

Control of the expression of the *Mrf4* and *Myf5* genes: a BAC transgenic approach

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ABSTRACT The muscle-specific transcription factors *Mrf4* and *Myf5* are two of the four myogenic regulatory factors (MRFs) involved in the transcriptional cascade responsible for skeletal myogenesis in the vertebrate embryo. *Myf5* is the first MRF to be expressed in mouse and avian embryos. We have previously described discrete enhancers driving *Myf5* expression in epaxial and hypaxial somites, branchial arches and central nervous system, and argued that additional elements are required for proper expression (Summerbell *et al.*, 2000). We have investigated the transcriptional regulation of these two linked genes using bacterial artificial chromosome (BAC) transgenesis. We show that sequences up to 140 kb upstream of *Myf5* are involved in the transcription of both genes and that these elements direct expression at different times and anatomical locations through development. In addition, we show that regulation of *Mrf4* requires at least four different elements one of which may be shared with *Myf5*.

In order to characterise all the regulatory elements involved in the regulation of *Mrf4* and *Myf5* expression, we have isolated a series of overlapping bacterial artificial chromosomes (BACs) that constitute a *de facto* 5' deletion series of the region. Homologous recombination in *E. coli* was used to introduce an *nlacZ* reporter cassette into *Myf5* (BAC-Z constructs) or both *alkaline phosphatase* (AP) and *nlacZ* into *Mrf4* and *Myf5*, respectively (BAC-APZ constructs). BAC modifications were performed as previously described (Carvajal *et al.*, 2001).

Results

Generation of transgenic mice using BAC clones

In order to identify potential long range regulatory elements for the *Mrf4* and *Myf5* genes, six overlapping BAC clones containing the locus were isolated from a murine BAC library (Research Genetics Ltd., USA). Clones were named according to the sequence length (in kb) upstream of the cap-site of the *Myf5* gene: BAC195, BAC140, BAC88, BAC81, BAC61 and BAC59. Using the same convention we refer to construct #1 of Summerbell *et al.* (2000), containing 8.8 kb of upstream sequences, as p8.8Z.

Identification of new *Myf5* regulatory elements

Transgene expression in BAC195Z, BAC59Z and p8.8Z lines starts before 8.5 dpc (Fig. 1 A-C). BAC59Z lines show that sequences 59kb upstream of *Myf5* repress the atypical dermomyotomal expression seen in p8.8Z lines (Fig. 1 E,F). BAC195Z is also able to drive correct expression in ventral dermomyotome (Fig. 1D). BAC59Z lines show expression in ventral myotome but not in ventral dermomyotome. Differences in dermomyotome expression are maintained through 10.5 dpc and later (Fig. 1 G-I). By 10.5 dpc

transgenic lines for both BAC clones show fore-limb expression and at 11.5 dpc (Fig. 1 J-L) both limbs are expressing the transgene. While at 12.5 dpc BAC lines equally maintain limb expression, note the difference in intensity at the intercostal level (Fig. 1 M-O). Differences in axial maintenance are clearer at later stages (Fig. 1 P-T).

BAC140 contains all the elements required for full *Myf5* expression

In order to delimit the additional elements identified above, we generated transgenic lines using the remaining BAC constructs. Deletion from BAC195APZ to BAC140APZ did not change gene expression between 8.5 and 14.5 dpc (e.g. Fig. 2 A,B), indicating that there are no additional elements in this 55 kb interval. At 10.5 dpc, BAC88Z (Fig. 2C), BAC81APZ (Fig. 2D) and BAC59APZ lines showed similar expression patterns in thoracic somites, while only BAC88Z lines consistently express in first and second branchial arches. BAC59Z does not drive expression in the hypoglossal cord. At later stages expression was maintained in BAC88Z lines (Fig. 2E) and downregulated in BAC81APZ lines (Fig. 2F).

Generation of double reporter-gene BAC constructs

We have modified the BAC-Z clones by the introduction of the *alkaline phosphatase* (AP) reporter gene into the *Mrf4* gene (BAC-APZ clones), and thus we are able to analyse the expression pattern of both reporter genes in the same genomic context and in the same embryo. Fig. 3A shows the double expression pattern in thoracic somites in a BAC59APZ embryo at 10.0 dpc (32 somites). The *Myf5* reporter marked the nuclei of the myotomal cells, aligned at the centre of the myoblasts, while the *Mrf4* reporter stained the surrounding membrane, occupying the entire width of the myotome.

