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Myogenesis in cultures of uniparental mouse embryonic stem cells: differing patterns of expression of myogenic regulatory factors

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ABSTRACT The expression of myogenic regulatory factors (MRFs) in cultures of androgenetic (AG) and parthenogenetic (PG) embryonic stem (ES) cells were analyzed to identify a role for imprinted genes in the myogenic program. The time course and levels of expression of *myf5, myogenin, MyoD1* and *myf6* were assessed by semi-quantitative RT-PCR. A more rapid induction of *myogenin* expression was seen in AG ES cell cultures compared to control D3 ES cells, and *myf6* was expressed by AG but not D3 cells. Persistence of *myf5* and *MyoD1* expression at late stages of AG cell culture suggests that proliferation and differentiation are maintained. Myogenic differentiation was delayed in PG ES cells, but abundant levels of *myogenin* and *myf6* transcripts were subsequently observed. Absence of *myf5* expression and only low *MyoD1* expression at later stages of culture demonstrate a decline in proliferation in PG cultures. *Igf2* was induced to high levels in the late phase of both AG and D3 but not PG cell cultures, indicating paternal allele-specific expression. *Igf2* expression correlated with expression of MRF genes associated with myoblast proliferation rather than terminal differentiation. *H19* was expressed at very low levels in both AG and PG ES cell cultures. The delay in myogenesis in PG cultures suggests that imprinted genes other than *Igf2* and *H19* play a role at early stages of the myogenic program.

KEY WORDS: *myogenic regulatory factors, androgenetic and parthenogenetic ES cells*

Introduction

Cells derived from uniparental mouse embryos display aberrant potential for skeletal muscle formation, suggesting that imprinted genes have an important role in the initiation and/or maintenance of the myogenic program. Parthenogenetic (PG) inner cell mass (ICM) or embryonic stem (ES) cells are progressively excluded from skeletal muscle when combined with normal embryonic cells in chimeric embryos (Fundele *et al.*, 1989, 1990; Nagy *et al.*, 1989; Mann, 1992; Allen *et al.*, 1994). In contrast, androgenetic (AG) embryonic cells contribute significantly to skeletal muscle in chimeras and in teratomas (Mann *et al.*, 1990; Barton *et al.*, 1991; Mann, 1992; Allen *et al.*, 1994; McKarney *et al.*, 1996).

The four basic helix-loop-helix myogenic regulatory factors (MRFs), myf5, myf6, MyoD1 and myogenin, that activate the myogenic program are not imprinted (Rudnicki and Jaenisch, 1995; Olson and Klein, 1994; Villar and Pedersen, 1994), and myf5, myf6 and myogenin are expressed in the somites of postimplantation PG embryos (Fundele *et al.*, 1994). Thus duplication or deficiency in the expression of MRF genes in uniparental embryos cannot be directly responsible for the effects on muscle development. A number of growth factor and oncogenic signals act as negative regulators of the muscle differentiation program (Olson, 1992; Sassoon, 1993), while other factors, particularly the insulin-like growth factors (Igfs), are positive stimulators of muscle development (Florini *et al.*, 1991; Florini and Ewton, 1992). Igf2 is highly expressed in fetal skeletal muscle (Lee *et al.*, 1990) and evidence implicates this mitogen as an important autocrine regulator of terminal muscle differentiation (Brown *et al.*, 1992; Kou and Rotwein, 1993). Since Igf2 is expressed exclusively from the paternal allele in skeletal muscle, liver and yolk sac (DeChiara *et al.*, 1991), aberrant expression in uniparental embryos is predicted to influence myogenesis. The maternally expressed gene, *H19*, is closely linked to *Igf2* on mouse chromosome 7 (Bartolomei *et al.*, 1991) and is expressed in fetal and adult muscle (Poirier *et al.*, 1991; Leibovitch *et al.*, 1995), but its role in the myogenic pathway is unclear.

