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Original Article

Fetal myoblast clones contribute to both fast and slow fibres in developing rat muscle

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ABSTRACT Retroviral cell lineage marking was used to investigate the role of cell lineage in fetal and neonatal rat muscle development. Clusters of infected cells, presumably myoblast clones, contribute cells to both slow primary and fast secondary fibres. Moreover, single clusters of marked cells contain both slow and fast primary fibres, suggesting that, at least during fetal life, single clones contribute nuclei to both fibres that are committed to remain slow and those that convert to a fast phenotype. The majority of fibres in individual fascicles of fetal muscle could be infected by a self-inactivating retroviral vector. Retroviral gene expression was markedly lower in non-muscle tissues, suggesting that fetal retroviral infection might target exogenous genes to mammalian muscle fibres during later life.

KEY WORDS: *muscle differentiation, retroviral vector, lineage analysis, rat, fiber type*

Introduction

Multinucleate muscle fibres are formed in two waves during the second half of rodent gestation (Stockdale, 1992). In rat hindlimb, primary fibres are thought to arise by terminal differentiation and fusion of an early population of embryonic myoblasts between embryonic days 15 and 17 (E15-E17) that generate slow muscle fibres (Kelly and Rubinstein, 1980; Condon *et al.*, 1990b). In contrast, secondary fibres arise in a more protracted later wave from E17 into the early neonatal period and generally express fast myosin heavy chain (MyHC) (Kelly and Rubinstein, 1980; Condon *et al.*, 1990b). Dividing myoblasts isolated from muscle tissue during secondary myogenesis differ from those isolated during primary fibre formation in a variety of tissue culture assays (Cossu *et al.*, 1988). There have also been indications of selective myoblast fusion during the period of the initiation of secondary fibre myogenesis (Duxson *et al.*, 1989; Harris *et al.*, 1989). Based on these and other observations in avian embryos (Stockdale, 1992; DiMario *et al.*, 1993), it has been proposed that cell lineage might underlie the diversification of muscle fibres.

To investigate the role of cell lineage during mammalian muscle development, while avoiding the potential problems of re-implanting cultured myoblasts (DiMario and Stockdale, 1995), we introduced retroviral myoblast lineage marking (Hughes and Blau, 1990). We showed that during postnatal life the progeny of single myoblast clones frequently fuse to multiple different fibres *in vivo* (Hughes and Blau, 1990). Moreover, these clones appear to contribute to the full range of muscle fibre types in their environment (Hughes and Blau, 1992). Use of the same lineage tracing technique during fetal life has suggested that clones of fetal mouse myoblasts contribute to both primary and secondary fibres (Evans *et al.*, 1994).

Retroviral vector technology has been continuously refined. Replication-incompetent vectors containing a marker gene, but lacking the viral *gag, pol* and *env* genes necessary for generation of infectious viral particles, permitted *in vivo* lineage tracing (Danos and Mulligan, 1988). Two or multiple distinguishable retroviral vectors can be employed to prove that clusters of cells are derived from single retroviral infection events (Galileo *et al.*, 1990; Hughes and Blau, 1990) and this procedure has been used to demonstrate that low titre retroviral infection of fetal rodent limbs generates clusters of marked fibres that result from single infection events, i.e., clones (Evans *et al.*, 1994). The discovery that first generation vectors could undergo genetic recombination events leading to production of replication-competent vectors, prompted development of vectors that minimised stretches of sequence homology with the wild type retrovirus (Miller and Rosman, 1989), and reagents of this type were used in the previous muscle lineage analyses (Hughes and Blau, 1990, 1992; Evans *et al.*, 1994).

Abbreviations used in this paper: MyHC, Myosin heavy chain; LTR, long terminal repeat.

