Transgenic Research 3, 59–66 (1994)

Tissue specific expression of an α-skeletal actin–*lacZ* fusion gene during development in transgenic mice

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Received 22 February 1993; revised 13 May 1993; accepted 17 May 1993

Transgenic mice carrying a chimaeric transgene containing 730 bp of the 5′-flanking sequences and the entire first intron of the rat α-skeletal actin gene fused to the *lacZ* reporter gene have been produced by microinjection. The *lacZ* reporter gene was used to verify the suitability of using the rat α-actin promoter elements to target expression of genes of agricultural and therapeutic value exclusively to skeletal and heart muscle cells and fibres of transgenic mice. Expression of the transgene indicates a tightly regulated developmental and muscle specific control of the rat α-skeletal actin gene, making it a useful promoter for gene targeting to muscle tissues. The cells destined to form muscle tissues in these transgenic mice are readily visualized in intact embryos by staining for β-galactosidase activity, making them a suitable animal model for studying the origin and development of skeletal and cardiac muscle tissues.

Keywords: α-actin, transgenic mice; gene expression; muscle; embryos; *LacZ*

Introduction

The ability to introduce foreign DNA into the germline of mice has permitted molecular approaches to the understanding of biological processes at the organism level (Palmiter and Brinster, 1986; Jaenisch, 1988). This transgenic technology is a powerful research tool which has a wide variety of practical biological applications. One such useful application is in the area of gene therapy. Gene therapy is a promising new concept based on the assumption that definitive treatment for genetic diseases should be possible by directing treatment to the mutant gene rather than to secondary or pleotropic effects of mutant gene products (Friedman, 1989).

A successful use of transgenic technology in gene therapy experiments requires the selection of a suitable disease model and a tissue or cell specific expression of transgene sequences which wherever possible must be developmentally regulated in a manner resembling their endogenous counterparts (Palmiter and Brinster, 1986). For instance, Duchenne Muscular Dystrophy (DMD) which is an X-chromosome linked recessive disorder and one of the most common lethal genetic diseases of humans (Rojas and Hoffman, 1991; for a review see Worton *et al.*, 1992) has a useful animal model in the form of the *mdx* mutant (Bulfield *et al.*, 1984), making DMD amenable to genetic manipulation experiments. The direct injection of human dystrophin expression plasmid DNA into dystrophin deficient *mdx* mice resulted in dystrophin expression about 5 mm proximal to the site of injection (Acsadi *et al.*, 1991). Other groups have expressed a recombinant DNA based dystrophin minigene in *mdx* mice under the control of a viral promoter (Wells *et al.*, 1992). Their results demonstrated that expression even at low levels was correlated with a reduction in skeletal muscle pathology in young transgenic *mdx* mice. The low level of expression makes a case for using a strong muscle specific eukaryotic promoter such as the rat α-skeletal actin promoter (Shani, 1986) for targeting expression of the dystrophin gene exclusively to the heart and skeletal muscle of *mdx* mice.

The actin family is a group of highly conserved proteins involved in cell motility, cytoskeletal structure and muscle contraction. Six different actins have been identified in vertebrates, including two striated forms which are

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encoded by two closely related genes, α-skeletal and α-cardiac actin (Sassoon *et al.*, 1988). Comparison of the α-skeletal and α-cardiac amino acid sequences reveals only four amino acid substitutions within 375 residues, among the highest conservation found in vertebrate actins (Vandekerckhove and Weber, 1979). Although these two genes are expressed in both cardiac and skeletal striated muscle the relative abundance of each isoform is dependent on the developmental stage of the embryo (Vanderkerckhove *et al.*, 1986).

The mode of expression of the rat α-actin promoter was examined in transgenic mice (Shani, 1988), but the use of chloramphenicol acetyl transferase (CAT) as a reporter gene meant that whole-mount demonstration of expression through the developmental time course was not possible. A recent report (Brennan and Hardeman, 1993), also using CAT as a reporter gene, addressed developmental regulation of the human α-skeletal actin gene. Brennan and Hardeman (1993) however, presented developmental regulation in terms of fibre type maturation from birth to the adult in transgenic mice. We have therefore verified the muscle specificity and developmental regulation of the rat actin promoter in F_1 transgenic mice, in order to assess its suitability for targeting the dystrophin gene (to skeletal and heart muscles of *mdx* mice) and other genes of agricultural value such as insulin-like growth factor I (IGFI) and growth hormone receptor (GHR) genes exclusively to skeletal muscles of mice. The present study extends the work of Shani (1986, 1988) and that of Brennan and Hardeman (1993) in that by using the *lacZ* reporter gene it has been possible to demonstrate expression in whole-mount embryos and follow the developmental profile of the actin promoter visually in embryos.

