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A Screen for Genetic Loci on the X Chromosome Required for Body-Wall Muscle Development during Embryogenesis in *Caenorhabditis elegans*

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We have screened available chromosomal deficiencies on the X chromosome for genetic loci whose zygotic expression is required for body-wall muscle development during embryogenesis in *Caenorhabditis elegans*. Previously, it had been reported that no sign of muscle development was detected in nullo-X embryos arrested at an early stage of embryogenesis. Based on this observation, it has been suggested that genetic loci exist on the X chromosome whose zygotic expression is essential for body-wall muscle formation. In order to identify such myogenic loci, 9 chromosomal deficiencies covering approximately 45% of the X chromosome have been tested. Homozygous embryos from these deficiency strains were collected and terminal phenotypes of arrested embryos were observed by Nomarski microscopy. As a secondary assay, monoclonal antibodies against two myosin heavy chain (MHC) isoforms, the products of the *myo-3* and *unc-54* genes, were used to detect body-wall muscle differentiation. All the homozygous deficiency embryos were positively stained with both MHC antibodies and muscle twitching movement was observed in most cases. Combined with previously analyzed deficiencies, our deficiency screen has covered approximately 70% of the X chromosome. We conclude that the regions covered by the available deficiencies on the X chromosome do not include any myogenic locus required for body-wall muscle formation. Alternatively, the possibility that nullo-X embryo may not form body-wall muscle due to a general failure to differentiate during embryogenesis remains to be tested.

The nematode *Caenorhabditis elegans* is an excellent genetic model system to study muscle development. Its powerful genetics in combination with cell biological techniques have provided new insights into developmental processes during embryogenesis. In *C. elegans*, there are two major muscle types: pharyngeal muscles are used for grinding food and body-wall muscles are used for locomotion (Waterston, 1988). Body-wall muscles of *C. elegans* are similar to skeletal muscles of vertebrates in their ultrastructure (Epstein et al., 1974; Waterston, 1988) and they also show similar expression patterns of myogenic transcription factors during development (Chen et al., 1992).

Studying muscle development in *C. elegans* has several advantages. First, embryogenesis occurs rapidly over 14 h at 22℃ and can be observed under the differential interference contrast (DIC or Nomarski) microscope. Second, the complete cell lineage during embryogenesis has been determined (Sulston et al., 1983). In other words, all the body-wall muscle cells can be traced back to early blastomeres. Third, intensive genetic studies have accumulated a large number of mutations including chromosomal deficiencies. Taking advantage of these features, we have chosen a genetic approach which utilizes existing chromosomal deficiencies to identify genetic loci whose zygotic expression is required for myogenesis. Similar approaches have been used to examine zygotic requirements during embryogenesis in *C. elegans* (Storfer-Glazer and Wood, 1994) as well as to identify genetic loci specific for cellularization in *Drosophila* (Merrill et al., 1988; Wieschaus and Sweeton, 1988). In a previous study, 77 deficiencies were screened (Ahnn and Fire, 1994), resulting in the identification of two chromosomal regions that show distinct defects in myosin heavy chain (MHC) gene expression. In addition, nullo-X embryos (the entire X chromosome deleted due to meiotic chromosomal disjunction, Hodgkin et al., 1979) exhibited no signs of muscle development. This finding led us to a search for additional loci on the X chromosome which might be required for body-wall muscle development. Additional

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9 chromosomal deficiencies have become available since the previous screen, which increased the total coverage of the X chromosome to approximately 70%. We have tested these deficiencies by observing terminal phenotypes in homozygous deficiency embryos and by staining with antibodies against MHCs of body-wall muscle. Here, we report results from screening new chromosomal deficiencies on the X chromosome.

Materials and Methods

Mutant strains and maintenance

The strains used in this study were obtained from the *C. elegans* genetic center (CGC) and are listed in Table 1. Breeding *C. elegans* (Bristol) was carried out according to Brenner (1974) and all strains including wild type (N2) were grown at 20℃. Since the homozygous deficiencies used in this study are embryonic lethal, strains were maintained as heterozygous hermaphrodites.

