Differential gene expression of *Caenorhabditis elegans* grown on unmethylated sterols or 4α-methylsterols

Mark Merris,[1,*] Tongsheng Wang,[1,†] Patricia Soteropoulos,[†] and John Lenard[2,*]

Department of Physiology and Biophysics,* University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ 08854; and Center for Applied Genomics,† Public Health Research Institute, Newark, NJ 07103

Abstract Transcriptional profiles of *Caenorhabditis elegans* grown on unmethylated sterols (desMSs) or on 4α-methylsterols (4MSs) were compared using microarrays. Thirty-four genes were upregulated and 2 were downregulated >2-fold by growth on 4MSs, including 13 cuticle collagen (*col*) genes, 1 cuticulin gene (*cut-1*), 2 groundhog-like (*grl*) genes, and 1 groundhog gene (*grd-4*); *col-36* and *grl-20* were increased 12- and 19-fold, respectively. Fifteen of these 17 genes have been assigned to metabolic mountain 17, suggesting coordinate 4MS-mediated regulation of expression. Quantitative RT-PCR was performed on 27–51 h old animals grown on cholesterol (a desMS) or lophenol (a 4MS). *col-36* and *grl-20* showed similar cyclic peaks of expression in cholesterol and similar alterations in lophenol, suggesting coregulation. Of six additional *grl* genes, only *grl-3* was upregulated on lophenol; the rest were downregulated. Cyclicity of expression was lost or altered in all six. Nuclear receptor genes *nhr-23*, *nhr-25*, *nhr-41*, and *daf-12* all showed cyclic expression in cholesterol and significant downregulation in lophenol by RT-PCR. Expression of the insulin-like receptor *daf-2* was lower in lophenol, whereas that of its major downstream target *daf-16* was higher. Thus, major changes in gene expression accompany growth on 4MSs, but with surprisingly little effect on normal growth and development.— Merris, M., T. Wang, P. Soteropoulos, and J. Lenard. **Differential gene expression of *Caenorhabditis elegans* grown on unmethylated sterols or 4α-methylsterols. *J. Lipid Res.* 2007.** 48: **1159–1166.**

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Dietary sterol is required by *Caenorhabditis elegans* because this animal is incapable of synthesizing the four ring sterol nucleus (1, 2). Sterol deprivation results in the pleiotropic arrest of growth and development at whatever stage the animal is occupying when the (often considerable) store of maternally supplied sterol is exhausted (1–3). A difficulty in cuticle production and shedding has frequently been reported in sterol-deprived animals (4–6), but it is not clear whether this is a direct effect of sterol depletion or a secondary consequence of arrested growth.

The question of how sterols are used by *C. elegans* has been the focus of extensive research. Sterols are apparently not essential components of plasma membranes, the way cholesterol is in vertebrate cells, as shown by the following findings. *1*) Minute amounts of sterols, insufficient to modify plasma membrane properties globally, are required (3). *2*) Sterols accumulate in a few specific cells rather than being distributed uniformly in cellular plasma membranes (3, 7). *3*) The enantiomer of cholesterol (possessing the opposite stereochemistry at all asymmetric carbons) does not substitute for cholesterol in supporting growth, although sterol-lipid interactions in bilayers are generally not enantiospecific (8). *4*) The sterol requirement can be met by 4α-methylsterols (4MSs) containing 1% or less unmethylated sterols (desMSs) (3, 6), even though 4MSs are inferior to desMSs in their ability to modify membrane bilayers.

The finding that no 4MS can completely satisfy the *C. elegans* sterol requirement without the addition of a minute amount of a desMS indicates that at least two essential sterol-requiring pathways are present, one using 4MS and the other requiring desMS (3). *C. elegans* is unique in being able to methylate the 4α position of sterols and conversely in being unable to remove the 4α-methyl group; all animals, plants, and yeast that synthesize sterols de novo possess the opposite abilities (**Fig. 1**) (9). Not only are 4MSs synthesized by *C. elegans*, but they are present in significant amounts at all stages of growth and development, suggesting a continuing role (10).

The nature of the requirement for desMS was elucidated recently. Two desMS metabolites were identified in cholesterol-fed worms (11) and shown to bind and activate the nuclear hormone receptor DAF-12. Activation of DAF-12 by ligand binding inhibits the long-lived dauer

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[1] M. Merris and T. Wang contributed equally to this work.
[2] To whom correspondence should be addressed.
e-mail: lenard@umdnj.edu