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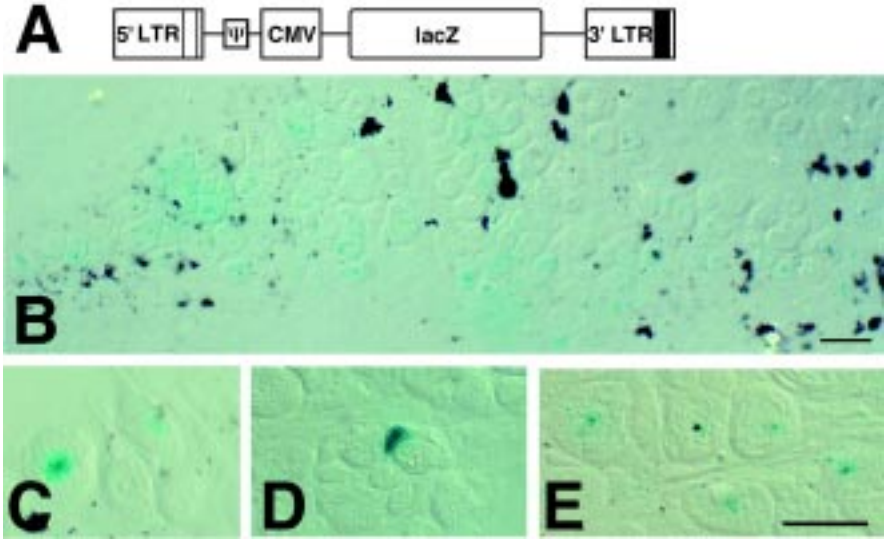

Fig. 1. Efficient somatic transgenesis *in utero* with a self-inactivating retroviral vector. (A) *Structure of αSCG Moloney murine leukemia virus-based retroviral vector. The lacZ gene is expressed from an internal CMV enhancer driving the α-globin promoter. The 3' LTR deletion (black box) is copied to the 5' LTR during infection such that infected cells are unable to synthesise full length viral RNA containing the Ψ packaging sequence. So, even if endogenous retroviruses in vivo were capable of providing packaging proteins, production of fresh viral particles carrying the lacZ gene is impossible.* **(B)** *Muscle fibres in the tibialis anterior of E21 rat marked with β–gal as a result of retroviral injection into the fetal hindlimb at E17. Note the diverse intracellular distribution of blue Xgal reaction product. Black particles are tattoo dye that was co-injected with the virus.* **(C)** *The nuclear/circum-nuclear location of Xgal reaction product in a large marked fibre.* **(D)** *A small marked secondary fibre adjacent to a large unmarked primary fibre, showing Xgal reaction product essentially filling the cytoplasm.* **(E)** *Large fibres showing punctate cytoplasmic Xgal reaction. Bar, 100 μm, C-E equal magnification.*

However, concern has remained that the presence of endogenous retroviruses within strains of laboratory rodents might permit repackaging of the infecting retroviral vector, allowing it to spread from cell to cell. To overcome this concern self-inactivating retroviral vectors have been developed (Yu *et al.*, 1986; Dhawan *et al.*, 1992; Hofmann *et al.*, 1996). These make use of the fact that the 3' long terminal repeat (LTR) is copied to generate the 5' LTR, which normally drives retroviral RNA production, during each round of infection. By engineering a 3' LTR capable of supporting vector infection but unable to drive retroviral RNA transcription vectors have been produced that can not be repackaged. In this paper, such a vector is used to examine the behaviour of fetal rat myoblasts. Single myoblast clones are found to contribute to both fast and slow, and primary and secondary, muscle fibres.

Results

Retroviral vectors efficiently infect fetal muscle cells

To examine the efficiency of retrovirally-mediated gene transfer into myoblasts, during fetal development, rat embryo hindlimbs were injected on embryonic day 17 (E17) *in utero* with the αSCG self-inactivating recombinant retroviral vector that expresses β–gal

TABLE 1

COMPARISON OF FREQUENCY OF MULTI-FIBRE CLUSTERS WITH AGE AT MYOBLAST INFECTION

Age at injection	Age at analysis	Incubation period (days)	Vector	Species (number of legs)	Number of bgal-labelled regions with		
					single marked fibre (%)	multiple marked fibres (%)	marked non-muscle cells (%)
E17	E21	4	αSCG	Rat (4)	23 (26)	53 (60)	13 (15)
P1	P21	20	MMuLVSVnlsLacZ	Rat (4)	9 (23)	26 (67)	4 (10)
P1	P15	14	LnβgeoCX	Mouse (1)	1 (50)	1 (50)	
P5	P21	16	LnβgeoCX	Mouse (3)	15 (83)	3 (17)	
P5	P18	13	BAG	Rat (1)	12 (55)	10 (45)	
P15	P37	22	MMuLVSVnlsLacZ	Rat (6)	89 (49)	92 (51)	