Genetic manipulation of deficiency strains

Five deficiency strains (SP266, SP268, SP271, SP273, and SP395) are carrying a duplication of the X chromosome. For example, SP271 [*mnDp1* (X; V))/+V; *mnDf10* X] has a deficiency (*mnDf10*) on the X chromosome and is constructed to be maintained in the presence of a complementing duplication gene *mnDp1*. These strains were propagated by picking heterozygous hermaphrodites. Self fertilization of these hermaphrodites should give rise to embryos homozygous for the deficiency.

The second group of strains (TY2137, TY2138) has a free duplication (*yDp13, yDp15*) which carries a *him* (high incidence of males) mutation (Akerib and Meyer, 1994). These strains produce a large number of dead embryos due to the loss of free duplication.

Translocation (*szT1*), which has a dominant *him* mutation and a *lon-2* (*e678*) mutation, was used as a balancer for the deletion in TY562 (*+/szT1[lon-2(e678)]*II; *unc-32(e189)*III; *yDf3/szT1* X). Because of this translocation, a large fraction of dead embryos arrested at various stages were produced. In order to analyze homozygous embryos for the deficiency (*yDf3*), *unc-10 (ad591)* males were crossed into this strain. In the F1 generation, animals showing uncoordinated phenotype, which are hemizygous for the *unc-10* gene due to the deficiency *yDf3,* were picked and we were able to obtain homozygous *yDf3* embryos in the F2 generation.

Collection of deficiency homozygous embryos

Ten to fifteen adult hermaphrodites were transferred to a fresh plate and allowed to lay eggs for 10 to 12 h. After removal of the hermaphrodites, the total number of embryos was counted and these embryos were allowed to hatch for 14 to 16 h at 20℃. Then the number of unhatched embryos was counted in order to calculate the percentage of arrested embryos. The arrested embryos were collected by mouth pipetting with pulled-out capillaries or by washing with EN buffer [100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA)] for further analysis.

Terminal phenotype analysis

Embryos were mounted on 2% agarose padded slides with 10-20 μl of EN buffer. Embryos were observed by Nomarski microscopy (or differential interference contrast) (Nomarski, 1955) and photographed at room temperature. Arrested embryos were examined for morphogenesis of pharynx and twitching behavior.

Immunofluorescent staining

Two monoclonal antibodies, 5-6 and 5-8, were used as primary antibodies for immunostaining. Antibody 5-6 recognizes Myo A (*myo-3* gene product) and 5-8 recognizes Myo B (*unc-54* gene product) (Miller et al., 1983). Both Myo A and Myo B are MHC isoforms specific for body-wall muscle (Epstein and Thomson, 1974). The *myo-3* and *unc-54* genes have been mapped to the middle of chromosome V and the right arm of chromosome I, respectively.

Immunostaining methods were modified from the previous procedures (Priess and Hirsh, 1986; Albertson, 1985; Ahnn and Fire, 1994). Embryos on the agar plates were washed with EN buffer and then transferred with capillary pipette to gelatin or polylysine coated slides. Embryos were permeablized by freeze-cracking and fixed in -20℃ methanol for 3 min. After serial rehydration in 75%, 50% and 25% methanol for 2 min each and washing with Tween TBS buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.8, 0.1% Tween 20), samples

