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Temporal reiteration of a precise gene expression pattern during nematode development

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The nematode *Caenorhabditis elegans* is contained within a multifunctional exoskeleton, the cuticle, that contains a large number of distinct collagens. As the nematode proceeds from the egg through four larval stages to the adult, transition between larval stages is marked by synthesis of a new cuticle and subsequent moulting of the old one. This is a cyclically repeated developmental event, frequently described as the moulting cycle. We have examined the temporal expression of a group of six genes encoding distinct cuticular collagens. As expected, mRNA abundance for each of the six genes tested is found to oscillate, peaking once during each larval stage. Unexpectedly, the periods of abundance for each gene do not coincide, different genes being expressed at different times relative to one another within the moulting cycle. We detect a programme of temporally distinct waves of collagen gene expression, the precise pattern of which is repeated during each of the four larval stages. This multiphasic pattern of oscillating cuticular collagen gene expression indicates an unexpected complexity of temporal control during the nematode moulting cycle and has implications for collagen trimerization and cuticle synthesis.

Keywords: *Caenorhabditis elegans*/gene regulation/moulting/oscillation

Introduction

The nematode cuticle is a complex multilayered structure which consists predominantly of a group of small collagen-like proteins that are encoded by the cuticular collagen multigene family (Cox *et al.*, 1984; Kramer, 1994a; Johnstone, 1994). In *Caenorhabditis elegans* this family has in excess of 50 members, the predicted encoded proteins of which share many features of primary structure, but do have differences that relate to function (Kramer, 1994b; Johnstone, 1994). In the wild-type worm a cuticle is synthesized five times during the life cycle, once in the egg prior to hatch and once during each of the four larval stages (Figure 1). Although the cuticles of different stages clearly have fundamental similarity, implying a common regulatory mechanism controlling their synthesis, they are not identical. They are morphologically and biochemically distinguishable, presumably relating to specializations on the basic plan of the cuticle advantageous to specific life cycle stages (Cox *et al.*, 1981a,b; Politz and Edgar, 1995; Ouazana and Herbage, 1995). This stage-specific specialization is also reflected in the pattern of hypodermal cell divisions that precede each cuticle synthesis, the precise pattern of divisions being different at different stages (Sulston and Horvitz, 1977; Ambros and Horvitz, 1984; Ambros, 1989).

Nematode cuticle synthesis is a phenomenon that is repeated cyclically during nematode development and occurs in discrete temporal compartments, known as the moulting cycle, which is the time from the synthesis of one cuticle to the synthesis of the next. During post-embryonic development this corresponds to a larval stage. The hypodermis, the tissue responsible for synthesizing the cuticle, secretes a new cuticle underneath the existing cuticle during a developmental phase termed lethargus, which occurs at the end of each moulting cycle, just prior to the moult and emergence of the next larval stage (Singh and Sulston, 1981). To investigate the similarity of each post-embryonic cuticle synthetic event, and hence each moulting cycle, we examined the precise temporal patterns of expression of a representative group of six of the cuticular collagen genes (Kramer *et al.*, 1982, 1988; von Mende *et al.*, 1988; Cox *et al.*, 1989; Park and Kramer, 1990; Johnstone *et al.*, 1992) through the four post-embryonic cuticle synthetic phases.

Results

Measuring relative transcript abundance during nematode development

Highly synchronous post-embryonic cultures of *C.elegans* can be generated by preparing embryos from gravid adults (Sulston and Hodgkin, 1988). The embryos are subsequently hatched in the absence of food, which causes development to arrest early in the L1 stage. Synchronous cultures are generated from populations of arrested L1 larvae. RNA was prepared from a synchronous culture of nematodes, samples being taken at 2 hourly intervals from shortly after hatch to young adult (Figure 1). Each of the L1 and L2 time points consisted of ~10 000 animals, the L3 and L4 of ~5000 and the adult of ~2500. Reverse transcriptase PCR (RT–PCR) (Kawasaki and Wang, 1989) was performed on the samples to measure the abundance of various RNA transcripts. The method of quantification relies upon the ability to amplify two independent gene transcripts simultaneously in each reaction. As the control transcript we used the *C.elegans* gene *ama-1*, which encodes RNA polymerase II (Bird and Riddle, 1989). The data we present here using this method (Figure 1), and other investigations also using *ama-1* as a control (Larminie and Johnstone, 1996), empirically support the choice of this gene as a control and indicate that during normal development in *C.elegans* the post-embryonic mRNA abundance of this gene is not subject to sufficient fluctuation to interfere with its use as a reference transcript. The

