Characterization of embryonic muscle development using twist in situ hybridizations in Manduca sexta

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Introduction

In insects there are two major periods of muscle development; embryonic and adult muscle development. In the moth Manduca sexta, adult muscles develop into two major types; body wall and visceral muscles (Bayline et al., 1998; Champlin et al., 1998). While some muscles require innervation for myoblast accumulation and proliferation, others do not (Bayline et al., 1998; 2001; Gardner and Bayline, 2005). Embryonic muscle development has not been characterized.

During both adult and embryonic muscle development, the muscle precursors are derived from mesodermal cells. Invertebrate mesoderm development is partially coordinated by the activity of the gene twist. This gene is present in mesoderm forming cells until late stages of development (Sommer and Tautz, 1994) Riboprobes for the twist gene in M. sexta would allow for the observation and identification of myoblast pools that will later develop into muscles. By studying the different genes involved in muscle development could help to determine how these muscles develop.

Isolating twist gene

Two rounds of Round PCR were used to isolate the Twist gene that would be used to make the probe. The sequence of twist was 600-700 base pairs long. The race PCR was run twice to increase the concentration of the twist. This slightly decreased the length of the sequence. Columns 2-7 contained the forward reaction, and 3&7 were used in the production of riboprobes. 8-10 and 2-5 contained the reverse reaction. 9 was eventually used in the production of the ribo probe.

Probes

The ribo probes were made using DIG labeled RNA. They were hydolyzed for final in situ. Ribo probes were hydolyzed in water using sodium carconate solution. This allowed a greatly increased signal, by allowing increased binding of the probe in the staining, while not increasing the background. Anti-sence strands are run in coulmn 2&3. Sense strands are shown in coulmn 4&5. The hydrolysed antisense strand is in coulmn 6 and the sence strand is in 7. The hydrolysis greatly increased the signal of the staining. Without hydrolysis embryos appeared much like D in figure 2.

Procedure

Using homology with Drosophila, a 700 bp sequence of twist was isolated from M. sexta (Bayline personal communication). The flow chart depicts the steps used to develop riboprobes, and carry out in situ hybridization for M. sexta embryos. The staining should characterize patterns of development for different muscles in the M. sexta embryo.

1) Isolate embryonic M. sexta RNA
2) Make cDNA from RNA
3) Design Twist PCR probes
4) Isolate Twist gene in M. sexta using RACE
5) Clone PCR fragment into plasmid vector
6) Sequence Twist
7) Make riboprobes
8) Perform in situ

Green steps performed in Lisa Nagy lab by Ben Daggett

cDNA production

RNA was isolated from 100-200 M.sexta embryos. The RNA was transcribed to DNA using enzyme reverse transcriptase. coulmn 1 has 2000 bp DNA ladder. Coulmns 3-5 have cDNA molecules. The cDNA sequences are aproximatly 600-700 bp long.

Probe Creation

1) Isolating embryos:
   Three techniques were tried to isolate embryos for obtaining embryos from eggs:
   all three first washed the embryos in 1:1 bleach manduca saline
   1. Direct dissection
   2. fix in 4% paraformaldehyde; dissect in 10% methanol
   3. fix in 4% paraformaldehyde; agitate in 1:1 heptane:PEMFA for 20 min: shake in 100% methanol till free
   The first technique produced optimum results. By providing more intact embryos.
2) Pretreatment
   1. rehydrate embryos in PBST; transfer to TEA; treat embryos with acetic anhydride (10 µl; 4ml TEA) for 20 min; return to PBST
   2.treat embryos with proteinase K; fix in 4% paraformaldehyde; transfer to PBST
   The first technique produced far lower background by blocking indoginous alkaline phosphotases. Before this procedure the young embryos were destroyed by the proteinase K, and all of the embryos appeared black for excessive staining.
3) Hybridization:
   It was performed at two successful temperatures: 50 & 60 deg C
   Embryos were washed 3*10 min in Blocking solution Hybridized over night in 1:2000 Dig-AP antibody: Hybridization buffer with block

Staining results:

M. sexta staining produced clear stains. The expression of twist was similar to that found in other insects. The central line shows the enzymatic color staining, and the difference can be seen between the day 2 embryos (figure 1) and the day 1 embryos (figure 2).

Discussion

The successful in situ hybridization will allow the identification of myoblast pools that will later develop into muscles. The staining in the day 1 and 2 embryos was found to be similar to staining found in other insects at a similar stage in development. This will be useful in the study of muscle development in both the embryonic and adult stage muscles. The characterization of twist expression using in situ hybridization can potentially be continued into day 3 and 4 embryos. Additionally the expression of other genes can be examined in this way. One of the major genes that will be studied is Dmufound (Du). This gene is active in founder cells of muscles (Bayles, Bate, and Gomez 1998). This could be a useful gene in the studying of a certain type of muscle found in Manduca sexta. The muscle does not form in the normal pattern of development. Instead of fusion competent cells massing around a myoblast and fusing to growing in a bundle certain muscles of M. sexta grow in sheet like patterns (Gardner and Baline). By studying the different genes involved in muscle development could help to determine how these muscles develop.

References


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