Table 1. Deficiency strains of *C. elegans* used in this study

Deficiency	Strain	Genotype	Reference
mnDf7	SP268	*mnDp1*(X; V)/+V; *mnDf7* X	Meneely and Herman (1979)
mnDf5	SP266	*mnDp1*(X; V)/+V; *mnDf5* X	Meneely and Herman (1979)
mnDf10	SP271	*mnDp1*(X; V)/+V; *mnDf10* X	Meneely and Herman (1979)
mnDf13	SP273	*mnDp1*(X; V)/+V; *mnDf13* X	Meneely and Herman (1979)
mnDf42	SP395	*mnDp1*(X; V)/+V; *mnDf42* X	Meneely and Herman (1979)
meDf6	TY2137	*meDf6* X; *yDp13* (X; f)	Akerib and Meyer (1994)
	TY2138	*meDf6* X; *yDp15* (X; f)	Akerib and Meyer (1994)
yDf3	TY562	*+/szT1[lon-2(e678)]* I; *unc-32(e189)*III; *yDf3/szT1* X	Meyer (personal communication)
nDf19	MT1401	*+/szT1[lon-2(e678)]* I; *nDf19/szT1* X	Ambros and Horvitz (1984)
stDf5	RW2551	*stDp2(X; II)/+II; stDf5* X	Hodgkin (1987)

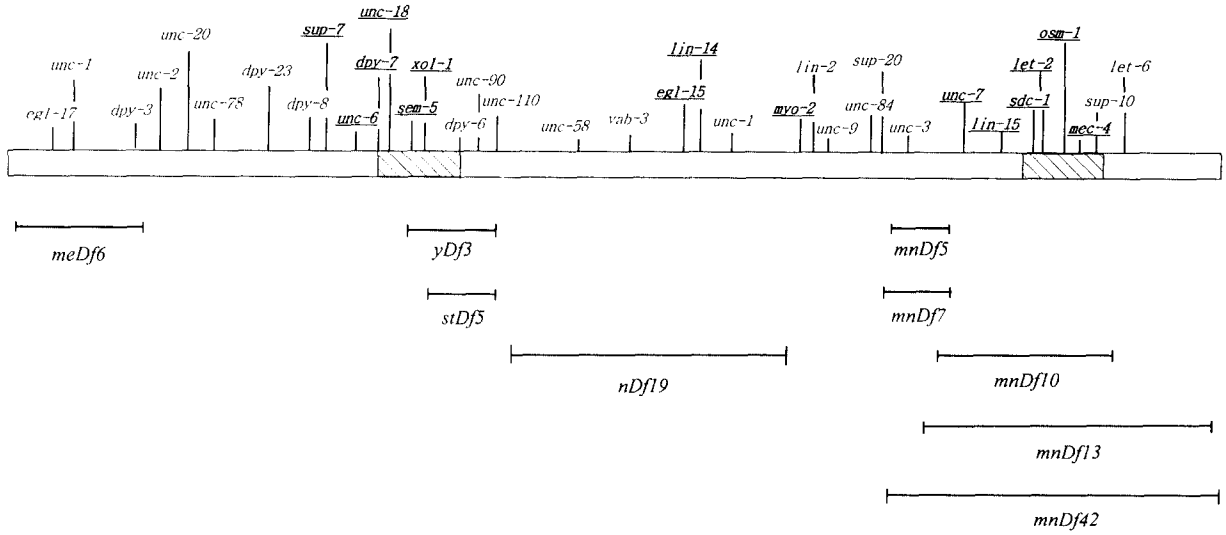

Fig. 1. Deficiency map of the X chromosome in *C. elegans*. A horizontal open bar indicates the X chromosome with selected genetic markers. Two shaded boxes show gene cluster regions. Below the X chromosome, the deleted regions of the 9 deficiencies are shown. Genetic loci appearing on both genetic and physical maps are underlined in boldface.

were incubated with primary antibodies for 5 to 6 h. After washing 3 times with TBS buffer, samples were treated with secondary antibodies (anti-mouse goat IgG antibodies conjugated with fluorescein purchased from Organon Teknika Co.) containing 1 μg/μl of diamidinophenolindole (DAPI) for nucleus staining.

When mounted on a slide, mounting medium (80% glycerol, 1% N-propyl gallate) was used to minimize photobleaching. Staining was observed with fluorescent microscope Olympus BX50.