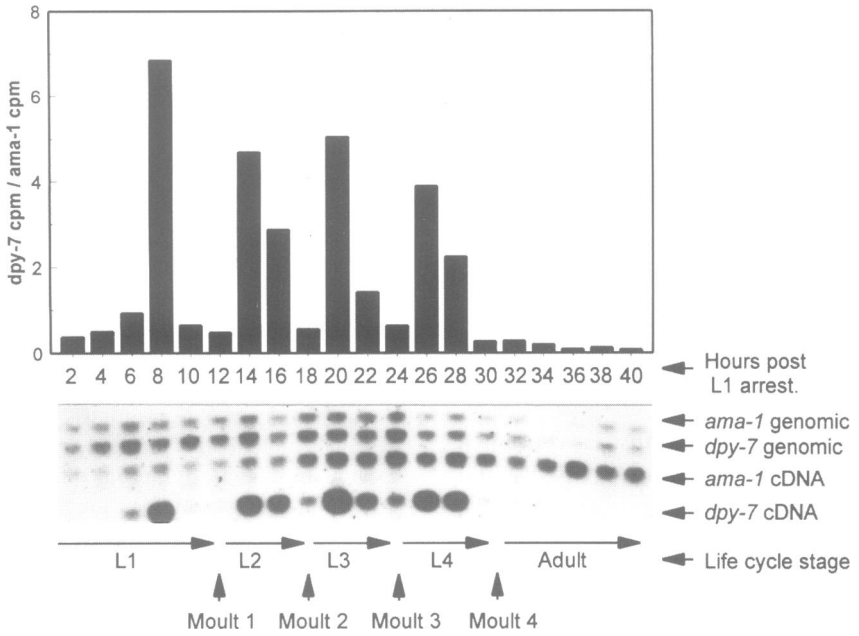

Fig. 1. The results of semi-quantitative RT–PCR for the cuticular collagen gene *dpy-7*, performed on a time course of RNA samples taken at 2 hourly intervals from early L1 to young adult. The *x*-axis of the graph indicates time in hours of development of the sampled worms post L1 larval arrest. The life cycle stage and timing of the moults relating to this time course are indicated at the bottom. The *y*-axis indicates the ratio of detected *dpy-7* mRNA over control *ama-1* mRNA. The graph was derived from the Southern blot presented below. The position on the blot of bands corresponding to amplified cDNA and genomic DNA for both *ama-1* and *dpy-7* are indicated. A peak of *dpy-7* mRNA abundance (relative to *ama-1*) is detected during each of the four larval stages.

abundance of the test transcript is expressed as a ratio of the amount of its amplified product to that from the control transcript. Such relative measurement means the assay is independent of the total amount of cDNA in each reaction, and hence RNA in each sample.

Post-embryonic oscillations in mRNA abundance of the cuticular collagen gene dpy-7

Fluctuations in transcript abundance of the cuticular collagen gene *dpy-7* were measured through the L1 to adult developmental time course (Figure 1). The oligonucleotides used for PCR amplification of the *dpy-7* and control *ama-1* cDNAs (and for all other genes tested) were designed to span an intron permitting product amplified from cDNA to be distinguished from that amplified from contaminating genomic DNA. Four separate peaks of mRNA abundance of the *dpy-7* transcript are detected, one peak during each intermoult period (Figure 1). Contrary to what we expected, the peaks in abundance do not coincide with lethargus and moulting, when the new cuticles are deposited, but are much earlier in the intermoult period (Figure 1). Since translation can occur only in the presence of transcript, we presume that *dpy-7* collagen is also synthesized early in the intermoult period, during the period when its mRNA is abundant.

Multiple overlapping temporal waves of collagen gene expression

Fluctuations in the abundance of transcripts from an additional five cuticular collagen genes, *sqt-1*, *col-1*, *dpy-13*, *col-14* and *col-12*, were measured throughout the L1 larva to adult developmental time course. Graphs generated from the processed data for all of these genes together with that for *dpy-7* are given in Figure 2. The abundance of transcripts for each of the six cuticular collagen genes tested was found to oscillate, peaking once during each larval stage. However, the timing of expression of these genes is distinct from that described above for *dpy-7*, and from each other (Figure 2). Rather than being expressed simultaneously, different genes are expressed at different times relative to one another within the cycle. The abundance of the *col-12* transcript peaks at the end of each larval stage during a lethargus (Figure 2c), the time when the new cuticle is secreted and deposited under the old (Singh and Sulston, 1981). Conversely, and as described above, the *dpy-7* transcript peaks early during each larval stage (Figure 2a), ~4 h prior to lethargus and the expression of *col-12*. Indeed, *col-12* and *dpy-7* are effectively expressed in anti-cyclical fashion to one another (Figure 2d). *col-14* is expressed slightly earlier than *col-12* (Figure 2c). The three genes *sqt-1*, *col-1* and *dpy-13* have very similar patterns to one another (Figure 2e), peaking after *dpy-7* (Figure 2a) and before *col-14* (Figure 2b). Figure 2f shows two independent sets of data for the gene *sqt-1*, indicating the reproducibility of the method with respect to measuring the timing of expression. For the six genes tested, the overall pattern is reproduced cyclically, being repeated during each larval stage. During the four larval stages each cuticle synthetic period is similar with respect to the timing and order of expression, resulting in a multiphasic pattern of oscillating collagen gene expression.