While cross-regulatory links between growth factors, MRFs, and other regulatory signals have been demonstrated (Olson,

Abbreviations used in this paper: MRFs, myogenic regulatory factors; AG, androgenetic; PG, parthenogenetic; ES cells, embryonic stem cells; ICM, inner cell mass.

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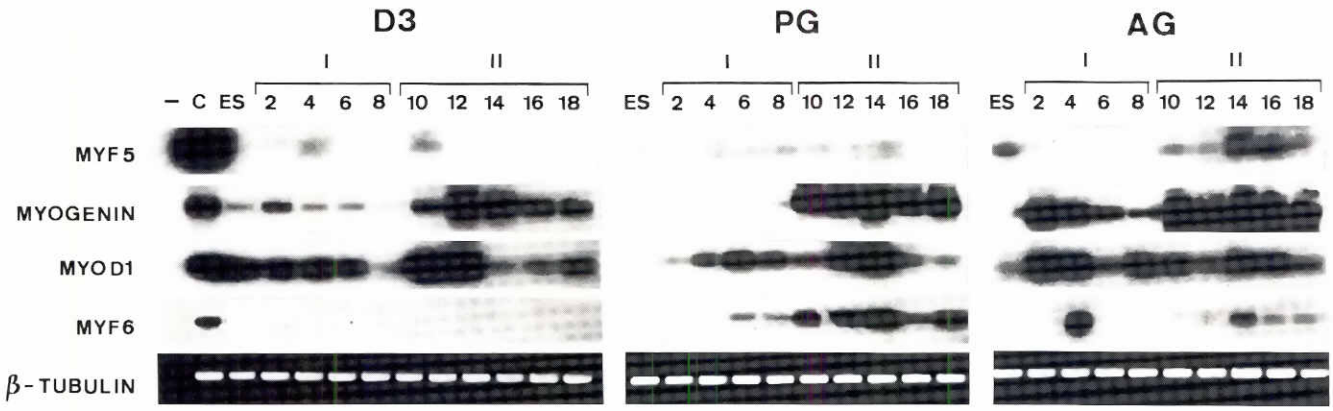

Fig. 1. Detection of myf5, myogenin, MyoD1 and myf6 transcripts in embryoid body cultures of D3, PG and AG ES cells by Southern blotting after RT-PCR analysis. *RNA was extracted from cells at 2 day intervals after plating of 5-day-old embryoid bodies, from 2 days to 18 days* **(lanes 2-18)**, *and from undifferentiated ES cells (ES) prior to embryoid body formation. Phase I* **(lanes 2-8)** *and phase II* **(lanes 10-18)** *of differentiation are indicated. The fetal control sample (C) is RNA extracted from day 10.5 whole embryos. A non-reverse transcribed negative control (-) is shown for the D3 sample, and was negative in all cases. Levels of β-tubulin amplification are used as a standard for the amount of cDNA template (see Materials and Methods). Sizes of PCR products for myf5, myogenin, MyoD1, myf6 and β-tubulin are 132, 517, 144, 117 and 317 bp respectively.*

1992; Sassoon, 1993), it is not fully understood how these multiple factors interact to precisely control the decisions for proliferation and differentiation of myoblasts. The aims of this study were to analyze the effects of a uniparental genome on the time-course and the levels of expression of MRFs. Previous studies have shown that the order of initiation of the four MRF genes in embryoid body cultures of normal diploid ES cells is similar to that during normal development and that these cells differentiate into fused myocytes that have electrophysiological properties of skeletal muscle cells (Miller-Hance *et al.*, 1993; Rohwedel *et al.*, 1994). In this study we have analyzed the pattern of MRF expression in embryoid body cultures of AG, PG and normal ES cells and compared these to levels of *Igf2* and *H19* expression in order to determine the potential roles of these imprinted genes in myogenesis.