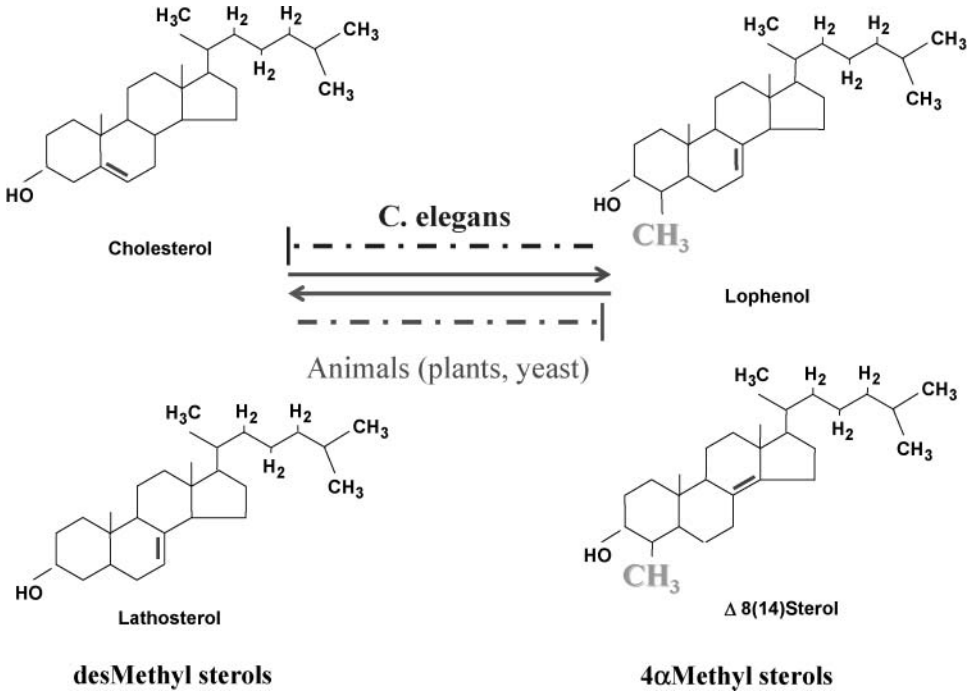

Fig. 1. How unmethylated sterols (left) are related to 4α-methylsterols (right). *C. elegans* is unique in being able to attach a methyl group to the sterol 4α position and being unable to remove it. Plants, animals, and yeast, which can synthesize sterol rings de novo, possess the opposite abilities.

pathway (induced by crowding and/or dietary stress) and promotes development through larval stages 3 and 4 to adulthood, possibly two or more distinct activities (11). The unliganded DAF-12 receptor, on the other hand, induces the pathway to dauer formation, enabling the worm to survive extended periods of adversity (12). DAF-12 ligands appear to be the only essential desMS derivatives, as dauer-deficient mutants of *daf-12* (i.e., mutants that behave as if they possess constitutive ligand-activated DAF-12 receptors) were maintained over several generations in 4MSs alone (6); the need for desMS is bypassed in this mutant.

It seems likely that 4MSs (or their metabolites), like desMSs, also regulate one or more nuclear receptors, presumably those controlling transcriptional programs required for normal growth and reproduction beyond the dauer-L2 branch point. No putative receptors have yet been identified, however, and no mechanisms of 4MS action are known. The present experiments were undertaken with the aim of generating more detailed hypotheses regarding the mechanisms of action of 4MSs in *C. elegans*.

MATERIALS AND METHODS

Wild-type N2 animals, Bristol type, were used for all experiments. Animals were grown on bacterial (*Escherichia coli* OP50) lawns on agarose plates using medium containing ether-extracted peptone for both nematode and bacterial growth (3). For microarray experiments, sterol-free medium contained one of the following four sterol supplements: 1 μg/ml cholesterol or lathosterol, or 1 μg/ml lophenol or 4α-methyl-cholesta-Δ8(14)-en-3β-ol [Δ8(14) sterol], each with 10 ng/ml cholesterol. For real-time RT-PCR experiments, only the cholesterol and lophenol supplements were used.

Unsynchronized populations of *C. elegans* were used for the microarray experiments. For the quantitative RT-PCR experiments, eggs were isolated by standard bleaching techniques and were allowed to hatch overnight in nutrient-free M9 medium. Synchronized populations were prepared by transferring the L1 hatchlings to nutrient-containing plates at time 0 and allowing development for the specified number of hours at 20°C.

RNA was isolated using Trizol essentially as described in WormBook (13). A TaqMan reverse transcription kit (Applied Biosystems) was used to reverse-transcribe mRNA into cDNA. Real-time quantitative RT-PCR was performed on Opticon (MJ Research) using a SYBR Green PCR core kit (Applied Biosystems). RT-PCR levels were normalized to the expression of *ama-1*, which encodes the large subunit of RNA polymerase II.

C. elegans whole genome oligonucleotide arrays were printed by the genome sequencing center of Washington University in St. Louis. The arrays contain 22,490 60-mers representing the 22,490 *C. elegans* genes and 124 control oligonucleotides. Array-specific information is available online (14).