Adult expression differs from larval expression

In addition to the waves of expression detected during the larval stages, both *col-12* and *sqt-1* have an additional wave of expression after the L4 to adult moult (Figure 2c and f). At this stage the earliest fertilizations and

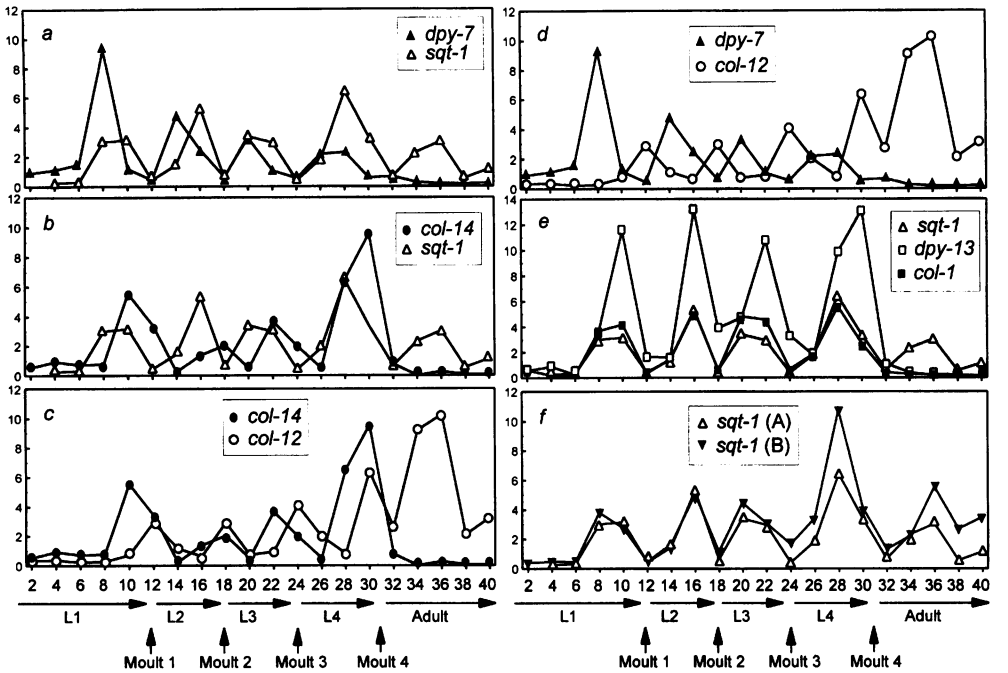

Fig. 2. Graphic representation of post-embryonic mRNA abundance of six different cuticular collagen genes. The data were generated using the same RNA time course and methods as described for the gene *dpy-7* (Figure 1). The *x*-axes of the graphs indicate time in hours of development of the sampled worms post L1 larval arrest. The life cycle stage and timing of the moults relating to this time course are indicated at the bottom of the figures. The *y*-axes indicate the ratio of detected signal for the test gene mRNA over control *ama-1* mRNA. (**a**) A comparison of the data for *dpy-7* with those for *sqt-1*; (**b**) *col-14* with *sqt-1*; (**c**) *col-14* with *col-12*; (**d**) *dpy-7* with *col-12*. (**e**) A comparison of the data for the three genes *sqt-1*, *dpy-13* and *col-1*. (**f**) Two sets of data for *sqt-1* derived from independent rtPCR reactions from the same RNA samples.

generation of embryos will occur within the young adults. For the following reasons we believe the expression we detect must come from the adults and not from the few embryos present. First, the level of expression detected is high by comparison with larval expression and is therefore extremely unlikely to originate from the small number of embryos present at this stage. Second, embryos are not disrupted by the method of RNA preparation used. Indeed, the method does not work for the preparation of embryonic RNA. Finally, from other experiments not presented here we know that *dpy-7* is expressed abundantly in embryos, but at a much later stage than the embryos contaminating these adult time points. We therefore conclude that this expression for *col-12* and *sqt-1* originates from adults and not embryos. This adult-specific expression is distinct from the cyclical larval expression and may relate to growth of the adult cuticle; the adult increases significantly in both length and circumference without moulting. If the function of this expression after the L4 to adult moult does indeed relate to growth of the adult cuticle, the result implies that only a restricted group of cuticular collagen genes are involved in such adult cuticle growth.