Results

Expression of myogenic regulatory factors

AG, PG and D3 embryoid bodies were analyzed for expression of *myf5, MyoD1, myogenin* and *myf6* mRNAs at 2 day intervals up to 18 days after plating. Since transcripts could not be detected by Northern blot analysis, the RT-PCR technique was used to determine expression profiles in a semi-quantitative manner, as described previously (Rohwedel *et al.*, 1994). Two distinct phases of differentiation could be distinguished in all three cell lines based entirely on the pattern of MRF expression, particularly obvious for expression of myogenin transcripts (Fig. 1). These two phases will be referred to as phase I, being from days 2-8, and phase II, from days 10-18 of culture (Fig. 1). These phases were consistent in duplicate experiments.

Myf5 transcripts were amplified at only two time points (days 4 and 10) in D3 cultures, indicating transient expression within phases I and II of the culture (Fig. 1). Expression was much lower than in the day 10.5 embryo control sample. *Myogenin* transcripts were detectable at low levels in undifferentiated D3 ES cells and during initial stages of phase I, but were not detected on day 8 of differentiation (Fig. 1). A rapid increase in expression was seen by day 10, and high levels were maintained throughout phase II. *MyoD1* transcripts were amplified from undifferentiated D3 ES cell cultures at levels equivalent to the day 10.5 embryo cDNA sample (Fig. 1). Low levels of *MyoD1* transcripts were amplified on day 8, and levels increased markedly at the start of phase II but declined from days 14-18 (Fig. 1). *Myf6* transcripts were not amplified at any stage of differentiation of D3 cell cultures.

Myf5 transcripts were detected at low levels in PG ES samples from day 4 onwards but levels declined towards the end of phase II (Fig. 1). *Myogenin* expression was not detected in undifferentiated PG ES cells nor from any stage of differentiation in phase I (Fig. 1). Transcript levels increased abruptly on day 10 and high levels were maintained throughout phase II. Unlike D3 ES cells, *MyoD1* transcripts were not detected in undifferentiated PG ES cells and levels of expression were much lower on day 2 of culture (Fig. 1). High levels were subsequently detected through to day 14 in phase II, after which a decline in amplification was seen. *Myf6* transcripts were amplified at low levels towards the end of phase I (days 6 and 8) and levels increased substantially in phase II when *myf6* expression exceeded that in the day 10.5 embryo sample (Fig. 1).

Myf5 transcripts were amplified from undifferentiated AG ES cells but were not detected at any stage of differentiation during phase I (Fig. 1). *Myf5* expression was detected throughout phase II at higher levels than in either D3 or PG ES cell cultures. *Myogenin* was not expressed by undifferentiated AG ES cells (Fig. 1). High transcript levels were detected in day 2 embryoid body cultures, and levels then declined through phase I. Increased levels of *myogenin* transcripts were induced at the start of phase II and this abundant expression was maintained until the end of phase II (Fig. 1). The levels of *MyoD1* transcripts amplified from undifferentiated AG ES cells were lower than those from undifferentiated D3 ES cells (Fig. 1). *MyoD1* transcript levels increased substantially at the start of differentiation in phase I and were maintained at high levels throughout phases I and II (Fig. 1). Transient high expression of *myf6* expression was detected on day 4 during phase I of AG ES