Rat or mouse fetuses or pups were injected in the lower hindlimb with the vector shown at various ages. After a period of growth retrovirally-infected clusters of fibres, or single fibres, were visualised by Xgal staining of tissue sections, and serial sections scored to analyse the numbers of isolated regions of label that contained either one (single marked fibre) or more than one (multiple marked fibres) labelled fibre. The percentage of putative clones in each category is shown in parentheses. Each row represents the summed total number of clusters for all animals in a single experiment. For E17 injections the median and mean number of fibres per cluster were 3 and 5.4, respectively. For P15 injections clones were smaller: the median was 2 and mean 2.3. χ^2 tests showed significant differences between early (P1 or earlier) and late (after P1) injections ($P<0.001$, all data; $P<0.05$ rat data only).

in the cytoplasm of infected cells. αSCG contains a deletion in the 3' long terminal repeat, which ensures that the vector can not spread from cell to cell within the animal after the initial round of infection (Dhawan *et al.*, 1992 and Robbins and Mulligan, unpublished, Fig. 1A). Injection of high titres of retroviral particles led to numerous labelled muscle cells in the region of the tattoo dye when fetal limbs were analysed four days later on E21 (Fig. 1B). On several occasions, whole fascicles of muscle were found in which most fibres showed β-gal activity. β-gal activity was more rarely detected in regions of injected limbs distant from tattoo dye. Uninjected contralateral limbs and mock-injected limbs never showed any β-gal reactivity (data not shown). To assess the frequency of infected fibres, the number of fibres expressing β-gal in several sections from a single highly-infected muscle was determined. Although some sections contained few marked fibres, in the middle region of the muscle around the site of the tattoo dye over 60% of fibres contained β-gal. Thus, injection of retroviral vectors can be an efficient method to introduce transgenes into numerous muscle fibres in developing fetal muscle.

In contrast to our previous findings in postnatal rodent muscle (Hughes and Blau, 1990), most detectable β-gal activity had a punctate distribution within the cytoplasm of muscle fibres: the fibre cytoplasm was not uniformly filled with β-gal. A variety of β-gal distributions were observed in distinct fibres (Fig. 1C-E). β-gal reactivity was most frequently located in dots adjacent to the nuclear membrane, but occasionally entirely surrounding the nucleus. Only rarely, and usually in smaller fibres that were strongly labelled, was the cytoplasm ever observed to be uniformly filled by β-gal reactivity. I have, however, been unable to correlate the distinct β-gal distributions with any other cytological property, for example fibre size, type or location. Nevertheless, the same individual fibre was frequently labelled in several serial sections with a similar β-gal distribution, suggesting that β-gal location is a property of the individual infected cell.

Fetal myoblast clones contribute to both primary and secondary fibres

In regions far from the site of injection marked by tattoo dye or when lower titres of retroviral vector were injected, many fewer cells were marked. Under these conditions, isolated marked single muscle fibres were often observed, but small clusters of marked fibres were more frequent (Table 1). Around 70% of muscle regions expressing β-gal contained more than one marked fibre. These findings, together with previous work by us and others using two distinguishable retroviral vectors (Hughes and Blau, 1990; Evans *et al.*, 1994), strongly suggests that clusters of marked cells represent clones derived from individual infected myoblasts that have proliferated and then fused to multiple muscle fibres in their environment. This view is consistent with the decline in the frequency of multi-fibre clusters observed when rats are infected with retroviral vectors at progressively later developmental stages. Injections on postnatal day 5 (P5) or later yield significantly fewer multi-fibre clusters and a lower median and mean cluster size, compared to fetal injections (Table 1). This was the case even though older animals were allowed longer periods of survival before analysis. This result is probably due to the increased rate of terminal differentiation, compared to proliferation, occurring among myoblasts in older animals. Thus, several lines of evidence indicate that the isolated clusters of marked fibres observed in infected fetuses represent clones.