Detailed timing of expression through L1 larval development

Data from the time course described above and presented in Figures 1 and 2 clearly differentiate four distinct waves of expression, the first represented by *dpy-7*, the second by *sqt-1*, *dpy-13* and *col-1*, the third by *col-14* and the last by *col-12*. To examine this in more detail, we generated a second synchronous culture of worms and sampled this every 15 min through L1 development. We included detailed measurements of the number of animals in lethargus at each time point. The same six genes were analysed (Figure 3). The results obtained using this independent synchronous culture of worms and set of RNA/cDNA samples confirmed the order of expression determined using the L1 to adult time course. Both from the earliest detectable induction above background and from the shape of the curves (Figure 3b–d) there is an indication that *sqt-1* is expressed very slightly earlier than *col-1* and *dpy-13*. The observations on lethargus (Figure 3g) provide some measure of the degree of synchrony of the culture used to generate the time course. If the culture was totally synchronous this curve would be rectangular, with transitions from 0 to 100% and back to 0%, with a duration equivalent to that of lethargus in one animal. In reality, the percentage of animals observed in lethargus at any one time point peaks at ~45%. Therefore, by this measure, there is significant asynchrony in the culture. Interestingly, the amount of time from the first detection of lethargic animals to the highest point of the curve (Figure 3g) is approximately the same as the time from the first detection of induction for each of the genes tested to the highest point of induction (Figure 3a–f). This probably indicates that the measured slope of each curve (implying rate of induction) is largely the result of asynchrony in the culture and that the real induction in any one animal is much quicker, virtually instantaneous within the 15 min intervals of the assay. There is one condition which could partially invalidate this conclusion. Cuticular collagen mRNA abundance is a biochemical phenomenon. Lethargus is a behavioural phenomenon that is closely related to cuticle synthesis. It is during lethargus that cuticular material is secreted apically from the hypodermis, underneath the existing cuticle and terminating with the removal of the

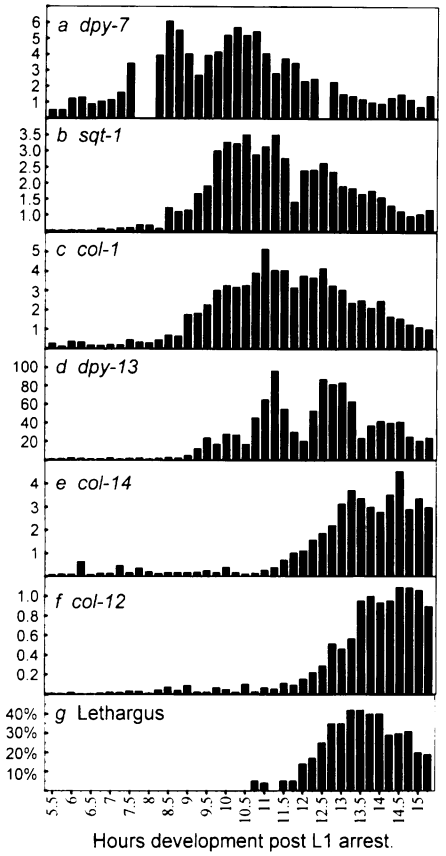

Fig. 3. The results of semi-quantitative RT–PCR for six cuticular collagen genes performed on an RNA time course, samples being taken every 15 min from early L1 to the L1/L2 moult. The *x*-axes of the graphs indicate time in hours of development of the sampled worms post L1 larval arrest. For graphs (**a–f**) the *y*-axes indicate the ratio of detected test gene mRNA over control *ama-1* mRNA. The *y*-axis in graph (**g**) gives the percentage of animals in each time point observed to be in lethargus at the time the sample was prepared. Shed cuticles, indicating the presence of some L2 larvae, were first observed at the 14.5 h time point.

old cuticle. The entire process takes ~1 h. It is possible that there is greater synchrony with respect to the biochemistry of collagen mRNA abundance, but that the precision of timing of the lethargic behaviour is more plastic in individual animals within a population. If this were the case, the actual rate of induction of mRNA abundance would be proportionally slower.