Results

Locations and extent of deficiencies on the X chromosome

Extensive genetic studies of *C. elegans* have accumulated several hundred chromosomal deficiencies (J. Hodgkin, R. Durbin, and S. Martinelli, personal communication). Close to 40 deficiencies have been mapped to the X chromosome. In the previous study, homozygous embryos of five deficiencies and nullo-X embryos were analyzed for their terminal phenotypes (Ahnn and Fire, 1994). We have chosen additional 9 deficiencies for this study.

The exact locations and the extent of the deficiencies used in this study are shown in Fig. 1. This deficiency map was drawn based on the physical and genetic map of *C. elegans* (Coulson et al., 1986, 1991; A, Coulson, Y. Lutterbach, R. Shownkeen, J. Sulton and R. H. Waterston, personal communication). Physical mapping data (Coulson et al., 1986, 1991) were used to approximate distances among the chromosomal markers shown in Fig. 1. The end points of each deficiency are based on the genetic mapping data. The simplest interpretation of our deficiency screen is that a deleted region would represent myogenic loci if defects in muscle formation be found in homozygous embryos from that particular deficiency.

Nullo-X embryos which lost the whole X chromosome due to meiotic disjunction, displayed no signs of muscle development (Ahnn and Fire, 1994). This can be interpreted in two ways. First, a myogenic locus might be located on the X chromosome and could be identified by a deficiency screen. Second, since nullo-X embryos arrested in early embryogenesis, the lack of muscle development could be due to a general failure to differentiate.

In order to identify possible myogenic loci on the X chromosome, additional 9 chromosomal deficiencies shown in Fig. 1 have been analyzed. Terminal phenotypes in homozygous embryos were observed by Nomarski microscopy and muscle formation was assayed by antibody staining. The extent of this screen was calculated from the approximate regions deleted by these 9 deficiencies. Approximately 45% of the X chromosome have been covered by this screen and, combined with the previous study, a total of about 70% of the X chromosome have been examined.

Analyses of arrested embryo phenotypes

Homozygous embryos from each deficiency strain were obtained as described in Materials and Methods. To observe the phenotypes of arrested embryo, the following developmental aspects during embryogenesis were considered: a. degree of morphogenesis, b. extent of differentiation, and c. tissue formation.

a. Degree of morphogenesis: Embryogenesis of *C. elegans* occurs rapidly and can be divided into three

Table 2. Terminal phenotypes and antibody staining results

Class[a]	Deficiency		Terminal phenotypes[c]			Antibody staining[d]	
	Df	Embryos unhatched (%)[b]	Morphogenesis	Muscle twitching	Pharynx formation	5-6	5-8
1	*mnDf7*	370/1470(25.0)	2-fold	+	+	+	+
	mnDf10	546/2244(24.3)	2-fold	+	+	+	+
2	*mnDf5*	782/2629(29.7)	Before comma	-	-	+	+
			3-fold	+	+	+	+
	mnDf13	833/2036(40.9)[e]	Before comma	-	-	+	+
			1.5-fold	+	+	+	+
	mnDf42	707/1985(35.6)	Before comma	-	-	+	+
			1.5-fold	-	+	+	+
	nDf19	311/465(66.0)[f]	Before comma	-	-	+	+
			Comma to 1.5-fold	+	+	+	+
3	*meDf6 (TY2137)*	1283/2040(62.9)[g]	Before comma	-	-	+	+
			Comma to 2-fold	+	+	+	+
			3-fold	+	+	+	+
	meDf6 (TY2138)	2121/3335(63.6)	Before comma	+	-	+	+
			Comma to 2-fold	+	+	+	+
			3-fold	+	+	+	+
	yDf3	1382/2420(57.1)[h]	Before comma	-	-	-	-[i]
			1.5 to 2-fold	+	+	+	+
			3-fold	+	+	+	+
	stDf5	229/2164(10.6)	Before comma	+	-	+	+
			Comma to 1.5-fold	+	+	+	+
			3-fold	+	+	+	+