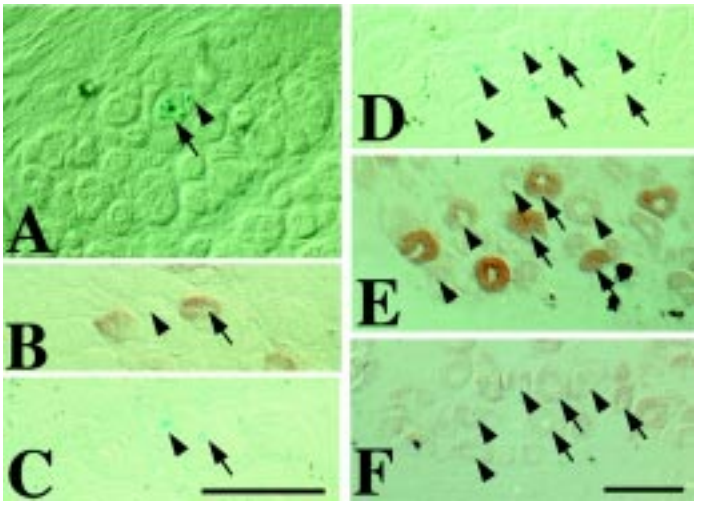

Fig. 2. Clusters of marked fibres contain both primary and secondary fibres and fast and slow fibres. (A) *A single cluster of fibres in the lateral gastrocnemius marked with Xgal, consists of one large fibre with classic primary fibre morphology (arrow) and one immediately adjacent small fibre with nascent secondary fibre morphology (arrowhead). This cluster extended through six sections, contained another secondary fibre and was over 0.5 mm from the next nearest marked muscle cells and, therefore, probably represents two fibres to which a single marked myoblast clone has contributed nuclei through fusion.* **(B,C)** *Another isolated cluster in the biceps femoris muscle consisting of at least two marked fibres (B), one with a primary fibre morphology (arrows) and the other with a smaller maturing secondary fibre (arrowheads). Only the primary fibre expresses slow MyHC reactive with the N2.261 anti-neonatal slow MyHC antibody on the adjacent section (C).* **(D-F)** *A cluster in which several large primary-type fibres are marked in the superficial region of the peroneal muscle (D). Three of these fibres express the N2.261 slow MyHC epitope (arrows, E) whereas another four fibres do not express slow MyHC (arrowheads, E), and no fibres in this superficial region have yet begun to express the N1.551 neonatal fast MyHC epitope (F), although the N1.551 epitope is detected in deeper regions of the same section. Note that several of the marked fibres have the large diameter, centrally-cored morphology of primary fibres. Bars, 50 μm for both A-C and D-F.*

Isolated clusters of marked muscle fibres contain a variety of fibre types. Initial visual inspection showed that, consistent with the results of Evans *et al.* (1994) on fetal mouse muscle, clusters of marked cells contain fibres with both primary and secondary fibre characteristics (Fig. 2A). Primary fibres can be distinguished from secondary fibres by their larger size and 'doughnut' morphology with a central core devoid of myofibrils and frequently containing nuclei. Secondary fibres, by contrast, have a smaller size and are initially located at the periphery of primary fibres. Subsequently, secondary fibres increase in size and separate from the primary fibres to become morphologically indistinguishable from them during early postnatal life. In the E17 rat thigh and calf, primary fibre formation is completed in most muscles but secondary fibre formation is about to be initiated (Condon *et al.*, 1990a). By E21 numerous secondary fibres have been formed, some have separated from primary fibres, but others are still in the early stages of development. Marked cells contribute to both large primary fibres and small secondary fibres (Fig. 2A). An analysis of all isolated