GFP reporter gene fusions with dpy-7 and col-12

In the RT–PCR-based experiments described above we investigated the four post-embryonic cuticle synthetic events. This method relied on the ability to generate synchronous cultures. This cannot easily be done for embryos, in which the first cuticle synthesis (L1 cuticle) occurs. To provide some indication of the similarity of the embryonic and the post-embryonic syntheses, and as an independent assay to the RT–PCR method, two of the cuticular collagen genes analysed by RT–PCR, *dpy-7* and *col-12*, were selected for the construction of gene fusions with the reporter gene *GFP* (green fluorescent protein). *dpy-7* and *col-12* were chosen as being the earliest and latest expressed of the six genes we analysed using RT–PCR and the most likely to have sufficiently different temporal patterns of expression to be detected by the gene

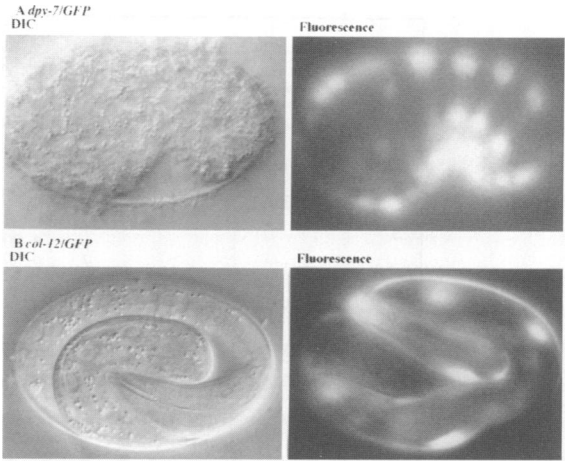

Fig. 4. Examples of the earliest detected fluorescence for *GFP* fusion constructs in transgenic animals. (**A**) *dpy-7–GFP* gene fusion; (**B**) *col-12–GFP* gene fusion. The panels on the left are photomicrographs taken using DIC optics, the panels on the right show GFP fluorescence of the same animal. The fluorescence image for (B) *col-12–GFP* does not superimpose on its DIC image. The images were taken from live animals and at this stage the worm is moving.

fusion method. Fusions were constructed using the vector pPD95.67 (A.Fire, S.Xu, J.Ahnn and G.Seydoux, personal communication) as source of the *GFP* gene. The upstream sequences, including the native ATG start codon for *dpy-7* and for *col-12*, were fused to the *GFP* sequences. This resulted in translational fusions between the cuticular collagen genes and *GFP*. Transgenic strains of *C.elegans* were generated with both gene fusions. Selection for transformation was achieved by co-transforming with the dominant marker *rol-6*(su1006) (Mello *et al.*, 1991). *rol-6* is also a cuticular collagen gene; the (su1006) allele is mutant and causes a dominant visible phenotype (Kramer *et al.*, 1990). Plasmids co-injected with a plasmid carrying this marker concatenate into large extrachromosomal 'free arrays' that are inherited in a non-Mendelian manner. Arrays are typically transmitted to between 10 and 60% of progeny per generation. Because the *rol-6* cuticular collagen gene and the test transgene fusion are present *in cis* on the same extrachromosomal array, there is a possibility that enhancers present in *rol-6* could interfere with *dpy-7–GFP* or *col-12–GFP* transgene expression. We have performed extensive analysis of the spatial expression patterns of *dpy-7* using the *rol-6* co-transforming marker and have found no evidence for interference from *rol-6* sequences (data to be published elsewhere).

The embryonic expression of the *dpy-7–GFP* and the *col-12–GFP* transgenes were analysed. We observed in excess of 100 fluorescing transgenic embryos for both gene fusions. The earliest *dpy-7–GFP* gene fusion fluorescence is observed at around the 'comma' stage (Figure 4A). This is about 4 h before the developing embryo secretes the cuticle of the L1 larva. The earliest *col-12–GFP* gene fusion fluorescence is observed after elongation is complete, in '3-fold' embryos (Figure 4B). This is around the time of cuticle secretion. These results indicate that the start of *col-12* expression occurs at around the time of cuticle secretion and ~4 h after that of *dpy-7*. This result is concurrent with the RT–PCR data obtained for

these two genes in the post-embryonic cuticle syntheses and indicates a similarity in the temporal pattern of expression within the embryonic synthesis, at least for *dpy-7* and *col-12*.

dpy-7–GFP fluorescence diminishes after the L4 to adult moult, however, loss of fluorescence is very gradual and can still be detected for several hours. *col-12–GFP* expression is very strong in adults and continues even in relatively old adults. The RT–PCR data indicate a rapid reduction in the abundance of wild-type *dpy-7* mRNA between 2 and 3 h prior to the L4/adult moult and no adult expression (Figures 1 and 2). The RT–PCR data for *col-12* indicate elevated abundance of the mRNA several hours after the L4 to adult moult (Figure 2). Hence the *GFP* gene fusion data for *dpy-7* and *col-12* are basically consistent with the differences seen between the mRNA abundance of the two genes in the L4 and adult stages, but with the probable indication that *GFP* takes longer to turn over than the wild-type mRNA. The expression of both gene fusions is detected throughout late embryonic and larval development with no obvious fluctuations in the intensity of fluorescence that could be accredited to peaks and troughs of transgene mRNA abundance. We conclude that the *GFP* is too stable (or abundant) to permit detection of the oscillating patterns of cuticular collagen gene expression with the gene fusions used here. It is, therefore, only of value in confirming the time of first onset of expression and confirming a difference between the adult expression of *dpy-7* and *col-12*.