Identification of additional elements driving *Mrf4* expression

Mrf4 expression was first detected in all BAC lines around 9.0 dpc. The expression pattern in BAC88APZ, BAC81APZ and BAC59APZ lines (Fig. 3D) is consistent with published *in situ* data (Bober *et al.*, 1991; Hinterberger *et al.*, 1991). An 8.5 kb fragment immediately upstream of the rat *Mrf4* gene contains all the elements required for the second wave of *Mrf4* expression but only a subset of the first wave restricted to the thoracic somites (Pin *et al.*, 1997). *Mrf4* expression in the BAC lines was not restricted to the thoracic somites, indicating that two elements are required for the first wave of expression. BAC195APZ and BAC140APZ lines revealed a new domain of ventral expression in thoracic somites (22-25 somite stage, Fig. 3 B,C). At the 38-44 somite stage transgene expression in the thorax extends more ventrally in BAC195APZ (Fig. 3E) or BAC140APZ lines than in other BAC lines (Fig. 3F).

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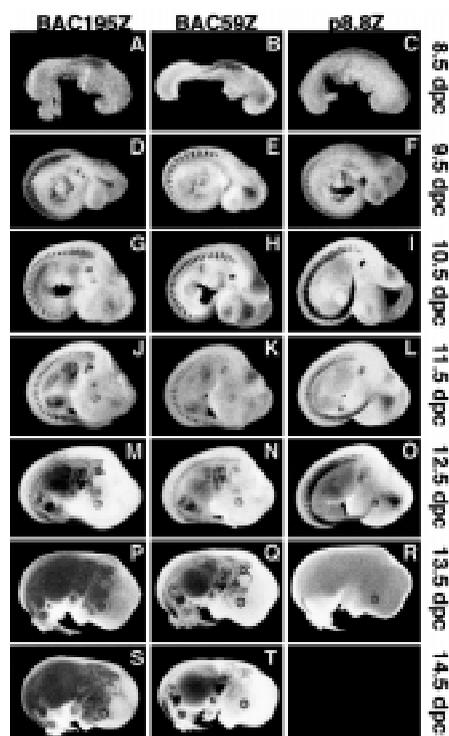


Fig. 1. Time course of *Myf5* transgene expression in BAC195Z, BAC59Z and p8.8Z lines.

Conclusions

Four distal regions are involved in the regulation of *Myf5*

The -58.6 kb to -8.8 kb region directs expression in the limbs, suppresses the atypical dermomyotomal expression and blocks the suppression of second arch expression seen in p8.8Z lines (Summerbell *et al.*, 2000); the -81.0 kb to -58.6 kb region directs expression in the hypoglossal cord; the -88.2 kb to -81.0 kb region drives expression in the first arch and is responsible for the maintenance of expression; the -140 kb to -88.2 kb region directs ventral expression from 9.5 dpc in the dermomyotome of thoracic somites. Similar results have been obtained by Hadchouel *et al.* (2000) using a YAC-based approach.

Four regions are required to recapitulate the pattern of *Mrf4* expression

The -15.3 kb to -8.8 kb region drives limb and the second wave of expression (Patapoutian *et al.*, 1993); the -17.3 kb to -15.3 kb region (Pin *et al.*, 1997) drives early expression in the myotome of thoracic somites; the -58.6 kb to -17.3 kb region drives the remainder of the somitic expression; the -140 kb to -58.6 kb region is required for *Mrf4* expression at the most ventral part of thoracic somites.

Are control elements shared between the two genes?