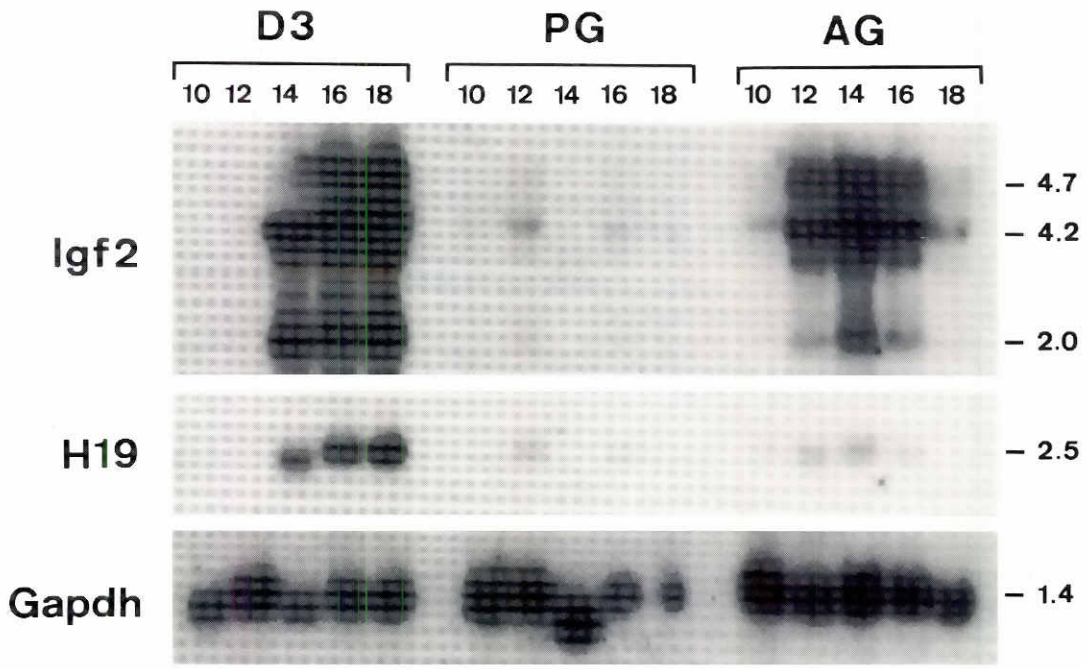

Fig. 2. Northern blot hybridization *of Igf2, H19 and Gapdh mRNA in total RNA isolated from D3, PG and AG ES cell cultures at 10, 12, 14, 16 and 18 days after plating of embryoid bodies. Transcript sizes are indicated (kb).*

cell differentiation, and transcripts were subsequently amplified at low levels in phase II, from days 14-18 (Fig. 1).

Expression of Igf2 and H19 genes

Northern blot analysis showed high levels of *Igf2* mRNAs by D3 and AG ES cells during phase II of the embryoid body cultures, from days 10-18 (Fig. 2). In D3 cultures, high levels of expression were induced on day 14, and these levels were maintained until day 18 (Fig. 2). In AG ES cell cultures, *Igf2* transcripts were detected at low levels on day 10 and were abundant by day 12. Levels peaked at day 14, with a gradual decline thereafter (Fig. 2). *Igf2* transcripts were detected only at very low levels in PG cultures on days 12-16 (Fig. 2).

H19 transcripts were evident only in samples in which *Igf2* mRNAs were also detected (Fig. 2). Highest levels of *H19* mRNA were seen in D3 ES cells between days 14-18. Only low levels were detected in AG ES cell cultures between days 10-16, and in PG ES cell cultures on days 12 and 16 (Fig. 2).

Discussion

These studies demonstrate that when AG and PG ES cells are cultured under conditions known to induce myogenic differentiation in normal ES cell lines (Rohwedel *et al.*, 1994), they express the four MRF genes, *myf5*, *MyoD1*, *myogenin* and *myf6*, that activate muscle differentiation. Variations between the cell lines in the levels of MRF transcripts and in the temporal sequence of their expression indicates that a uniparental genotype influences the regulation of myogenic proliferation and differentiation.