Materials and methods

Construction of rat α-skeletal actin–lacZ *transgene*

The actin promoter was derived from plasmid pCV (Shani, 1986) which contains the 5′-flanking region and exons I to VI of the rat α-skeletal actin gene fused to the 3′ end of human fetal ε-globin. Briefly this plasmid was digested with *Eco* RI–*Bam* HI to isolate the 3 kb fragment representing the α-actin gene and subcloned into Bluescribe. A *Dra* III–*Bam* HI restriction fragment was replaced with a 17 bp synthetic oligonucleotide linker (Fig. 1). This manipulation deleted the coding sequence for α-skeletal actin (exons II to VI) and the translation initiation signal in exon II. The remaining 1.8 kb *Eco* RI–*Bam* HI fragment contained the 5′-flanking region, exon I, the entire first intron and the 5′-end of exon II. This DNA fragment was sub-cloned into the *Eco* RI–*Bam* HI restriction site of pBluescript II sk^+ (Stratagene), to create the muscle-specific expression vector, pJB5, which is essentially −730intCAT (Shani, 1988), but with the CAT reporter gene substituted for *LacZ*.

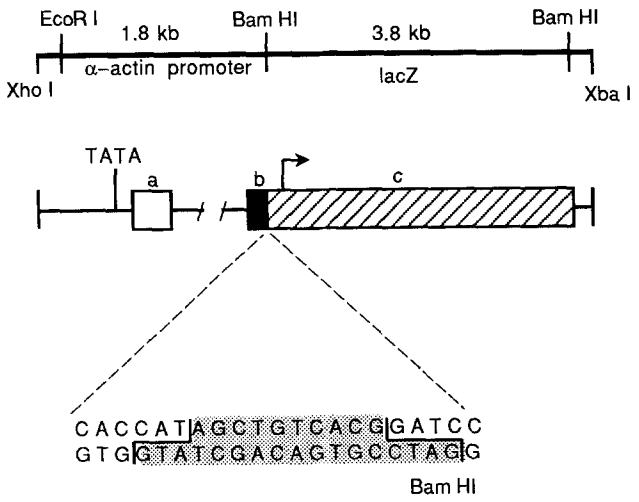

Fig. 1. A schematic representation of the α-skeletal actin–*lacZ* transgene. The 5.6 kb *Xho* I–*Xba* I fragment was excised from its vector sequences and used to generate transgenic mice. (a) untranslated exon I of the rat α-skeletal actin gene (b) 5′-end of exon II excluding the translation initiation site (c) coding sequences of the *LacZ* gene. Arrow marks the translation start site.

A *lacZ* reporter gene derived from pCH110 (Pharmacia-LKB) was used to verify the tissue-specificity of the rat actin promoter. The plasmids pCH110 and pUC8 (BRL) were each digested with *Hin* dIII, ligated together and then cut with *Bam* HI. A 3.8 kb *Bam* HI fragment was isolated and cloned into the *Bam* HI site of pJB5 (Fig. 1). A double digest of *Xba* I–*Xho* I released a 5.6 kb DNA fragment representing the α-skeletal acting-*lacZ* chimaeric gene, which was purified by gel electrophoresis and introduced into pronuclei of fertilized mouse eggs by microinjection.

Production of transgenic mice

F_1 females (C57BL/6 × CBA) were superovulated with 5 IU of pregnant mare serum gonadotropin (PMSG), followed by administration of 5 IU of human chorionic gonadotropin (hCG) 48 h later to induce synchronized ovulation. Females were mated with fertile F_1 males. One cell zygotes were collected and microinjected with the α-skeletal actin–*lacZ* transgene at 1.5 μg ml^{-1}. Injected eggs were cultured overnight and surviving two-cell zygotes were transferred into pseudopregnant recipients (Hogan *et al.*, 1986). The resulting offspring were weaned at 3 weeks of age. Transgenic mice were identified by Southern Blot analysis of high molecular weight DNA prepared from tail biopsies taken at 5 weeks of age. The 3.8 kb *Bam* HI *lacZ* fragment was used as a probe for the α-skeletal actin-*lacZ* transgene.