a. Deficiencies are grouped into three classes based on their arrest phenotypes.
b. Percent unhatched shows the actual numbers counted (number of unhatched embryo/total number of embryos examined) and the percentage. A fraction of 25% deficiency homozygote is expected except where noted. In most cases a somewhat larger number of arrested embryos was observed, due presumably to deleterious effects of the heterozygous deficiency and genetic markers used.
c. Terminal phenotypes of each deficiency were scored for morphogenesis, muscle twitching and pharynx formation. Morphogenesis was scored as the stage of the arrested embryos. Muscle twitching and pharynx formation were scored either as positive "+" or negative "-", based on Nomarski observation of deficiency embryos. Examination of each deficiency was carried out multiple times and consistent results were observed in all deficiencies analyzed.
d. Antibody staining: antibody 5-6 and 5-8 were used to assess body- wall muscle differentiation (see Materials and Methods). Staining was examined under a fluorescent microscope and scored as positive "+" or negative "-". In all cases except one (see below "i") embryos showed positive staining with both antibodies.
e. More than 25% of the arrested embryos were observed. We can not definitely rule out the possibility that the smaller class of embryos (1.5-fold) actually represent the homozygous deficiency.
f. Due to a translocation in this strain a larger number of arrested embryos was observed.
g. Two different strains were used to study the *meDf6* deficiency. A very similar fraction of arrested embryos were observed, which is comparable to the frequency of a duplication loss.
h. Due to a background translocation a larger number of arrested embryos was observed.
i. A small number of early arrested embryos failed to stain with both antibodies (see Discussion).

stages (Wood, 1988). First, from the first cleavage to the 28-cell stage, founder cells and embryonic axes are established. Second, from the 28-cell stage to the 550-cell stage, most of cell proliferation and cell division takes place. The last stage begins with a constriction of the embryo to form characteristic shapes such as "comma" or "two-fold". During this last stage, most of the morphogenesis and differentiation are completed. For our purpose, we scored the arrested stages as "before comma", "comma", "1.5-fold", "2-fold", and "3-fold" stages (Table 2). Embryos arrested before the comma stage fail to undergo any significant morphogenesis.

b. Extent of differentiation: Since we were interested in muscle formation in the arrested embryos, any indication of muscle differentiation was carefully examined. The best indication of muscle differentiation during embryogenesis is the twitching movement of the embryo. In a wild type embryo, this twitching movement can be observed only after the formation of functional body-wall muscle or pharyngeal muscles.

c. Tissue formation: Signs of differentiation can also be found by observing tissue formation during embryogenesis. In particular, pharyngeal tissues and hypodermal tissues are relatively easy to examine. Therefore, we scored pharyngeal tissue formation as another criterion for tissue differentiation.

Results of these phenotype analyses are summarized in Table 2. Based on the complexity of arrested stages, deficiencies were grouped into three classes. The deficiencies *mnDf7* and *mnDf10* belong to class 1: Embryos of these two deficiencies arrested at the 2-fold stage as shown in Fig. 2B. These embryos showed both muscle twitching movement and the formation of pharyngeal structures as summarized in Table 2. Class 2 includes the deficiencies *mnDf5*, *mnDf13*, *mnDf42* and *nDf19*. Embryos from this group arrested at two different stages. A representative *mnDf13* embryo arrested at about 1.5-fold stage is shown in Fig. 2C and its phenotypes are summarized in Table 2. The third class contains the *meDf6* and *yDf3* deficiencies, which give rise to multiple arrest phenotypes. Phenotypes of each arrested group are summarized in Table 2. Deficiency of *meDf6* was analyzed in two different strains (TY2137