At the ventral domain, expression of *Mrf4* and *Myf5* seem to coincide in time and the regions involved in regulating this expression overlap. It is thus possible that a single element controls the expression of the two genes. The two genes are linked in all species

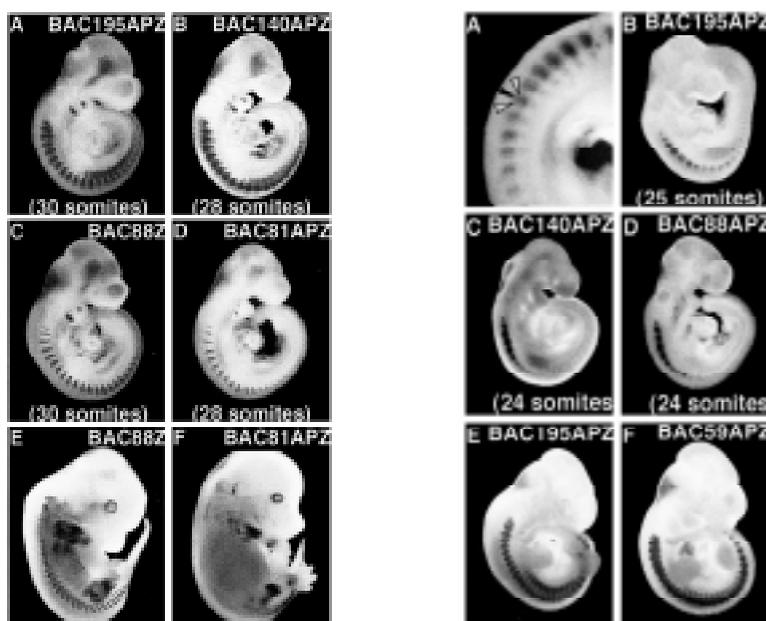


Fig. 2. (Middle) Expression patterns in the BAC deletion series. *Myf5* expression at 10.0 dpc in BAC195APZ (A), BAC140APZ (B), BAC88Z (C) and BAC81 (D). BAC88Z lines (E) lack expression in the ventral half of tail somites. BAC81APZ lines fail to maintain expression (F).

Fig. 3. (Right) Analysis of *Mrf4* expression using double reporter gene constructs. Doubly stained 10.0 dpc embryo from a BAC59APZ line (A) *Mrf4*, grey arrows; *Myf5*, black arrow. *Mrf4* expression at 9.5 dpc in BAC195APZ (B), BAC140APZ (C) and BAC88APZ lines (D). *Mrf4* expression at 11.5 dpc in BAC195APZ (E) and BAC59APZ (F) lines.

analysed and may have been maintained by co-regulation of this aspect of the genes' expression. The coincident activation of the two genes in this domain suggests that *Mrf4* may not be functionally downstream of *Myf5* and that in this case it might act earlier in myogenesis than previously thought.

References

- BOBER, E., LYONS, G. E., BRAUN, T., COSSU, G., BUCKINGHAM, M. and ARNOLD, H. H. (1991). The muscle regulatory gene, *Myf-6*, has a biphasic pattern of expression during early mouse development. *J Cell Biol* 113: 1255–1265.
- CARVAJAL, J. J., COX, D., SUMMERBELL, D. and RIGBY, P. W. J. (2001). A BAC transgenic analysis of the *Mrf4/Myf5* locus reveals interdigitated elements which control activation and maintenance of gene expression during muscle development. *Development* 128: 1857–1868.
- HADCHOUËL, J., TAJBAKSH, S., PRIMIG, M., CHANG, T. H., DAUBAS, P., ROCAN COURT, D. and BUCKINGHAM, M. (2000). Modular long-range regulation of *Myf5* reveals unexpected heterogeneity between skeletal muscles in the mouse embryo. *Development* 127: 4455–4467.
- HINTERBERGER, T. J., SASSOON, D. A., RHODES, S. J. and KONIECZNY, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev Biol* 147: 144–156.
- PATAPOUTIAN, A., MINER, J. H., LYONS, G. E. and WOLD, B. (1993). Isolated sequences from the linked *Myf-5* and MRF4 genes drive distinct patterns of muscle-specific expression in transgenic mice. *Development* 118: 61–69.
- PIN, C. L., LUDOLPH, D. C., COOPER, S. T., KLOCKE, B. J., MERLIE, J. P. and KONIECZNY, S. F. (1997). Distal regulatory elements control MRF4 gene expression in early and late myogenic cell populations. *Dev Dyn* 208: 299–312.
- SUMMERBELL, D., ASHBY, P. R., COUTELLE, O., COX, D., YEE, S.-P. and RIGBY, P. W. J. (2000). The expression of *Myf5* in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors. *Development* 127: 3745–3757.