Estimation of gene copy number

To construct a standard curve, 0–50 genomic equivalents of the α-skeletal actin/*lacZ* DNA were added to 10 μg nontransgenic mouse DNA. These standards and 10 μg of DNA from transgenic mice were digested with 10 units of *Bam* HI (Boehringer) for 16 h at 37°C. The DNA was precipitated with ethanol, separated on 0.9% agarose gel and analysed by Southern Blotting using the 3.8 kb probe described above. Subsequently the filter was washed at high stringency in 0.5 × SSC (1 × SSC: 0.15 M sodium chloride, 13 mM trisodium citrate, pH 7.0) and 0.1% SDS at 65°C for 1 h, and autoradiographed. The radioactive bands were excised and counted in a liquid scintillation counter.

Staining embryos for β-galactosidase activity

Control and hemizygous F_1 embryos were recovered from pregnant females (Hogan *et al.*, 1986) between 6.5 and 17.5 days *post coitum* (*pc*). Midday after detection of a vaginal plug was designated as 0.5 day *pc*. The embryos were washed in phosphate buffered saline (PBS) and fixed in a solution containing 1% paraformaldehyde, 0.25% glutaraldehyde, 0.25% nonidet P-40, 50 mM NaCL in 1 × PBS for 2 h with two changes of fixative. Fixed embryos were washed in 1 × PBS for 30 min and stained at 37°C overnight with gentle agitation, in a solution containing 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal), 20 mM $K_3Fe(CN)_6$ and 20 mM $K_4FE(CN)_6.3H_20$ in 1 × PBS pH 8. The embryos were washed in PBS for 30 min to remove Xgal crystals and then transferred to ethanol glycerol (2:1) for 20 min to fix the blue stain in tissues. They were cleared in PBS glycerol (1:1) and then stored in PBS glycerol (1:9) to clear further before being photographed.

Staining neonates for β-galactosidase activity

A number of pregnancies were allowed to continue to term and the offspring were killed at 1 day, 2 days, 9 days, 3 weeks, 9 weeks and 3 months of age. One and two day old mice were fixed intact for 1 h, skinned and fixed for a further 2 h. Various organs (heart, lung, liver, spleen, kidney, testes and brain) and limbs were dissected from older mice and fixed for 3 h with two changes of fixative. Fixed whole mice and dissected tissues were washed in PBS for 30 min. Skin was subsequently removed from intact mice and limbs and the specimens were stained for β-galactosidase activity. The dissection of older mice and skinning was to facilitate penetration of fixative and staining solution.

Histological analysis

Embryos and neonatal organs for histological analysis were transferred to PBS to wash off excess glycerol, then dehydrated in increasing strengths of ethanol for 2 h each in a Tissue Processor. Embryos and tissues were embedded in paraffin wax, 4 μm thick sections cut and counterstained with eosin.

Results

Production of actin–lacZ transgenic mice

To verify the muscle-specific expression of the rat α-skeletal actin promoter mouse lines were created that carry hybrid α-skeletal rat actin-*lacZ* transgene. A 5.6 kb *Xba* I–*Xho* I DNA fragment containing the 5′ region of the rat α-skeletal actin gene fused to the *lacZ* reporter gene (Fig. 1) was microinjected into pronuclei of fertilized mouse eggs and cultured overnight to the two cell stage. A total of 43 mice were born out of 254 two-cell stage embryos transferred to pseudopregnant recipient females. Of these, 12 founder animals were identified as being transgenic by Southern transfer analysis of DNA prepared from tail biopsies (data not shown). Determination of transgene copy numbers showed a range from 1 to 40 copies (Table 1). Only nine putative transgenic founder mice were tested for transmission of the transgene by hemizygous backcross to F_1 mice (C57/BL6 × CBA).