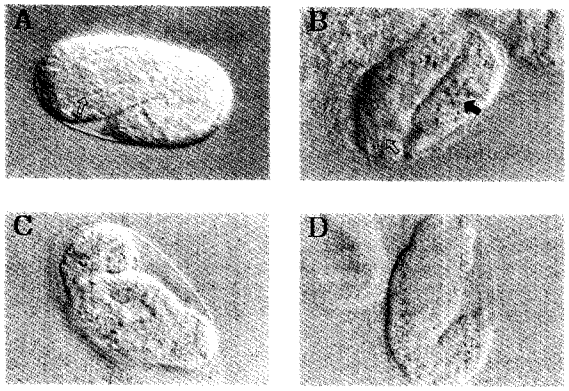

Fig. 2. Nomarski images of wild type (N2) and arrested deficiency embryos. A, A wild-type embryo developing at 1.5-fold stage. This image shows the separation of basal membrane to form a pharynx in the head region (⇩). B, *mnDf10* embryo arrested at the 2-fold representing class 1: Pharyngeal structures (⇩) and rough gut granules (⬆) are identified. C, *mnDf13* embryo of class 2 arrested at the 1.5-fold stage. Gut granules and programmed cell death (button-shape structures) can be recognized. D, *meDf6* embryo of class 3 arrested at the 1.5-fold stage.

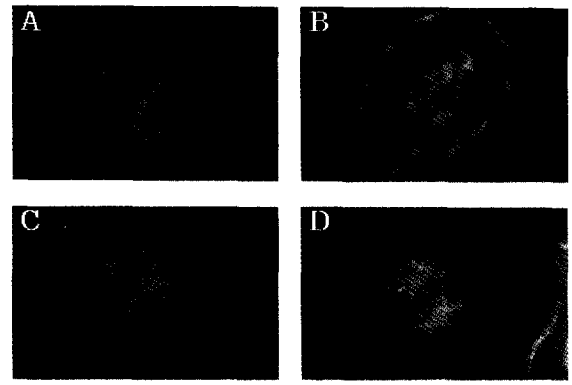

Fig. 3. Antibody staining of wild type and deficiency embryos. Embryos were stained with antibody 5-8 which recognize the *unc-54* gene products. A, Wild type (N2) embryo showing muscle cell staining along dorsal and ventral body lines. B, *mnDf10* embryo showing very similar staining patterns as in the wild type embryo. C, *mnDf42* embryo arrested before comma stage showing disorganized pattern. D, *meDf6* embryo arrested before comma stage showing muscle fibers, but disorganized pattern.

and TY2138). Very similar, if not identical, phenotypes were observed except that occasional twitching movement was seen only in TY2138 (Table 2). An embryo representing *meDf6* arrested at 1.5-fold stage is shown in Fig. 2D.

Antibody staining of homozygous deficiency embryos

In order to detect any muscle differentiation, which may not be observed by Nomarski microscopy (Nomarski 1955), embryos were stained with muscle specific antibodies. Two monoclonal antibodies (5-6 and 5-8) against body-wall myosin heavy chain isoforms (*myo-3* gene and *unc-54* gene products, respectively) were used to stain deficiency embryos as described in the Materials and Methods. Results from the antibody staining are summarized in Table 2.

Embryos from all 9 different deficiencies were positively stained with both antibodies indicating that they expressed myosins specific for body-wall muscle. Embryos representing each group of deficiencies are shown in Fig. 3. In wild type embryos, myosin heavy chains are expressed after cell differentiation begins (Miller et al., 1983). The expression of body-wall muscle specific myosin is only detected in body-wall muscle cells. The timing of myosin expression corresponds to the stages where embryos begin to elongate to become a comma shape embryo (Priess and Thomson, 1987). A wild type embryo stained with myosins in body-wall muscle cells is shown in Fig. 3A. In some deficiencies, exemplified by *mnDf10* (Fig. 3B), the myosin staining pattern looked quite similar to that of wild type (Fig. 3A). In contrast, other deficiency embryos showed disorganized and misoriented staining patterns (Fig. 3C, D). While the majority of *yDf3* embryos were positively stained, a small fraction of embryos which arrested before the comma stage failed to be stained with both antibodies (Table 2). Our interpretation is that the genetic background of this strain may result in some fraction of aneuploid embryos. The possible genotype of these embryos is discussed in detail in the Discussion.