Total RNA sample labeling and microarray hybridization were performed using the Genisphere Array 350 kit (Genisphere, Hatfield, PA). Briefly, first-strand cDNA was synthesized from 5 μg of total RNA. RNA in the DNA/RNA hybrid was denatured, and the cDNA of the two samples was then combined. The combined sample was purified and concentrated using a Millipore Microcon YM-30 centrifugal filter device. Microarray hybridization was performed in two steps. First, the concentrated cDNA was hybridized to the array overnight at 42°C. The array was washed to remove the cDNA, and a second hybridization was performed with the 3DNA capture reagents for 3 h at 50°C. The array was then washed and spun dry at 1,000 rpm for 2 min. The microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). The complete labeling and

hybridization protocol is available online (15). Feature intensities were extracted from scanned images using GenePix Pro 5.1 (Molecular Devices).

The raw data from 12 chips (four comparisons in triplicate) were normalized by the Print-tip LOWESS method (16). The normalized data were then filtered using the following criteria: *1*) the feature diameter was ≥50 μm; *2*) the spot was not flagged as bad; and *3*) the sum of median intensities (Cy3 and Cy5) was >500. The normalized and filtered data were analyzed using a *t*-test (standard Bonferroni correction; adjusted $P < 0.001$) and Significance Analysis of Microarray (median false discovery rate of 0, delta = 1.03) (17) tools in The Institute for Genomic Research MeV software (18). We considered genes to be upregulated or downregulated only if they were determined to be significant by both the *t*-test and the Significance Analysis of Microarray and had on average at least a 2-fold change.

RESULTS

RNA from animals at all stages of development was isolated for microarray analysis from unsynchronized populations grown in four different sterol conditions: 1 μg/ml cholesterol, lathosterol, lophenol, or Δ8(14) sterol (Fig. 1). The first two are desMSs, and the latter two are 4MSs, to which cholesterol (10 ng/ml) was added, because a small amount of a desMS is required for normal growth when 4MSs are supplied as the major sterol (3). Unsynchronized populations were used to facilitate recognition of only the largest and most persistent 4MS-induced transcriptional effects. Visual observation of these animals showed no obvious differences in their overall health or distribution of developmental states in any of the sterol conditions. All four of the possible 4MS versus desMS comparisons were run in triplicate, and all 12 results were averaged. Thirty-four genes were found to be upregulated >2-fold in 4MS, and only two were downregulated (**Table 1**). Of the upregulated genes, 19 belonged to recognized gene families. Interestingly, 14 of the 19 named genes encoded structural components of the cuticle: 13 *col* family genes and *cut-1*, which encodes cuticlin, a dauer-specific cuticular component (19). In addition, C14C6.5 is identified in Wormbase as a "secreted surface protein" (Table 1). It is striking that 12 of the14 genes encoding cuticle structural proteins are assigned by Kim et al. (20) to the same "metabolic mountain," mount 17. Metabolic mountains represent groups of genes that tend to be coordinately upregulated or downregulated regardless of the specific parameters being compared. The finding of so many upregulated genes of similar function assigned to the same metabolic mountain suggests that they may be coordinately regulated through a common 4MS-based signaling pathway.

Three additional genes from mount 17 were also upregulated in 4MS-grown animals: *grl-20*, *grl-17*, and *grd-4* (Table 1). These genes of unknown function have been assigned to the same superfamily as hedgehog, a developmental morphogen found in flies and vertebrates. Hedgehog is the only protein known to be covalently linked to a sterol. It is activated by internal proteolysis catalyzed by its "hog," or intein domain, followed by the covalent attachment of a cholesterol molecule to its newly formed C terminus (21). There is no evidence for any covalently sterolated protein in *C. elegans*, and no close homolog of hedgehog in its genome. A hedgehog-related superfamily of genes has been identified in worms, however, and divided into three families: warthog (*wrt*), groundhog (*grd*), and groundhog-like (*grl*) (22). It is intriguing that three members of this distantly sterol-related group of genes are found to be upregulated by growth on 4MS, and furthermore, that they are assigned to mount 17 along with the upregulated cuticle genes. This suggests that they may all participate in a 4MS-dependent regulatory network governing cuticle construction.

Additional genes that were upregulated in 4MS included three genes encoding enzymes involved in lipid metabolism: *fat-7*, a stearoyl-CoA Δ^9 desaturase that is strongly controlled by the nuclear hormone receptor NHR80 (23), and two putative cholesterol esterases. Other upregulated genes included two encoding putative permeases, two encoding putative "defense-related proteins," and seven encoding mostly small proteins of unknown function (Table 1).