Discussion

We have shown that multiple discrete temporal waves of larval collagen gene expression exist, reflected in the expression patterns of the six genes detailed here. It is conceivable that additional timings exist, as we have assayed only ~10% of all the cuticular collagen genes in *C.elegans* (Cox *et al.*, 1984), but what is striking is that the same ordered pattern is reiterated during each larval stage, and hence moulting cycle. This analysis has revealed what appears to be a precise programme of ordered expression of cuticular collagen genes. The ordered expression must have implications for the process of assembly of the cuticle. As translation can occur only when mRNA is present, it is likely that the observed pattern results in several waves of translation of different collagens. This could provide a mechanism for co-ordinating what is thought to be a critical step in cuticle formation (Johnstone, 1994), the restricted trimerization of compatible collagen monomers. The predicted products of the cuticular collagen genes are all very similar to one another in overall structure, however, they do vary in certain key features, including the precise spacing and patterns of clusters of cysteine residues believed to be involved in trimerization via interchain disulphide bridging (Kramer, 1994b; Johnstone, 1994). The various genes have been classified based on these differences (Kramer, 1994b; Johnstone, 1994). Interestingly, there is a correlation between these sequence classes and the expression waves we have detected. The predicted encoded collagens of the two late genes *col-12* and *col-14* belong to the same structural class (Johnstone, 1994). The two genes *col-1* and *dpy-13*, which are expressed at an intermediary time, belong to a second structural class (Johnstone, 1994). *sqt-1*, which is also expressed at an intermediary time (but possibly slightly earlier than *col-1* and *dpy-13*), belongs to a third and the early gene *dpy-7* is a slightly distinct member of this third class (Johnstone, 1994). The precise nature of the primary interactions between different cuticular collagen chains in cuticle formation is not known, however, there is some evidence from the behaviour of mutant collagen genes that trimerization is restricted either to limited sets of gene products or to homotrimer formation (Johnstone, 1994). Given the large size of the cuticular collagen multigene family, possibly in excess of 50 different genes (Cox *et al.*, 1984), the temporally distinct waves of gene expression may provide a mechanism for restricting the number of collagen gene products being translated and processed during any one time interval. This could assist in the promotion of appropriate interchain interactions by limiting the complexity of the pool of collagen monomers free at any one time to form primary interactions.

Temporal compartmentalization and developmental complexity

Our data, that cuticular collagen mRNA abundance oscillates during development with the same periodicity as the observed moulting cycles themselves, are expected. However, the pattern of cuticular collagen mRNA abundance we detect is more complex than the observed periodicity of moulting. Although each gene tested peaks (and troughs) once during each moulting cycle, the timing of peaks (and troughs) for different genes is distinct, resulting in a multiphasic pattern, the precise nature of which is repeated once during each moulting cycle. Clearly some form of developmental control process, presumably culminating in the activity of transcription factors, must be responsible for generating this patterned expression. Although the results do not elucidate the nature of the mechanism(s) responsible, the process is one of temporal pattern formation. Regardless of the mechanism, there are two distinguishable levels of pattern formation operating. The primary one is the generation of the discrete waves of structural gene expression within a single intermoult period. The secondary is the repetition of this basic pattern during each intermoult period and as such is presumably part of the same developmental mechanism that generates the nematode's repetitious multilarval life cycle itself.

Parallels can be drawn between this and other examples of the evolution of structural complexity. Familiar examples include the repeated use of common sets of developmental instructions at different points in space, resulting in the generation of multiple copies of an ancestral structure, as in the formation of vertebrae, flower formation in plants and the body segmentation of insects (Akam, 1987; Ingham and Martinez Arias, 1992; Weigel and Meyerowitz, 1994; Krumlauf, 1995). In the case of the discrete larval stages in nematode development, the repeated use of a common ancestral set of developmental instructions leads to multiple life cycle stages and thus operates in time rather than space.

How might the temporal control be achieved?