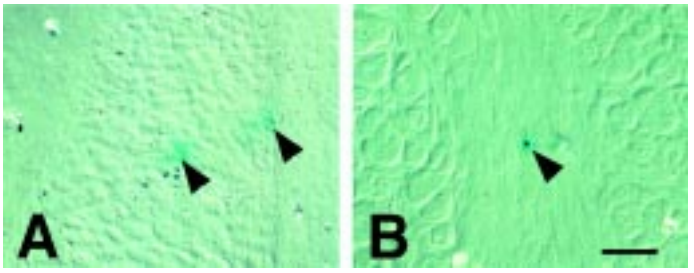

Fig. 3. Retroviral infection of non-muscle cells in fetal rat limbs. *Fetus injected with αSCG at E17 and analysed for β-gal at E21.* **(A)** *Cluster of marked cells within knee cartilage.* **(B)** *An interstitial cell marked in the fascia between soleus and peroneal muscles. Arrowheads indicate blue β-gal reaction product. Bar, 50 μm.*

marked clusters of fibres showed that fusion to fibres with both primary and secondary type morphology was common. Of 53 multi-fibre clusters analysed, 21 (40%) contained fibres of both primary and secondary morphology. This is likely to be an under-estimate because a) ambiguous clusters were scored as containing only a single class of fibre, and b) only clusters in which primary and secondary fibres were marked in the same section were scored as containing both primary and secondary fibres. However, as a two vector analysis of clonality could not be performed (due to the punctate cytoplasmic β-gal), some multi-fibre clusters may not represent clones. Nevertheless, over the period between E17-21, at least some myoblast clones fuse to both primary and secondary fibres.

To confirm that marked cells fuse to both primary and secondary muscle fibres, I distinguished these fibres using monoclonal antibodies to distinct isoforms of MyHC. Primary fibres generally express slow MyHC, whereas secondary fibres in fast muscles do not express this isoform (Condon *et al.*, 1990a). Large slow MyHC-containing fibres were marked in the same cluster as fibres not containing slow MyHC (Fig. 2B,C). Owing to the irregular morphology of fetal muscle and the frequently short length of nascent secondary fibres, it was rarely possible to follow clusters of marked fibres over many serial sections. Hence, it has proved difficult to observe both fast and slow MyHC in the same cluster of labelled cells. Nevertheless, as neonatal fast and slow MyHC-containing fibres are near-reciprocal subsets of fibres in fast muscles at E21 (Condon *et al.*, 1990a; Maggs *et al.*, unpublished results), these data unambiguously demonstrate fusion of myoblasts in clusters to both slow and fast fibres. Whereas clusters of labelled fibres frequently contained large slow and small non-slow fibres, on occasion clusters of marked fibres that contained two large primary fibres one of which expressed slow MyHC and the other of which did not were also observed (Fig. 2D,E). I conclude that it is likely that single myoblast clones can contribute to both primary and secondary fibres and to distinct types of primary fibres present in fetal rat muscle.

Retroviral vectors preferentially mark myogenic cells

The αSCG retroviral vector gave rise to marked non-muscle cell clusters after embryonic injection (Table 1). However, such clones were rare, raising the possibility that the vector was displaying tropism: the tendency to infect particular cell types. To investigate this possibility, rat limbs into which a retroviral vector had been introduced were examined for infection of clones in non-muscle tissues. Infected cells were observed within cartilage, tendon, dermis and in interstitial tissue within the muscle masses or between muscles (e.g., Fig. 3). However, these were rare compared to infection within muscle (Table 2), despite the widespread distribution of the co-injected tattoo dye (Fig. 2B), and the abundant presence of Hoechst 33258-stained nuclei in non-muscle regions (data not shown). Scoring of marked cells in serial sections of a single infected limb demonstrated the high frequency of marked muscle cells and the larger cluster sizes compared to non-muscle clusters (Table 2). We have previously observed rare infected clones of adipocytes within interstitial fat tissue of postnatal rats using a distinct retroviral vector after neonatal infections (S.M. Hughes and H.M. Blau, unpublished observation). However, when postnatal mice were injected with a variety of other Moloney murine leukemia virus-based retroviral vectors, infection of non-muscle cells was observed in neonates, but was not in more mature animals (Table 1), suggesting that susceptibility to infection may drop as non-muscle tissues mature.