One out of the nine founder animals tested failed to transmit the transgene to its offspring, and also did not express *lacZ* in adult tissue. Of the three remaining founder animals not tested for transmission, one AL31, was used directly to assay for transgene expression in adult tissues. The other two, AL18 and AL41 were not mated nor analysed for expression. Expression of the transgene was measured by Xgal staining of embryonic or adult tissues. On the basis of this criterion five out of the 10 founder lines (including AL31) tested were found to express the *lacZ* gene. Though the levels of *lacZ* expres-

Table 1. Genomic copy number and relative levels of *LacZ* expression in transgenic mice

Mouse (AL)	*Sex*	*Copy number*	LacZ *Expression*
2	M	1	+
6	M	5	+
21	F	1	+
22	F	2	+++
31[a]	M	1	+
7[b]	M	1	−
18[c]	M	1	ND[d]
23	F	2	−
24	F	40	−
25	F	2	−
26	M	27	−
41[c]	M	2	ND[d]

[a]AL31 was used directly for expression studies in adult tissues.
[b]AL7 did not transmit transgene and therefore not analysed further.
[c]AL18 and 41 were only analysed for integration.
[d]Not determined.

sion varied, the temporal and spatial patterns of expression were constant between and within lines. Transgenic line AL2 was selected as a representative line and the pattern of expression and developmental profile of the α-skeletal actin promoter in embryos from this line is described in detail below.

Expression of lacZ *in embryonic tissues*

The pattern of expression and developmental profile of the α-skeletal actin-*lacZ* transgene in whole-mount embryos are presented in Fig. 2. Although embryos were examined from 6.5 days *pc* *lacZ* expression is first detected at 8.5 days *pc* predominantly in the heart and also the cranial region (Fig. 2a). At 9.5 days *pc* (Fig. 2b) expression in the heart persists but becomes reduced and remains patchy throughout prenatal development (Fig. 3b). The neck region begins to stain and remains high until 12.5 days *pc* after which it disperses (Fig. 2e).

Segmentation of the paraxial mesoderm to form somites becomes apparent at 9.5 days *pc* and at 10.5 days (Fig. 2c) *pc* more pronounced staining of the somites is seen particularly in the anterior and mid trunk regions. By 11.5 days *pc* (Fig. 2d) the somites become more discrete (separated by unstained intersomitic bands) and differentiate around a central cavity with the appearance of the myotomes. The myotomes are the first skeletal muscles to form in the embryo and they develop in a rostrocaudal gradient (Lyons *et al.*, 1991). The limb buds which are visible by 9.5 days *pc* do not express *lacZ* until 12.5 days *pc* (Fig. 2e) when expression of the trunk myotomes becomes diffuse and progressively reduced in a rostrocaudal direction. Facial muscles stain prominently at this stage and by 13.5 days *pc* (Fig. 2f) the discrete expression in the head and neck regions has been replaced by diffuse staining of mature muscle fibres in the face and neck. The discrete staining of myotomes along the entire length of the longitudinal axis have also been replaced by expression in the limbs, flank and intercostal musculature.

The expression pattern in the 15.5 days *pc* (Fig. 2g) and older embryos (data not shown) is similar to that seen at 13.5 days *pc*, but with progressive increase in staining of individual muscle fibres of the limbs.

Expression of lacZ *in neonatal tissues*

Tissues were analysed for *lacZ* expression at 1 day, 2 days, 9 days, 3 weeks, 9 weeks and 3 months of age. Expression of *lacZ* was detectable in all skeletal muscles of 1 day old mice. In particular, expression in the limb muscles peaked at this stage (Fig. 4b). Sectioning of the hind limb shown in Fig. 4b reveals that *lacZ* staining is confined to skeletal muscle fibres with no staining of bone (Fig. 3a). The extent of *lacZ* staining in a head and two hearts of 1 day old mice are presented in Fig. 4a and Fig. 4c respectively. Expression in the head is confined to muscles such as the masseter and pre-muscle tissues. The two hearts are presented to show consistency of patchy expression which became apparent in the early embryonic stages.

Evisceration and sectioning to score internal organs and the brain did not reveal staining in organs other than the heart which showed patchy expression (data not shown). From postnatal day 2 onwards β-galactosidase activity in the limbs and heart declined drastically, and by 9 days old *lacZ* staining in the heart was scarcely detectable (data not shown). At 3 weeks of age β-galactosidase activity in both the heart and limbs is still visible to the naked eye but greatly reduced. From 9 weeks old onwards patchy Xgal staining of the leg muscle fibres could only be seen from wax sections (data not shown).