There are two levels of temporal control of cuticular collagen gene expression; generation of discrete waves of

expression and the number of repeats of this pattern. Clearly the second of these is intrinsically associated with the control of the number of larval stages. The timing of many post-embryonic developmental events in *C.elegans* is controlled by a group of genes termed heterochronic, mutations in which alter the timing of such events (Ambros and Moss, 1994). *lin-29* is a downstream member of the heterochronic regulatory pathway whose activity is specifically required to terminate the moulting cycle and hence specify the number of larval stages (Ambros, 1989). In *lin-29* loss of function mutants the moulting cycle is not terminated after the L4 larval stage, resulting in extra larval stages and hence repeats of cuticle synthesis. This phenotype is consistent with the existence of an underlying regulatory mechanism whose activity drives the repetitious use of the various sets of developmental instructions that constitute a moulting cycle, and hence generate a larval stage with its accompanying cuticle synthetic event. The periodic nature of cuticular collagen genes expression, the cyclical nature of moulting and generation of larval stages and the multiple extra larval stages generated in a *lin-29* mutant suggest that the mechanism which drives the moulting cycle has characteristics of a biological oscillator. The *lin-29* gene product is necessary for the termination of the activity of this mechanism.

Such an oscillator may not readily explain the primary level of temporal control of cuticular collagen gene expression, that which generates the discrete waves of expression of different genes repeated during each larval stage. The control mechanisms which generate this pattern may also regulate, or integrate with, other events that occur within the hypodermis at the same time and in a repeated fashion during each moulting cycle. Most notable are the sets of divisions of a group of specialized hypodermal cells, the seam cells, that yield some daughter cells which fuse with the major hypodermal syncytium (Sulston and Horvitz, 1977). These divisions and fusions occur in a basically repeated fashion during each moulting cycle. In those heterochronic mutants which alter the number of larval stages, such as *lin-29* discussed above, each additional larval stage, and hence repeat of the moulting cycle, is accompanied by an additional repeat of the seam cell divisions and fusions (Ambros and Horvitz, 1984; Ambros, 1989). This basically synchronous progression through the cell cycle, cell division and fusions to the major hypodermal syncytium occurs concurrently with the progression through the various waves of collagen gene expression during each repeat of the moulting cycle. Although this does not prove association between these two phenomena, it is at least conceivable that cell cycle events and cell fusions within the hypodermis could in some fashion relate to the timing of expression of different collagen genes.

Materials and methods

Synchronous cultures

In all experiments wild-type *C.elegans* Bristol strain N2, supplied by The *Caenorhabditis* Genetics Stock Center, was used. A synchronous culture of *C.elegans* was prepared by hatching alkaline hypochlorite-purified embryos (Sulston and Hodgkin, 1988) in the absence of food. The resulting synchronously arrested L1 larvae were inoculated onto 90 mm plates of NGM agar (Sulston and Hodgkin, 1988) containing a lawn of bacteria (*Escherichia coli* OP50). Plates which were to be sampled as time points during L1 and L2 development were seeded with ~10 000 arrested L1 larvae each. Plates for L3 and L4 time points were seeded with ~5000 larvae; plates for adult time points were seeded with ~2500 larvae. The reduction in the number of larvae per plate for later time points ensured that the cultures did not run out of bacterial food during the experiment. For each time point the larvae on one single plate were collected, therefore, the reduction in numbers for later time points also partially compensated for the increase in worm biomass, and hence RNA, as the animals grew. The plate cultures were incubated at 25°C and every 2 h the worms contained on a single plate were harvested, until 40 h, when all the animals had reached adulthood. RNA was prepared from the 20 time points. Worms (and bacteria present on the plate) were washed off in water and pelleted by centrifugation for 1 min in a microfuge. Each pellet was resuspended in 0.5 ml 0.5% SDS, 5% 2-mercaptoethanol, 10 mM EDTA, 10 mM Tris–HCl, pH 7.5, and protease K was added to a final concentration of 0.5 mg/ml. Samples were incubated at 55°C for 1 h. Samples were organically extracted with 1 vol phenol/chloroform (1:1) equilibrated to pH 5.2 with 10 mM sodium acetate, pH 5.2, 50 mM NaCl, 3 mM EDTA. RNA was precipitated with ethanol, collected by centrifugation and resuspended in water. The RNA samples were adjusted to 6 mM NaCl, 6 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 1 mM DTT and treated with DNase I (final concentration 50 μg/ml) at 37°C for 1 h. The samples were re-extracted with phenol/chloroform and ethanol precipitated as above. The samples were finally dissolved in 0.2 ml water.

cDNA synthesis and RT–PCR

First strand cDNA was generated from 1 μl of the RNA for each time point using reverse transcriptase priming with random hexamers (Kawasaki and Wang, 1989). Reactions were performed in 20 μl volumes using Superscript II from Life Technologies. Buffers and conditions were as supplied and recommended by Life Technologies. cDNA synthesis was performed at 42°C for 1 h. After synthesis, each cDNA reaction was diluted by the addition of 0.4 ml TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA).