To better understand the developmental regulation of these genes, quantitative RT-PCR was performed using RNA from synchronized populations of animals grown on

TABLE 1. Upregulated and downregulated genes (>2-fold) in 4α-methylsterol versus unmethylated sterol microarrays

Gene	Name	Average ± SEM	Mount	Wormbase Annotation
Upregulated genes				
C23H5.9	*grl-20*	18.9 ± 8.0	17	Groundhog-like
C27H5.5	*col-36*	12.3 ± 3.7	17	Cuticle collagen
C17F4.7		9.7 ± 5.1		
T13B5.4	*col-40*	9.5 ± 3.1	17	Cuticle collagen
W01B6.7	*col-2*	7.5 ± 2.5	17	Cuticle collagen
C18H7.3	*col-102*	7.1 ± 2.1	17	Cuticle collagen
ZK863.2	*col-37*	6.7 ± 1.9		Cuticle collagen
D2023.7	*col-158*	6.6 ± 1.6	17	Cuticle collagen
T05H10.3		6.5 ± 2.5		Permease
ZC513.8	*col-43*	6.5 ± 1.6	17	Cuticle collagen
ZK355.3		6.3 ± 1.5	15	
C47G2.1	*cut-1*	6.2 ± 2.0	17	Cuticlin
C35A5.3		5.2 ± 1.5	19	Permease
D2024.8	*col-114*	4.7 ± 2.9	17	Cuticle collagen
Y51F10.7		4.3 ± 0.3		
H10D18.2		3.7 ± 1.1	15	Defense-related ptn
T21D9.1	*col-164*	3.5 ± 0.7	17	Cuticle collagen
C56A3.1	*grl-17*	3.4 ± 0.6	17	Groundhog-like
T13B5.6		3.2 ± 0.7		Cholesterol esterase
F55G11.4		3.2 ± 0.9		
T21B4.2	*col-85*	3.1 ± 1.4	17	Cuticle collagen
F55A3.5		3.1 ± 0.9	10	Glutathione peroxidase
ZK6.7b		2.9 ± 0.4	8	Cholesterol esterase
B0024.1	*col-149*	2.9 ± 0.7	16	Cuticle collagen
C14C6.5		2.9 ± 0.7	19	Secreted surface ptn
F22A3.6		2.8 ± 1.0	19	
W08D2.6	*col-123*	2.7 ± 1.0	17	Cuticle collagen
F02D10.1	*col-183*	2.7 ± 0.8	17	Cuticle collagen
T06E4.11	*pqn-63*	2.6 ± 1.1	29	Q/N-rich prion-like
H10D18.4		2.5 ± 1.0	15	Defense-related ptn
F49E11.6		2.5 ± 0.7	15	
F10D2.9	*fat-7*	2.4 ± 0.4	8	Acyl-CoA dehydrogenase
T01B10.1	*grd-4*	2.4 ± 0.6	17	Groundhog family
F10D2.8		2.1 ± 0.7		
Downregulated genes				
F42A1 0.7		0.25 ± 0.07	6	
F59D8.2	*vit-4*	0.44 ± 0.08	22	Vitellogenin

either 1 μg/ml cholesterol or 1 μg/ml lophenol plus 10 ng/ml cholesterol. Populations were harvested at 3 h intervals from 27–51 h after transferring newly hatched L1 larvae from M9 medium to bacterial plates. This corresponds to the period from approximately mid-L3 larval stage to early adulthood.

We first compared expression levels of *grl-20* and *col-36*, the two genes most strongly upregulated by growth on 4MSs according to the microarrays (Table 1). Previous studies showed cyclic expression of several cuticle collagen genes in conventionally grown, cholesterol-fed animals, peaking at similar times within each larval stage (24). Therefore, a cyclic expression profile was expected for *col-36*, with a similar peak of expression for *grl-20*, if these two genes are indeed coordinately regulated. As shown in **Figs. 2**, **3**, similar expression profiles were seen for both genes in cholesterol-grown animals, with one peak of expression at 39 h and a second increasing into adulthood at 48–51 h. The expression profiles were altered and upregulated by growth on lophenol but remained similar for *col-36* and *grl-20*, consistent with their involvement in a common 4MS-regulated pathway. Peak expression was seen at 33–36 h in the lophenol animals, possibly reflecting a slightly different rate of development. The increase seen at the later times in cholesterol animals was absent in lophenol animals, however. Results for these two genes provide confirmation for the microarray results (Table 1).