Discussion

The data show that early myoblasts that contribute to many, if not all, types of muscle fibres can be infected by retroviral vectors delivered by simple intramuscular injection. Many of these dividing myoblasts go on to generate progeny that drop out of division, terminally differentiate into myocytes and fuse with other myocytes to form myotubes that express the β–gal protein from the inserted retroviral genome. The clonal analysis suggests that single myoblast clones are capable of generating cells that fuse with both primary and secondary fibres during the fetal period. This confirms in younger animals the lack of selectivity of myoblast fusion we

TABLE 2
FREQUENCY OF DISTINCT CLUSTERS OF RETROVIRALLY-MARKED MUSCLE FIBRES IN EMBRYONIC RAT

Tissue	Number of clusters (%)	Number of cells (%)	Cells/cluster
Muscle	**76 (85)**	**407 (95)**	**5.4**
Tendon	**5 (6)**	**9 (2)**	**1.8**
Dermis	**7 (8)**	**9 (2)**	**1.3**
Interstitial	**1 (1)**	**3 (1)**	**3**
Total	**89**	**428**	

Rat fetuses were injected with αSCG retroviral vector at E17 and analysed by Xgal staining of fixed alternate serial sections through the entire hindlimb at E21. Each section was analysed for the presence and location of fibre clusters and the number and nature of each cell containing Xgal reaction product recorded. Marked cells in the same location within the same muscle on adjacent sections were considered to be within the same cluster. It was only rarely possible to follow the same fibre through multiple sections owing to the meandering route taken by fibres, the short length of nascent secondary fibres, the rapid changes in fibre shape and area along their length and the distortion of the unfixed tissue during sectioning. To avoid the possibility of over-estimating cluster size the number of marked fibres was taken as the largest number of marked fibres detected in any single section of that cluster. This approach may lead to under-estimation of the number of fibres in a cluster, although this error is probably small as clusters rarely extended over more than four serial sections.

have previously observed in postnatal rats (Hughes and Blau, 1992) and reported by Evans *et al.* (1994) in fetal mice. Consistent with this view, the data show that single marked fibre clusters contain both large slow primary fibres and small fast secondary fibres. Moreover, most clusters contained at least some marked large primary-type fibres, suggesting that fusion to primary fibres is frequent among the myoblast population present in late fetal rodent limbs. In this type of experiment, the possibility that some myoblast populations might be refractory to retroviral infection can not be ruled out (although I have observed no indication that this is the case from tissue culture infection of myoblasts). Nevertheless, the data suggest that retrovirally-infectable myoblasts fuse at random to fibres in their environment during the late fetal period. Thus, it is likely that at least some clones of myoblasts can contribute to both primary and secondary fibres.

A second striking observation was that clusters of labelled large fibres contain large primary-type fibres some of which do, and others of which do not, express slow MyHC isoforms. So clones do not appear to be restricted to fusion with particular fibre types during late fetal life. This observation also suggests that single clones can fuse to both slow and fast primary muscle fibres, the two major divisions of primary fibres, which are proposed to originate from distinct clonal myoblast populations (Stockdale, 1992). All primary fibres are thought to express slow MyHC at their inception (Kelly and Rubinstein, 1980; Narusawa *et al.*, 1987). Subsequently, some primary fibres lose slow MyHC and express fast MyHCs (Condon *et al.*, 1990a). To avoid the potential criticism that the large slow MyHC-expressing fibres contributed to by these clones were in the process of converting to fast fibres, I used an antibody (N2.261) that we have shown to detect a maturational slow MyHC epitope that is not expressed in primary fibres undergoing conversion to a fast character, but accurately marks slow fibres that are in the process of maturing in future slow muscles and muscle regions (Hughes *et al.*, 1993; Maggs *et al.*, unpublished results). In the study presented here, positive markers of fast fibres were not employed on clones for three reasons. First, all fibres express embryonic fast MyHC and no fibres express adult fast MyHCs before birth in rats, so these markers are uninformative. Second, neonatal fast MyHC is expressed both in slow fibres undergoing transformation into fast and in definitive fast fibres. Third, in superficial regions of muscle (which constitutes the majority) neonatal fast MyHC is still expressed at low levels at birth. So, as all fibres express some form of MyHC, slow MyHC markers provide the most definitive method of distinguishing future-slow from future-fast fibres. Thus, single marked myoblast clones appear to contribute to fibres with the morphological characteristics of primary fibres that express either fast or slow MyHC.