The pattern of appearance of MRF transcripts in the control D3 ES cell line showed a much earlier activation of *MyoD1* and *myogenin* expression than that reported previously for the BLC6 and D3 cell lines (Miller-Hance *et al.*, 1993; Rohwedel *et al.*, 1994). In addition, two distinct phases of MRF expression could be distinguished in each cell line, which has not been previously reported (Rohwedel *et al.*, 1994). These phases do not correspond to any changes in the culture environment (e.g. serum withdrawal) and must reflect intrinsic developmental phases within the cultures. Previous studies of cultured myogenic cell lines, and tissues from normal embryos and from mice with null mutations have implicated roles for each MRF at specific stages of myogenesis (Montarras *et al.*, 1991; Sassoon, 1993; Olson and Klein, 1994; Rudnicki and Jaenisch, 1995). MyoD1 and myf5 expression is associated with the initial determination and proliferation of myoblast cells, while myogenin and myf6 expression are induced at later stages of differentiation when cell fusion occurs. Myf5 and MyoD1 were predominantly localized to mononucleated cells in ES cells undergoing myogenesis *in vitro* (Rohwedel *et al.*, 1994). Myogenin marks the exit of proliferating myoblasts from the cell cycle as they terminally differentiate into myotubes, although proliferating adult skeletal muscle cells can also express myogenin (Maley *et al.*, 1994). *Myogenin* transcripts were predominantly localized to elongated cells resembling primary myotubes in ES cell cultures, but were also seen in mononucleated cells (Rohwedel *et al.*, 1994). *Myf6* is transiently expressed in early somites and expression is later induced in secondary myofibers. *Myf6* is the major MRF to be expressed in postnatal skeletal muscle. The patterns of MRF expression are thus useful markers to characterize different stages of myoblast differentiation and proliferation in the ES cell lines.

High levels of *MyoD1* transcripts in undifferentiated D3 ES cells suggest a spontaneous commitment of these cells to the myogenic lineage even in the presence of LIF. Transient expression of *myf5* and expression of *MyoD1* through both differentiation phases demonstrates continued proliferation of myoblasts in these cultures but at declining rates at later stages of the culture. The significantly higher levels of *myogenin* expression in phase II compared to phase I suggest that a higher degree of terminal differentiation into fused myotubes occurs during phase II. How-

ever, absence of *myf6* expression shows that these cultures had probably not yet advanced to the secondary myofiber stage of terminal differentiation.

Like D3 ES cells, spontaneously commitment of AG ES cells to the myogenic lineage occurs in the presence of LIF. Differentiation of myoblasts into *myogenin*-expressing cells occurs rapidly at early stages of embryoid body culture, and the abundant expression of all four MRFs in phase II indicates continued proliferation and differentiation of myogenic cells in these cultures, including the possible formation of secondary myofibers. These data demonstrate that AG ES cells have a higher capacity for proliferation and terminal differentiation than the control D3 ES cells, which is consistent with the predominance of AG ES cell-derived muscle cells in chimeras produced from AG cells and normal embryos and the prevalence of skeletal muscle in AG ES cell-derived teratomas (Mann *et al.*, 1990; Barton *et al.*, 1991; Mann, 1992; Allen *et al.*, 1994; McKarney *et al.*, 1996). Absence of *MyoD1* expression by undifferentiated PG ES cells, and delayed induction of expression after embryoid body plating suggests that these cells have a reduced potential for myogenesis when compared to the D3 and AG ES cell lines. A decline in the proliferative capacity of myoblasts is suggested by the marked reduction in *MyoD1* and *myf5* transcripts towards the end of phase II. While *myogenin* expression was significantly delayed in comparison to both D3 and AG ES cell lines, both *myogenin* and *myf6* transcripts were abundant throughout phase II. This pattern of MRF expression demonstrates a marked delay in the formation and terminal differentiation of myoblasts when compared to the other two cell lines, but a subsequently higher rate of differentiation. These studies suggest that PG ES cell-derived myoblasts have a limited capacity for self-renewal, which is consistent with the progressive reduction of PG-derived muscle cells in chimeras (Fundele *et al.*, 1989, 1990; Nagy *et al.*, 1989; Allen *et al.*, 1994). Previous studies have shown that PG cells are also defective in their ability to maintain precursor cell populations in other cell lineages (Jagerbauer *et al.*, 1992; Sturm *et al.*, 1994; Bender *et al.*, 1995; Newman-Smith and Werb, 1995), suggesting the possibility of common pathways for stem cell renewal.