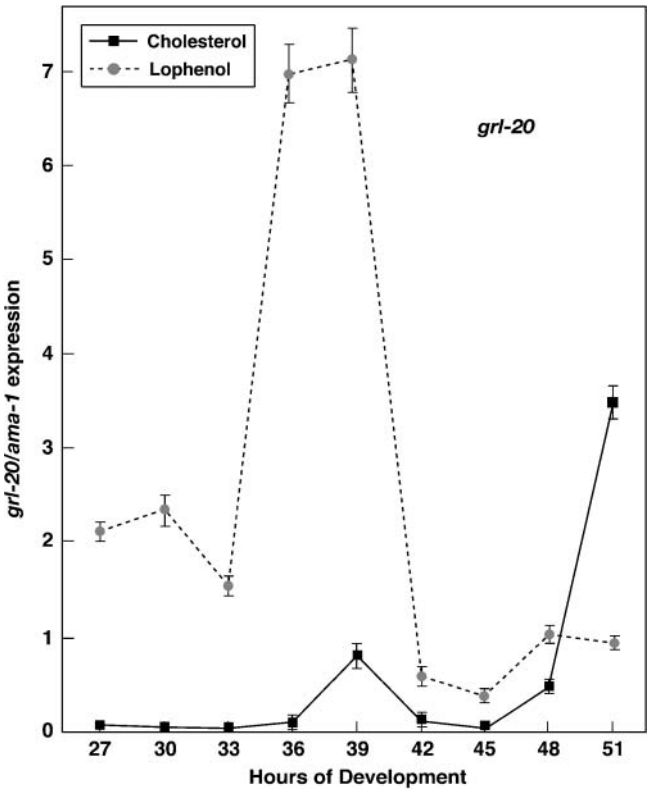

Fig. 2. Quantitative real-time RT-PCR analysis of *grl-20* expression levels in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

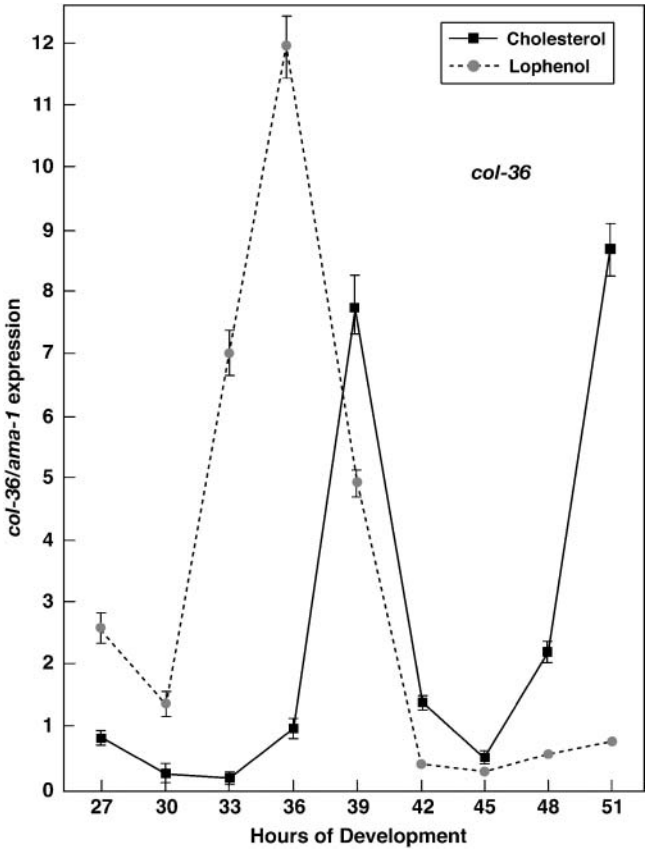

Fig. 3. Quantitative real-time RT-PCR analysis of *col-36* expression levels in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

RT-PCR results for *col-36* in cholesterol animals do not agree with an earlier report, however, which found expression of this gene restricted to L1, L2, and L2-dauer stages (25); it is possible that expression is much higher in these stages, which were not examined in the present study.

To compare another well-characterized cuticle collagen gene with *col-36*, we examined the expression of *dpy-7*. This gene (which is not on mount 17) has been shown to be cyclically upregulated during a second wave within each larval stage in cholesterol-grown animals (24). Expression of *dpy-7* was quite different from that of *col-36*. It exhibited a broad peak of expression at 33–42 h in cholesterol-grown animals and was downregulated in lophenol-grown animals with loss of cyclic expression (**Fig. 4**). This result suggests that *dpy-7* is regulated separately from *col-36* and *grl-20* in both cholesterol- and lophenol-grown animals.

It was of great interest to examine the expression of other *grl* genes, as this is the first report of regulated expression, and the first hint of function, for any member of the family. We examined the expression of *grl-3*, *-17*, *-19*, and *-23*, assigned to mount 17, and *grl-21* and *-27*, assigned to other mountains. Results for four of these six genes are shown in **Fig. 5**. Of these six genes, only *grl-3* was upregulated by growth on lophenol. Not even *grl-17*, which was identified as modestly upregulated in microarrays,