Discussion

The present study has confirmed that 1.8 kb of 5′ flanking region of the rat α-skeletal actin promoter contains sufficient sequence information for muscle specific expression *in vivo* and extends the results of previous *in vivo* studies (Shani, 1986, 1988). By using the *Eschericha coli* *lacZ* reporter gene instead of CAT used by Shani (1988) it has been possible to follow visually the temporal and spatial expression pattern of α-skeletal actin both during embryonic and postnatal mouse development, without the need to homogenize specific tissues for CAT assays. This is particularly important in early embryonic stages where cell populations have not yet differentiated into specific organs.

The *lacZ* gene offers the advantage of visualizing expression in specific organs such as the heart relative to other parts of the intact embryo. This whole mount analysis of actin gene expression (and any gene for that matter) is not possible with CAT assays.

The pattern of *lacZ* expression in five independent transgenic lines was qualitatively similar and restricted to the heart, skeletal muscles and muscle precursor cells in embryos. Shani (1988) demonstrated that although 145 bp of the 5′ flanking region of the rat α-skeletal actin gene was sufficient to permit preferential expression in striated muscle, low levels of CAT activity were also found in non-myogenic tissues such as the liver, spleen, thymus, brain and lung. By using a construct containing exon 1 and the first intron of the rat α-skeletal actin promoter however a more uniform and restricted expression was observed in striated muscles. Our results support those of Shani (1988) that the first intron probably contains the requisite elements for strict muscle specific expression. It has been suggested that intron sequences may be important for active chromosome structure (Brinster *et al.*, 1988) and that splicing may be associated with an increased efficiency of polyadenylation and transport to the cytoplasm (Huang *et al.*, 1990).

The *lacZ* expression seen in the cranial and neck regions between 8.5 and 13.5 days *pc* would at first sight