RT–PCR

For each gene tested (*dpy-7*, *sqt-1*, *dpy-13*, *col-1*, *col-12* and *col-14*) the resulting cDNA was amplified using two sets of PCR primers, one set specific to the test gene and the second set specific to an internal control, *ama-1*, which encodes the large subunit of RNA polymerase II (Bird and Riddle, 1989). In each case oligonucleotides were chosen to span an intron. The number of PCR cycles were chosen empirically, determined by preliminary reactions with each set of oligonucleotides, and reactions stopped before reagents were exhausted. Typically, 25–27 cycles were used. Cycling was performed under the following conditions: (a) 94°C 3 min, ×1; (b) 60°C 30 s, 72°C 30 s, 94°C 30 s, ×25–27; (c) 60°C 3 min, 72°C 3 min, ×1.

PCR reactions were electrophoresed in 2% agarose gels and Southern blotted. Blots were probed with the appropriate oligonucleotides end-labelled with γ-ATP. After autoradiography, bands corresponding to amplified test cDNA and control *ama-1* cDNA for each time point were cut out of the membrane and counted in scintillant. The relative abundance of the test gene is then expressed as a ratio of the signal from the test gene to that of *ama-1*. For methodological reasons this value is not a measure of the real difference in abundance of the test transcript to the control transcript, however, it does permit highly reproducible measurement of fluctuations in the relative abundance of the two transcripts between different samples. It is essential to compare only values obtained from within a single experiment. This is indicated in our own data for *dpy-13*. The L1 peak of expression of this gene is measured as ~12 times that of *ama-1* in Figure 2 and ~100 times that of *ama-1* in Figure 3. This is the result of experimental variables such as the precise specific activity of the probes used to detect the control and test gene transcripts in different experiments and the precise conditions used for washing the blots. In our own experience using end-labelled oligonucleotides as probes for detection we find the blot washing step particularly prone to generating these differences between experiments. Although this results in peaks of different absolute values for one gene between different experiments, it does not interfere with the determination of which samples within a series have more or less relative abundance of a particular transcript.

The oligonucleotides used were: *ama-1* 5′-TTCCAAGCGCCGCTGCGCATTGTCTC-3′, 5′-CAGAATTTCCAGCACTCGAGGAGCGGA-3′; *dpy-7* 5′-GGGCCAACCACGTGGCAAAAGCCACC-3′, 5′-TTGGTTGTCGGATTGAGCACTATC-3′; *col-12* 5′-ACTTGGCTTCTAAAGTCCAGTGACA-3′, 5′-TCCGCATGAGCAGCATGATCCTCCA-3′;

dpy-13 5′-ATGGACATTGACACTAAAATCAAGGCC-3′, 5′-TTGTGGGCATGGCTTGCATGGTGGTGGG-3′; *col-1* 5′-ATGGAAACTGACGGTAGGCTCAAAGC-3′, 5′-CCTGGAAGGCAGCATCCCTCGCATTG-3′; *col-14* 5′-GCATTATATTCAAACTTTGGAATCTCAAG, 5′-CCTGGTGGAGCCTCACATGGACATTG-3′; *sqt-1* 5′-TGACGGCTTCGGTCACTGTCGCC-3′, 5′-TGGGCACTTGTTATCAGCATTGC-3′.

Observations on lethargus

Culture plates were removed briefly from the incubator and observed under a bench microscope. Approximately 50 animals were observed for signs of lethargus, which include a lack of mobility and cessation of pharyngeal pumping. In each case the next plate to be sampled was observed. For the L1 to adult time course observations were made every hour. For the L1 detailed time course observations were made from each plate immediately prior to harvesting.

GFP gene fusions

The vector pPD95.67 (A.Fire, S.Xu, J.Ahnn and G.Seydoux, personal communication) was used for the construction of cuticular collagen–*GFP* gene fusions. For *dpy-7* sequences from –431 to +18 bases relative to the ATG translational start (Johnstone *et al.*, 1992) were fused into the *Bam*HI site in vector pPD95.67, generating a translational fusion between *dpy-7* and *GFP*. A similar construct was generated for *col-12*. In this case sequences were subcloned from the plasmid pys124 (a gift from J.Kramer), containing the *col-12* gene. Sequences extending from a *Sal*I site ~1 kb upstream of the ATG to base +18 (relative to ATG) were cloned into the *Bam*HI site of pPD95.67, generating a similar translational fusion to that for *dpy-7*.

Plasmid DNA was prepared for microinjection using Qiagen tips. Gene fusion DNA was mixed for co-transformation with plasmid pRF4, which contains the *rol-6*(su1006) allele for detection of transgenic lines. The mixes contained 50 μg/ml gene fusion clone plus 100 μg/ml pRF4. DNA was injected into the syncytial gonad of adult N2 worms and transgenic lines isolated using standard methods (Mello *et al.*, 1991). Transgenic animals were observed as live specimens using a Zeiss Axioplan with filter set 15 (BP 546/12, FT 580, LP 590 for observation of GFP fluorescence).

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