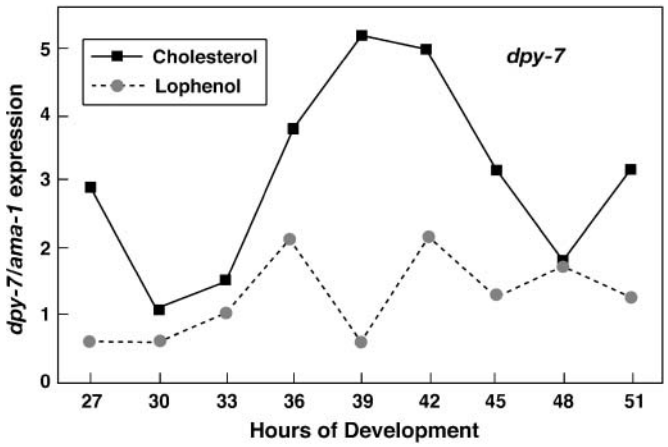

Fig. 4. Quantitative real-time RT-PCR analysis of *dpy-7* expression levels in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

showed increased expression in lophenol animals during this 27–51 h time period (Fig. 5). All six genes showed roughly similar patterns of expression in cholesterol animals, with more or less well-pronounced peaks at 30 and 39 h and a variable tendency to increase during the last two time points. Cyclicity of expression was completely lost in lophenol for *grl-3* and *grl-27* and was muted and shifted to ~36 h in the other *grl* family genes examined. Different genes in this family thus respond differently to alterations in sterol growth conditions.

Several studies have implicated nuclear hormone receptors in the regulation of cuticle biosynthesis, most notably *nhr-23*, *nhr-25*, and *nhr-41*, which are all homologous to members of the "ecdysone cascade" that controls molting in *Drosophila* (26–28). In light of the results described above suggesting a connection between growth sterol and cuticle biosynthesis, we examined the expression pattern of these genes (none were observed on our microarrays, and none are on mount 17). Results for *nhr-23* and *nhr-25* are shown in **Fig. 6**. Both of these genes showed very similar patterns of expression, with a peak of activity at 39 h in cholesterol-grown animals, quite similar to those of *col-36* and *grl-20* in cholesterol (Figs. 2, 3) and much as reported previously (26–28). Expression was much lower, and lacked cyclicity, in lophenol-grown animals (Fig. 6). Patterns of expression for *nhr-41* were similar to those shown in Fig. 6, except that expression was much lower overall, peaking at ~2% of *ama-1* expression at 39 h in cholesterol-grown animals (data not shown). It should be noted that although RT-PCR of the synchronized populations showed >2-fold downregulation of all three *nhr* genes during the

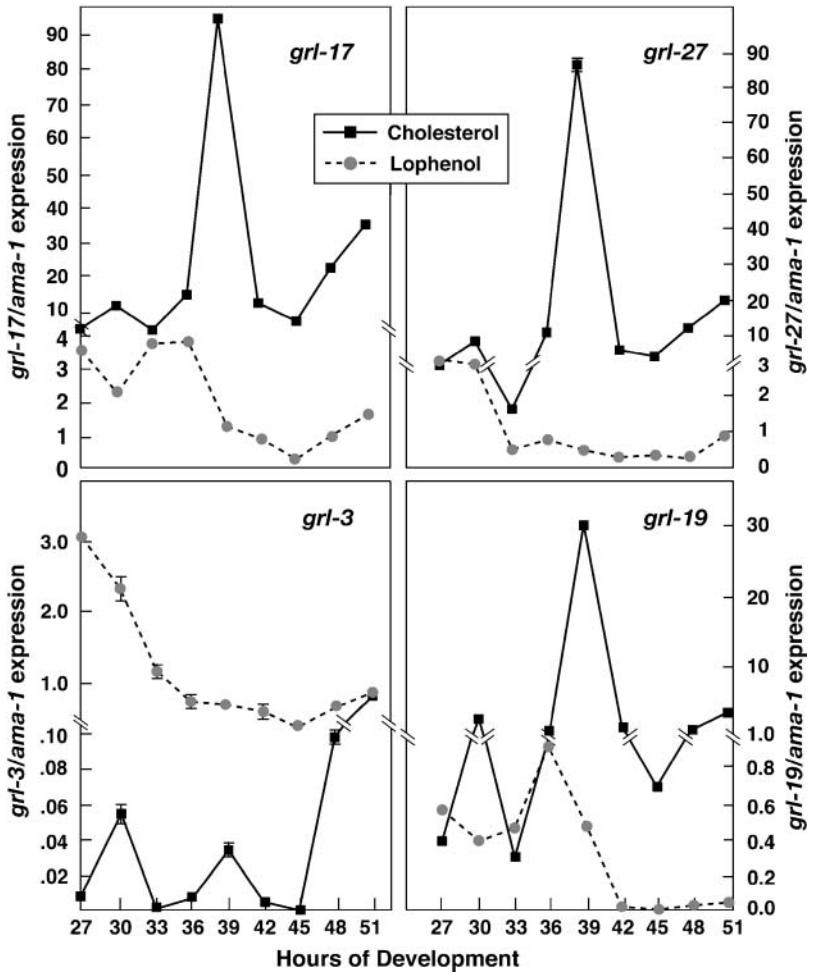

Fig. 5. Quantitative real-time RT-PCR analysis of expression levels of *grl* family members in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