Discussion

Genetic screening of nine deficiencies on the X chromosome

Several approaches are available to identify genetic loci whose zygotic expression is required for myogenesis. The approach that we have taken in this study utilizes existing chromosomal deficiencies to assay zygotic requirements during embryogenesis. In *C. elegans*, this approach is especially useful because many general metabolic components are present in the oocyte and are encoded by the maternal genome. Thus, relatively large genetic regions can be deleted with only minor effects on the events of embryogenesis. Another advantage of this approach is that the genome can be searched in a systematic and comprehensive way. The regions that have been screened once can be excluded from the following screens. Therefore, the data we have accumulated in this study will be able to contribute to the future mapping of myogenic loci on the X chromosome.

We have analyzed nine deficiencies in order to search for genetic loci on the X chromosome that are required for myogenesis. We obtained homozygous deficiency embryos through genetic manipulations from heterozygous animals. The morphogenesis of arrested embryos, especially the degree of differentiation of body-wall and pharyngeal muscle was examined by microscopic observation. For better assessment of body-wall muscle formation, muscle specific monoclonal antibodies were used. As summarized in Table 2, all the embryos from nine different deficiencies expressed muscle specific genes, which indicates muscle cell differentiation. In some strains, higher frequency of

lethality and various levels of morphogenesis were observed due to their complex genetic background. Therefore, it was difficult to assign which types of arrested embryos indeed resulted from homozygous deficiency. Nevertheless, robust staining of body-wall myosin was observed in all types of embryos from every deficiency except for *yDf3*.

In the case of *yDf3*, embryos arrested before the comma stage failed to be stained with either antibodies. These embryos might arrest before the comma stage due to an aneuploidity caused by *szT1* or simply because they are *yDf3* homozygotes. In order to separate *yDf3* from the *szT1* background, *unc-10* males were crossed in and arrested embryos from the F2 generation were analyzed. Although we were able to reduce the frequency of aneuploidity caused by *szT1*, we could not obtain embryos completely devoid of the *szT1* background. For this reason, we analyzed another small deficiency (*stDf5*) which deletes most of the regions covered by *yDf3*. These *stDf5* homozygous embryos also arrested at various stages but all of them were stained with antibody 5-6 (Table 2). This result suggests that the subset of *yDf3* embryos which were not stained is probably *szT1* aneuploid in their genotype. Two additional reasoning support our interpretation that these unstained embryos could be aneuploid caused by *szT1*. First, it is difficult to comprehend how the relatively small region deleted by the *yDf3* deficiency causes such severe lethal effects as seen in embryos arrested before the comma stage. Second, *szT1* is a translocation in which almost the entire X chromosome has been translocated to the left half of chromosome I. Therefore, it is most likely that *szT1* aneuploidity may cause the defects of early arresting. However, this needs to be proven by more elaborate genetic studies.

Is there any genetic locus on the X-chromosome required for myogenesis during embryogenesis?

In the previous study, nullo-X embryos showed severe muscle defects. Two possibilities were suggested. An important gene required for myogenesis could be located on the X chromosome. Alternatively, nullo-X embryos may lack muscle due to a general failure of differentiation. More than 30 different deficiencies on the X chromosome have been isolated and characterized (J. Hodgkin, R. Durbin, and S. Martinelli, personal communication). Five deficiencies had been characterized in the previous study and additional nine deficiencies were analyzed in this study. Together, these fourteen deficiencies cover approximately 70% of the X chromosome. Homozygous embryos for the deficiencies that were analyzed so far showed positive staining with muscle specific antibodies. We conclude that the regions covered by these deficiencies on the X chromosome do not include any loci essential for proper myogenesis. The remaining regions will be tested when other deficiencies become available. The possibility that nullo-X embryos could not form body-wall muscle because of a general failure in embryogenesis still remains until all the regions of the X chromosome are screened.

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