Igf2 expression was induced to high levels only in D3 and AG ES cell cultures coinciding with terminal differentiation of muscle cells in phase II. *Igf2* expression was not induced in PG ES cells, indicating imprint-dependent inactivation of the maternal *Igf2* alleles. Induction of *Igf2* expression in AG and D3 ES cells was preceded by increased expression of *myogenin*, in agreement with previous studies showing that endogenously produced Igf2 stimulates muscle differentiation after induction of *myogenin* gene expression has occurred (Florini *et al.*, 1991; Brown *et al.*, 1992; Rosen *et al.*, 1993). It is proposed that the autocrine action of Igf2 and autoregulatory and cross-regulatory interactions between Igf2 and MRFs amplify the expression of these factors above the threshold necessary to fully activate the synthesis of muscle contractile proteins (Florini and Ewton, 1992; Olson, 1992; Rosen *et al.*, 1993; Black *et al.*, 1995). However, studies demonstrate that terminal muscle differentiation can proceed in the absence of high Igf2 expression, as seen by the abundant expression of *myogenin* and *myf6* in phase II of PG ES cell cultures. Our data suggests that absence of Igf2 induction correlates with a reduced proliferative capacity of the myoblast population rather than having an effect on their differentiation potential. It has been suggested that differences in the relative timing of expression for each MRF in different myogenic cell populations may control distinct muscle phenotypes (Hinterberger *et al.*, 1991; Olson, 1992; Smith *et al.*, 1993) and it will be of interest to determine whether the phenotype of PG ES cell-derived muscle cells differs from that of D3 and AG ES cells.

Our studies do, however, suggest that imprinted genes other than *Igf2* and *H19* play a role at early stages of the myogenic program, since commitment of myoblast cells and *myogenin* expression were significantly delayed in PG ES cell cultures when compared to the other two cell lines. This delay is unlikely to be due to differences in *H19* expression, since the levels of *H19* gene expression were much lower in both AG and PG ES cell cultures when compared to D3 controls. Decreased transcription of *H19* in AG ES cell cultures is consistent with downregulation of paternal alleles during differentiation (McKarney *et al.*, 1996). Low level expression of *H19* in PG myogenic cells suggests abnormal regulation of this maternally-active gene, since *H19* transcripts are normally abundant in terminally differentiated skeletal muscle (Poirier *et al.*, 1991; Leibovitch *et al.*, 1995). The actual function of *H19* transcripts is not known, although some studies suggest a role in downregulation of cellular growth control (Hao *et al.*, 1993). No effects of H19 on either growth or differentiation of myoblast cells

TABLE 1

OLIGONUCLEOTIDE PRIMERS AND PROBES USED FOR RT-PCR ANALYSIS OF MRF GENE EXPRESSION

Gene		PCR primers (5' - 3')	Hybridization probe (5' - 3')	Size of cDNA product
β-tubulin	Forward:	GGAACATAGCCGTAAACTGC	GATGTCCATGAAGGAGGTGG	317 bp
	Reverse:	TCACTGTGCCTGAACTTACC		
myf5	Forward:	GTTCTTTCGGGACCAGACAGGGCTG	CATTCAGGCATGCCGTCAGAGCA	132 bp
	Reverse:	GAGCTGCTGAGGGAACAGGTGGAGA		
myogenin	Forward:	GGGCTCTCTGGACTCCATCT	1.5 kb cDNA (Wright *et al.*, 1989)	517 bp
	Reverse:	AGCTCCCTCAACCAGGAGGA		
MyoD1	Forward:	CCTGGACTCGCGCACCGCCTCACT	1.7 kb cDNA (Davis *et al.*, 1987)	144 bp
	Reverse:	CACTACAGTGGCGACTCAGACGCG		
myf6	Forward:	GGAGGCTGAGGCATCCACGTTTGC	CCAAGTGTTTCGGATCATTCCAG	117 bp
	Reverse:	GAGGGTGCGGATTTCCTGCGCACC		