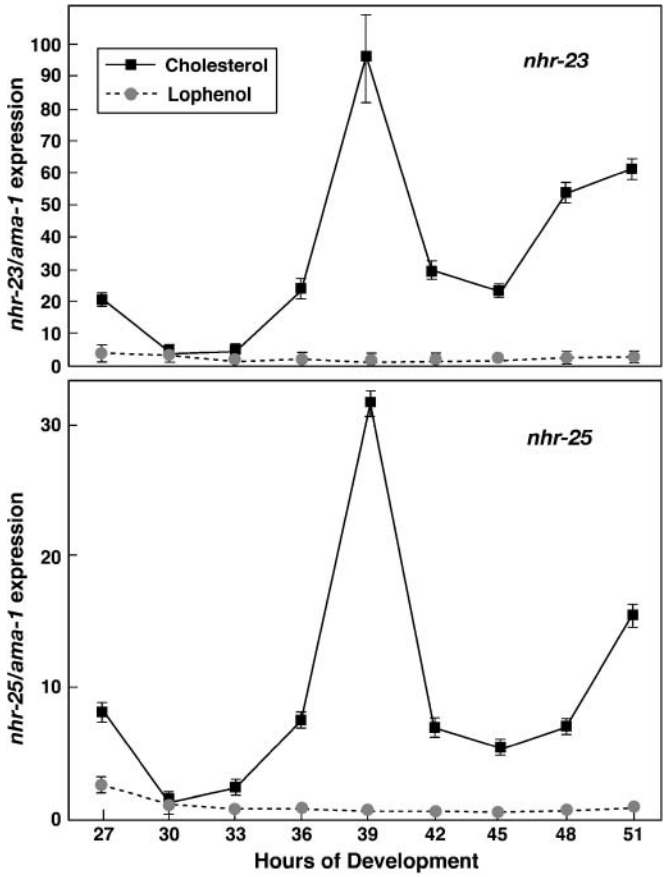

Fig. 6. Quantitative real-time RT-PCR analysis of *nhr-23* and *nhr-25* levels in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

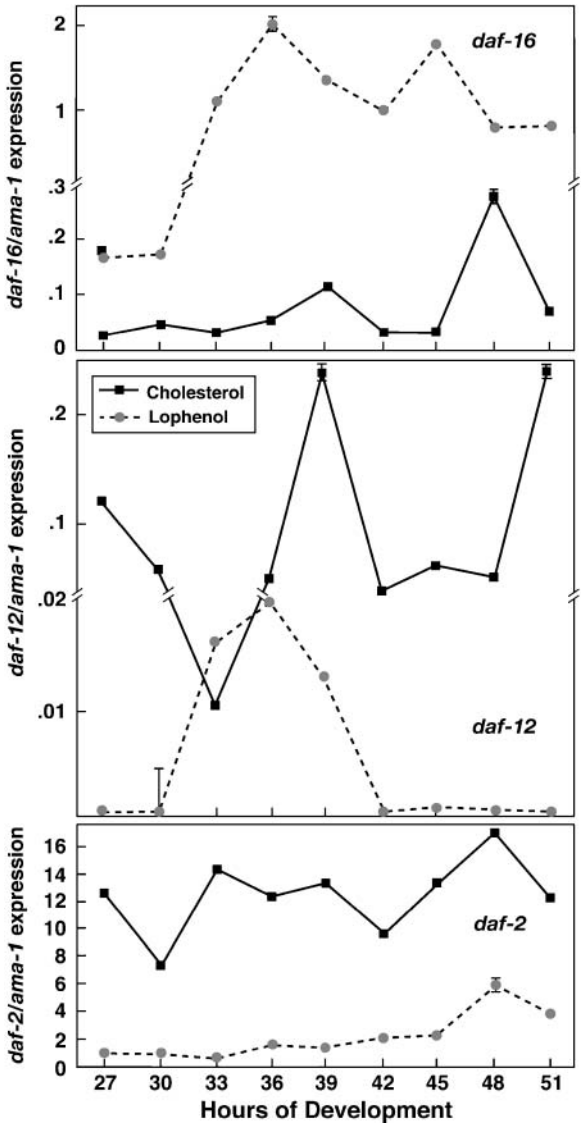

Fig. 7. Quantitative real-time RT-PCR analysis of *daf-2*, *daf-12*, and *daf-16* levels in synchronized populations of animals aged 27–51 h grown in cholesterol or in lophenol relative to *ama-1*. n = 3. Error bars represent means ± SD.

27–51 h period, no genes of this family were observed in the microarray analysis; perhaps expression is much higher at stages of the life cycle other than those studied here.