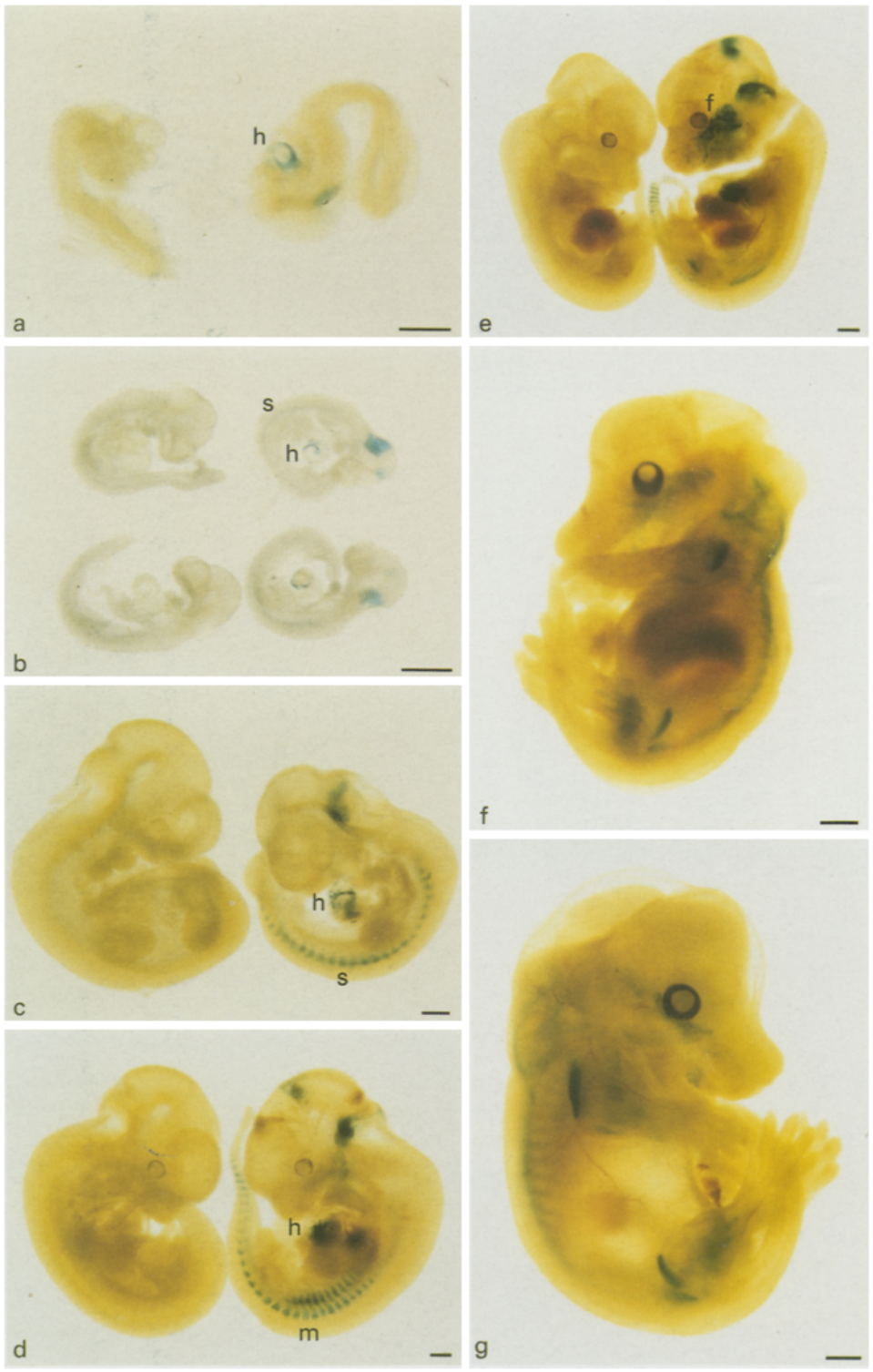

Fig. 2. Temporal and spatial expression of the α-skeletal actin–*lacZ* transgene in mouse embryos. Transgenic and nontransgenic control embryos were stained for β-galactosidase activity as described in the Materials and methods section. Pictures of transgenic whole mounts and their nontransgenic controls: (**a**) 8.5 days *pc*; (**b**) 9.5 days *pc*; (**c**) 10.5 days *pc*; (**d**) 11.5 days *pc*; (**e**) 12.5 days *pc*; (**f**) 13.5 days *pc*; (**g**) 15.5 days *pc*. (h) heart (s) somites (m) myotomal muscle (f) facial muscle. (Scale bar = 1 mm).

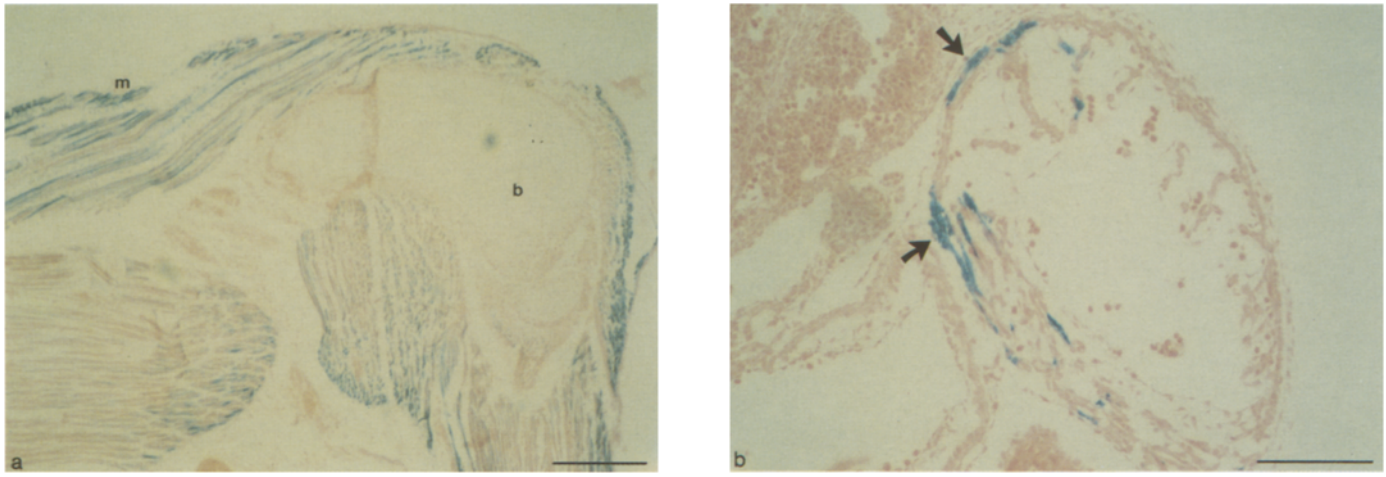

Fig. 3.