can be inferred from our study. Our data suggest that delayed myogenesis in PG ES cell cultures could be due to the absence of positive regulators that are normally expressed from paternal alleles, or increased expression of negative regulators by the diploid maternal genome. None of the negative regulators of myogenesis, e.g. members of the FGF and TGFβ growth factor families and their receptors, are known to be imprinted, but this analysis has not been done for all these genes. Seventeen imprinted genes have now been identified, including novel genes and transcription factors of unknown function (John and Surani, 1996). Myogenesis in uniparental ES cell cultures could provide a useful system for identifying the imprinted genes involved in the myogenic program.

Materials and Methods

Embryonic stem cell culture and differentiation

AG (LB1) and PG (LG1) ES cells were kindly provided by Drs. Jeff Mann and Colin Stewart (Mann *et al.*, 1990; Mann, 1992) and normal D3 ES cells (Doetschman *et al.*, 1985) were used as controls (McKarney *et al.*, 1996). Undifferentiated cells were cultured with 10^3 U/ml Leukemia Inhibitory Factor (LIF) (Amrad, Australia) in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (P.A. Biologicals, Australia) and 10^{-4} M β-mercaptoethanol (McKarney *et al.*, 1996). Myogenesis was induced as described previously except that FBS was not treated with dextran-coated charcoal (Rohwedel *et al.*, 1994). In brief, 800 ES cells per 20 μl of medium were aggregated in hanging drops for 2 days (in the above medium without LIF), followed by suspension culture in bacteriological plates for 3 days. Each 20 μl drop of 5-day-old aggregates (approximately 40 embryoid bodies) were then plated onto 10 cm gelatin-coated tissue culture plates and cultured for up to 18 days with medium changed every 2-3 days. Duplicate plates were analyzed at each time point.

Northern blot analysis

Total RNA was extracted from undifferentiated ES cells prior to embryoid body formation, embryoid bodies at 2 day intervals after plating (2-18 days), and also day 10.5 mouse embryos. Embryonic tissue and single plates of each cell culture were homogenized in 7.5 M guanidine HCl and total RNA was extracted as described previously (Chirgwin *et al.*, 1979). Ten μg of total RNA were electrophoresed through 1% agarose gels containing formaldehyde and transferred to Hybond-N filters (Amersham) by standard procedures (Sambrook *et al.*, 1989). Filters were sequentially hybridized with ^{32}P-dCTP-labeled (Boehringer-Mannheim) cDNA probes to mouse *Igf2*, mouse *H19* and rat *Gapdh* as described previously (McKarney *et al.*, 1996). Hybridized filters were exposed to Kodak XAR-5 film.

Reverse transcriptase-PCR

Five μg of total RNA were reverse transcribed using AMV reverse transcriptase (Promega). The exponential phase of PCR amplification of β-tubulin (primers listed in Table 1; Miller-Hance *et al.*, 1993) was determined in order to standardize the concentration of cDNA in each sample. Samples (5% of the reverse-transcribed product) were amplified with *Tth Plus* polymerase (Biotech International) in 15-35 PCR cycles of 93.5°C, 55°C and 72°C for 60 sec at each temperature. Amplification of each sample was determined to be within the exponential phase at 30 cycles. Sample concentrations were adjusted to amplify approximately equivalent amounts of *β-tubulin* cDNA after 30 cycles. Oligonucleotide primers for amplification of *myf5, myogenin, MyoD1* and *myf6* transcripts were identical to those used by Montarras *et al.* (1991) and Miller-Hance *et al.* (1993) and are listed in Table 1. PCR conditions for all four genes were identical to those for *β-tubulin*. Southern blotting was performed to increase the sensitivity of detection of PCR products, using ^{32}P-dCTP labeled cDNA or oligonucleotide probes to detect each amplified transcript (Table 1).

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