However, the desmin in these cells may have been synthesized subsequent to S phase, in the G_2 period, during which these cells were treated with Colcemid. Yablonka-Reuveni and Nameroff (41), using the same antiserum against desmin as that of Duglosz *et al.* (42) and Hill *et al.* (45), showed that about 5% of myoblasts from 18-day chicken embryo hindlimb cells both incorporated [^{3}H]thymidine and stained with anti-desmin antibodies. They also raised their concern, in the absence of any independent marker, whether these labeled cells were indeed skeletal myoblasts or were of smooth muscle origin. As with the other studies of chicken cells referred to above, it is possible that the cells in these experiments that expressed desmin did so after they had incorporated [^{3}H]thymidine. The relatively low frequency of replicating, desmin$^+$ chicken embryo cells in these and in our experiments, the possibility that desmin expression followed terminal replication, and the lack of an independent marker for chicken skeletal myogenic cells leave unresolved the question of whether or not replicating chicken myoblasts express a distinct phenotype.

Our analysis of fetal rat skeletal muscle has avoided the ambiguities of the avian system and allowed the definition of a population of replicating myoblasts with a muscle-specific phenotype. The frequency of desmin expression and of replication in newborn rat myoblasts suggests that most if not all myoblasts at this stage of development express desmin.

Table 3. Expression of desmin in replicating mouse and chicken myoblasts

	n	% BrdUrd$^+$	% desmin$^+$	% desmin$^+$ also BrdUrd$^+$	% BrdUrd$^+$ also desmin$^+$	% of total cells desmin$^+$ BrdUrd$^+$
Mouse						
Experiment 1	1,889	53.5	40.2	48.5	14.6	19.5
Experiment 2	1,987	50.2	32.3	51.6	33.2	16.7
Chicken						
Experiments 1–6	9,998*	16–35†	11–18†	ND	ND	0.45 ± 0.27‡
Experiment 7	≈18,000	30	18	ND	ND	<0.1

For experiments with mouse myoblasts, cells from newborn mouse hindlimb were cultured for 24 hr and grown in 40 μM BrdUrd for the last 90 min of incubation. The frequency of BrdUrd incorporation and of desmin expression was determined by immunofluorescence in 15 fields on each of two coverslips. For chicken myoblasts, data were pooled from six experiments in which 11-, 12-, and 18-day chicken embryos were used; an additional experiment (no. 7) used cells from 11-day embryos. n, Number of cells scored; ND, not determined.

*Total in six experiments.

†Range in six experiments.

‡Mean ± SD from six experiments.

Expression of desmin in replicating rat myoblasts was unaffected by inhibition of protein synthesis during the S phase in which myoblast DNA was labeled with BrdUrd, indicating that desmin expression is truly characteristic of these replicating cells. H36, an independent cell surface marker specific for skeletal and cardiac smooth muscle myogenic cells, was readily identified on replicating ($BrdUrd^+$) rat myoblasts, removing any ambiguity in identifying skeletal myoblasts. Myoblasts enriched by flow cytometry based on expression of H36 continued to replicate and to express H36 and desmin. Since the coincidence in expression of H36 and desmin in newborn rat myoblasts is >99%, each marker provides an independent confirmation of the myogenic nature of these cells.

Due to the species specificity of the anti-H36 antibody for rat cells, detection of a distinct myogenic phenotype in replicating myoblasts from other species is presently limited to desmin. Our results show that replicating mouse myoblasts also generally express desmin. This is also true of human satellite myoblasts (data not shown), and thus it may be true of mammalian myoblasts in general. The low frequency of expression of desmin in replicating chicken myoblasts may reflect differences in the regulation of desmin expression during avian and mammalian myogenesis or differences in the proliferative capacities of these cells *in vitro*. Nevertheless, in all these species the regulation of synthesis of desmin does appear to be distinct from that of myofibrillar proteins such as myosin heavy chain, titin, and creatine kinase (45). That anti-integrin antibody maintains chicken myoblast proliferation and increases the frequency of expression of desmin (but not myosin heavy chain) (46) also suggests such a distinct regulatory mechanism. Conversion of $H36^-$ precursors into $H36^+$ cells (47) and conversion of C3H/10T½ cells into replicating myoblasts by transfection with a *myd* genomic clone (26) and MyoD1 cDNA (27) further substantiate that there are early regulatory events in the myogenic lineage.

It is probable that desmin and H36 are just two of many properties characteristic of replicating skeletal myoblasts. The C2C12 line of mouse myoblasts expresses muscle-specific trans-acting transcription factors in replicating myoblasts (48); these cells also express desmin (unpublished data). Additional muscle-specific traits expressed in replicating myoblasts are likely to be found associated with their stringent control of replication, myoblast motility and interaction with extracellular matrix proteins, regulation of receptor-mediated endocytosis during myogenesis (49), and the activation of promoters of tissue-specific transcription and posttranscriptional mechanisms.

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