Two distinguishable retroviral vectors expressing cytoplasmic or nuclear β-gal were employed with the aim of determining the frequency with which clusters of infected cells were clones. However, I found that cytoplasmic β-gal was frequently punctate in distribution in fetal rat muscle, raising doubts of the utility of this marker and preventing statistical analysis of clonality. Clusters of labelled cells were, therefore, identified as clones based on their isolated position within the limb and on the demonstration by Evans *et al.* (1994) that such clusters in fetal mouse muscle are clonally-derived. So, in the absence of a formal demonstration of clonality in fetal rat muscle, conclusions based on clusters observed at low frequencies must be regarded as suggestive, rather than definitive.

Although no tendency of selective fusion can be distinguished at the developmental stage examined, the data does not rule out the possibility that at earlier stages, during the formation of primary muscle fibres, there could be some selective fusion processes at work. However, data from *Drosophila* suggest that selective fusion of myoblasts does not generate the homogeneous myofibre phenotypes, but rather that the first differentiating myoblast (the so-called founder cell) determines the gene expression pattern of other nuclei that subsequently become located in the same cytoplasm through myocyte fusion (Rushton *et al.*, 1995). Such a view is consistent with reports that cells artificially fused with muscle to create heterokaryons take on the gene expression of the muscle cytoplasm they enter (Blau *et al.*, 1985), and with our observations from implantation of myoblasts of known character into mice. We showed that the implanted cells take on the character of the fibres with which they fuse (Hughes and Blau, 1992). It is also consistent with the finding that cloned avian myogenic cells can, on occasion, induce ectopic MyHC expression when implanted into very early limb buds, presumably by acting a founder cells (DiMario *et al.*, 1993). On the other hand, our observations with similar transplants using cloned murine fetal myoblasts suggest that there are signals present within the developing chicken limb bud that can induce correct regional MyHC expression patterns in implanted cells, even if those implanted cells fail to fuse with any endogenous myogenic cells, and therefore act as founder cells themselves (Robson and Hughes, unpublished results). In summary, if there is a role for cell lineage as a regulator of muscle fibre type *in vivo*, it would appear to act at the earliest stages of muscle development, possibly by specifying the fate of the founder myoblast that initiates each new fibre, as has been observed in *Drosophila* (Dohrmann *et al.*, 1990). However, whether mammalian homologues of genes known to regulate *Drosophila* founder cell character are differentially expressed within early developing vertebrate myoblasts in unclear, so it is as yet uncertain whether the invertebrate model is appropriate.

The numbers of putative clones that generated multi-fibre, as opposed to single fibre, clusters fell slightly when retrovirus was introduced postnatally, compared to prenatally. Moreover, the average size of labelled fibre clusters decreased. This could be explained if myoblasts in fetal limbs are proliferating more rapidly than other cell types. Retroviruses require nuclear envelope breakdown within a certain period after internalisation for the reverse-transcribed viral genome to integrate into a chromosome and achieve an infection. Once infected, more rapidly dividing cells would be expected to create larger clones in the four day incubation period. Consistent with our previous work (Hughes and Blau, 1990; Blake *et al.*, 1997), we observed lower rates of infection per injected viral particle, and smaller clones in postnatal animals, suggesting that postnatal myoblasts may proliferate less than fetal myoblasts. The large cluster sizes during fetal life could not be accounted for by longer incubation times because fetal rats were only infected four days before analysis as opposed to up to 22 days before analysis in postnatal rats. Nor could viral spread account for the results because self-inactivating vectors were employed. It is striking that the timing of this restriction in the spread of infected cells coincides with the time that new fibre formation is reported to decline (Ontell and Kozeka, 1984; Ontell *et al.*, 1988). This observation suggests that during the formation of new fibres single myoblast clones are free to contribute to several fibres, perhaps to

groups located within a single basal lamina sheath. In contrast, when new fibre formation declines, clones generally become restricted to fusing to one or two fibres. The observation that multi-fibre clusters are more abundant, and larger, in superficial rat fast muscles after postnatal infection (data not shown) is consistent with the possibility that *de novo* fibre formation persists in such regions after birth. Nevertheless, results presented in the current work and previously (Hughes and Blau, 1990, 1992), show that on occasion myoblast clones contribute cells to multiple fibres even in deep early-forming muscle regions. Therefore, even after myoblast proliferation declines, myoblasts appear capable of migrating between seemingly-intact fibres surrounded by a basal lamina sheath.