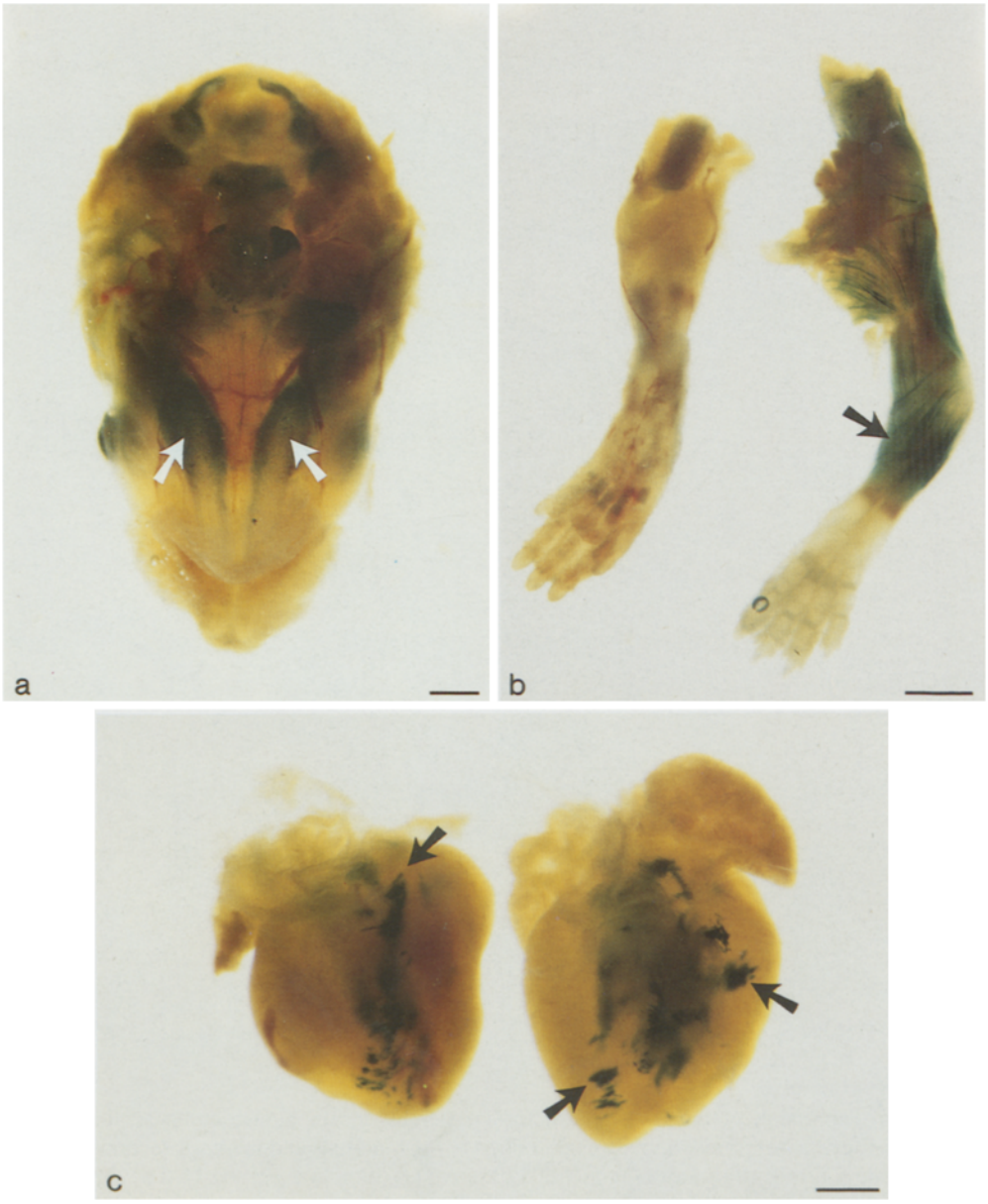

Fig. 4.

appear to be non-specific or leaky expression. But this pattern of staining was too consistent in all embryos examined to be discounted. This transient *lacZ* expression in the embryonic head region in the present study corresponds to cranial nerve regions III, IV and V (Noden and Lahunta, 1985) which are associated with extrinsic muscles of the eye, face and head regions and may be in early muscle precursor cells destined to contribute to the musculature of the face and head. Somitomeres were first discovered in the head of the chick embryo (Meir, 1979), with the use of stereo scanning electron microscopy. They first appear in the head, and the cranial somitomeres do not become somites, but disperse to contribute to the head the same products that the myotomes, sclerotomes and dermatomes of the somites make in the trunk region (Noden, 1983). In the mouse embryo there are seven head somitomeres that do not form somites and their relationships to head parts are the same as in the chick embryo (Meir and Tam, 1982). In the present study, these cranial somitomeres, like the myotomes in the trunk and caudal regions, are no longer seen as discretely staining cells once they have given rise to appropriate muscle tissues by day 13.5 *pc*.