Implications for retroviral gene therapy

The data presented show that replication-incompetent retroviral vectors can readily be employed to introduce novel gene products into large numbers of muscle fibres in developing rodents. Most β-gal is present within muscle fibres, suggesting that either the retroviral vectors preferentially infect muscle precursor cells, or that these cells are dividing more rapidly and hence are more susceptible to infection (as retroviral vectors can only infect dividing cells) or that the vectors express more highly in muscle cells than in other tissues. Whichever of these is the correct explanation, it raises the possibility that similar vectors could be used to introduce replacements for defective genes in developing human muscle cells. Retroviral infection of fetal muscle cells at an early stage might be an effective way of treating hereditary diseases of muscle, since infection of stem cells populations that may replenish muscle tissue throughout life might be achievable at early stages when stem cell precursors may be proliferating more rapidly than in later life (Cossu *et al.*, 1985). Indeed, this type of treatment might be essential in diseases like Duchenne muscular dystrophy (DMD) in which early cycles of degeneration and regeneration in muscle fibres of the fetus (Webster *et al.*, 1988) can lead to changes in myoblast populations that mimic the normal ageing process (Blau *et al.*, 1983; Webster and Blau, 1990). Regardless of the utility of such procedures in DMD, they may prove useful in therapy for other diseases that are not primarily muscle defects, as we have suggested previously (Dhawan *et al.*, 1992; Hughes and Blau, 1992).

Materials and Methods

Animals

Timed-mated Wistar rats were obtained from Simonsen, CA or bred in King's College London.

Retroviral vector injection

Retroviral vectors αSCG (Dhawan *et al.*, 1992), BAG (Price *et al.*, 1987), MMuLVSVnlsLacZ (Bonnerot *et al.*, 1987) and LnβgeoCX (Blake *et al.*, 1997), were used. All vectors are based on Moloney murine leukemia virus. αSCG is produced from the amphotropic Ψ_{CRIP} packaging cell line, whereas BAG and MMuLVSVnlsLacZ are from Ψ_{CRE} and LnβgeoCX from BOSC23 (Pear *et al.,* 1993) ecotropic producer cells. Although these two classes of packaging cell line produce viruses that utilize distinct cell surface receptors, there is no evidence that the route of infection influences subsequent behaviour of infected cells. Infectious viral particles were prepared as described previously (Hughes and Blau, 1992) and concentrated by either centrifugation or using Vivapor concentrators (LnβgeoCX). Concentrated αSCG viral stock (maximally, around 2000 infective particles/μl) containing polybrene and tattoo dye (to aid identification of successfully injected limbs and the subsequent likely location of infected cells) was injected into rat fetuses after maternal laparotomy. Usually, several embryos in the mid-region of each uterine horn were injected and their position within recorded. The amniotic sack was removed from the uterus and embryos injected with a 33 gauge needle (Hamilton) under back-illumination with a fibre-optic lamp into one hindlimb. After suturing of uterus and body wall, survival of injected embryos was 13/15 across all experiments. Accurate quantification of the number of injected viral particles was impossible due to visible leakage of much of the injected material from the wound, but may have been around 1000 infective particles. Postnatal injections were intramuscular as described previously (Hughes and Blau, 1992).

Analysis of limbs

Mothers were killed by CO_2 inhalation and limbs of virally-injected or control fetuses dissected, frozen and cryo-sectioned with alternately 30 μm and 10 μm sections. Analysis was largely as described (Hughes and Blau, 1992). Initially, 30 μm sections were stained fixed for β-galactosidase (β-gal) activity in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% Nonidet P40 in PBS overnight at 37°C. Once the location of the retrovirally-infected cells was identified, 10 μm sections flanking those containing marked cells were incubated unfixed with anti-MyHC antibodies A4.840, N2.261 (Hughes *et al.*, 1993) or N1.551 (Cho *et al.*, 1994) followed by biotin-conjugated goat anti-mouse IgG and Vectastain ABC reagent (Vector). After developing in diaminobenzidine sections were mounted in gelvatol. The number of Xgal-reactive cells was counted under DIC optics with a 40X objective on a Zeiss Axiophot.

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