The patchy expression in the heart seen in the present study is consistent with the observation by Sassoon *et al.* (1988) that in the embryonic heart α-skeletal actin is often expressed in clusters of cells or patches not obviously associated with particular structures in the heart.

The longitudinal section of a hind limb (Fig. 3a) reveals a heterogeneous staining of the muscle fibres within the thigh and leg muscles although all muscle fibres should have identical genotypes. Such mosaicism in *lacZ* staining has been found in transgenic tissues (Kothary *et al.*, 1989) and tissue culture cells (MacGregor *et al.*, 1987). A possible technical problem of penetration of the staining solution to inner fibres, cannot adequately explain why non-staining fibres lie in close proximity to deeply staining fibres (Fig. 3a). One possible explanation for our observations could be that as muscle fibres mature they no longer actively synthesize actin proteins and may therefore be switched off or down-regulated. The mosaicism may therefore reflect different phases of muscle development or actin protein synthesis. Such a hypothesis might be investigated by studying *lacZ* expression in regenerating adult muscle of actin-*lacZ* transgenic mice following mechanical damage.

Cells expressing the α-skeletal actin-*lacZ* transgene are identifiable by Xgal staining very early in development before they are committed to any particular muscle type, thus making it feasible to follow the fate of these cell types. These include the myotomal cells in the somites destined to form much of the skeletal muscle of the embryo, and the presegmented cells in the head region associated with cranial nerves III, IV and V (Noden and Lahunta, 1985) whose target tissues include muscles of the head such as the temporalis and masseter. Since α-actin proteins and mRNAs have been co-localized to the same embryonic mouse muscle tissue types (Lyons *et al.*, 1991) this system can be used to predict the localization of α-actin proteins. In mature muscle it could also be used to determine which cells express α-skeletal actin during muscle growth and regeneration. The expression of the transgene in the heart during early development may also prove useful in studies of hypertrophy of the heart in which a greater than 10-fold increase in α-skeletal actin is found in the right ventricle (Bakerman *et al.*, 1990).

Thus the present data confirm that the sequences of the rat α-skeletal actin promoter used in the construct can target expression of structural genes of agricultural and therapeutic value exclusively to muscle cells to provide invaluable physiological information on such genes.

Acknowledgements

We gratefully acknowledge the gift of plasmid pCV from Dr U. Nudel. We wish to thank Dr C. Gribbin for helpful advice on β-galactosidase assays, Dr P. Simons and R. Wallace for advice on transgenic techniques and D. Parnham and his staff for maintaining the mice. We also wish to thank R.K. Field and N. Russel for help with photography and Drs C. Goddard and D.J. Wells for their helpful comments on the manuscript. This work was supported in part by a grant from the Muscular Dystrophy Group (UK) awarded to G. Bulfield.

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Fig. 3. Expression of the *lacZ* transgene (scale bar = 100 μm). *pc* embryonic heart, showing patchy staining (←). (**a**) Longitudinal section of hind limb from a postnatal day 1 transgenic mouse. This section indicates *lacZ* expression in the muscle fibres (m) compared to lack of expression in bone (b). (**b**) Longitudinal section of the 10.5 day *pc* embryonic heart, showing patchy staining (←). (Scale bar = 100 μm).

Fig. 4. Expression of the *lacZ* transgene in postnatal day 1 mice. Transgenic and nontransgenic control neonates were stained for β-galactosidase activity as described in the Materials and methods section. (**a**)Parasagittal view of the head of a transgenic neonate, note staining of the masseter muscles (←); (**b**) Hind limb of a transgenic and nontransgenic neonate, note staining of muscle fibres (←); (**c**) hearts from two transgenic neonates, note the patchy staining (←). (Scale bar = 1 mm).

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