The best characterized and most intensively studied nuclear hormone receptor gene in *C. elegans* is *daf-12*. This gene was recognized early as a putative steroid receptor that is critically involved in the dauer versus nondauer development decision. The DAF-12 ligands Δ4 and Δ7 dafachronic acid, which are desMS metabolites, were recently identified and shown to activate DAF-12, directing the animal along the nondauer pathway to adulthood (11). DAF-12 is also part of the signaling pathway that has been shown to have important effects on aging (29). The insulin-like receptor DAF-2 is upstream of DAF-12 in this pathway, and the forkhead transcription factor DAF-16 is downstream. Therefore, it was of interest to learn whether the expression of *daf-2*, *daf-12*, and *daf-16* was affected by growth in lophenol, which has no significant effect on longevity (10). Results are shown in **Fig. 7**. Expression of *daf-12* in cholesterol-grown animals was typical of the other nuclear hormone receptors examined, with a peak at 39 h and higher levels of expression at the first and last time points. Growth on lophenol resulted in significant downregulation, in common with the *nhr* genes (Fig. 6). There was no clear cyclicity of expression of *daf-2* in either sterol, but its expression was slightly decreased by growth on lophenol. In contrast, *daf-16* was significantly upregulated by growth on lophenol.

DISCUSSION

Several conclusions can be drawn from our observations: *1*) Growth in excess 4MS results in a significant increase in the expression of numerous cuticle genes. It is noteworthy that this was observed in unsynchronized populations, in which contributions from larger animals are likely to predominate. 4MSs are found in *C. elegans* in substantial quantities at all life stages (10), suggesting that they may have a continuing function in the maintenance of the animal. Microarray results shown in Table 1 and

RT-PCR results shown in Figs. 2, 3 suggest that 4MSs control a transcriptional network responsible for late-stage cuticle synthesis and, furthermore, that this network involves certain *grl* and *grd* gene family members. This is the first indication of a function for any member of these gene families, although members of the hedgehog superfamily in other organisms function as developmental morphogens.

Although cuticle formation has been studied by microscopic techniques, the details of cuticle construction remain largely unknown. *C. elegans* constructs a new cuticle five separate times, during each of its four larval stages and during development into adulthood. There are close to 200 putative cuticle collagen genes in the *C. elegans* genome. The expression of fewer than a dozen of these has been studied in synchronized populations, but essentially all of those studied have shown periodic upregulation corresponding to discrete time points within each larval stage. The most thorough characterization has shown that different collagen genes are upregulated at different time points within each larval stage and that the corresponding proteins are found in different structural features of the cuticle (24). It is generally thought that cuticle structure and composition are similar, but not identical, at each larval stage, but the differences have not yet been systematically elucidated. Nor have the roles of the multitude of unstudied collagen genes been determined. The coordinate upregulation of several of these uncharacterized genes, along with several hedgehog superfamily genes all assigned to the same metabolic mountain (20), suggests that these genes may be organized in a common regulatory network controlled by a 4MS-liganded hormone receptor. It may be that different *grl* family genes are involved at each larval stage.

2) Putative 4MS-activated NHRs are probably not among those previously suggested to control cuticle biosynthesis. On the contrary, the two best-studied genes, *nhr-23* and *nhr-25*, are so strongly downregulated in lophenol, at least during the 27–51 h developmental period, that they may not be functional in lophenol-grown animals at all during this period. Yet, the animals are grossly normal, including possession of apparently normal cuticles that allow for normal movement. Although these two *nhr* genes have been associated with abnormal cuticle production, it is not clear whether this effect is direct or is a secondary consequence of a disrupted program of larval development. At the very least, the actions of these NHRs would seem to be redundant with others that are presumably activated by excess 4MSs.

Alternatively, it is unlikely but conceivable that NHR-23 and NHR-25 are liganded by 4MS derivatives but are downregulated in response to excess environmental 4MS and that their cyclicity is lost. The quantitative relationship between nuclear receptor levels, sterol availability, and functional output is a general problem that remains to be solved.

3) Many of the genes that are thought to exert important regulatory control can be profoundly altered in their expression patterns without significantly disrupting normal development. A great surprise was that none of the genes we investigated remained unaltered in expression during the 27–51 h period. Although this may be rationalized for the large and presumably redundant *nhr* family, it is more surprising for *daf-2* and *daf-16*. Indeed, it is even uncertain whether *ama-1* levels, the standard of reference for this and previous investigations, were unchanged by growth on 4MS, as suggested by a puzzling observation. Using a constant input of RNA (measured by OD_{260}), average real-time RT-PCR values for *ama-1* expression at all time points were 30.9 ± 0.5 cycles (n = 8/time point) for cholesterol-grown animals and 22.6 ± 0.8 cycles (n = 9/time point) for lophenol-grown animals. Also, *ama-1* values for the 48 and 51 h lophenol animals differed significantly from those of all other lophenol time points combined (25.0 vs. 21.9 cycles; $P = 0.000046$), whereas those from cholesterol animals did not (30.5 vs. 31.0 cycles; $P = 0.58$). Levels of *ama-1* expression are clearly better than OD_{260} values for normalization of our results, however, for three reasons: *i*) the nonspecific nature of OD_{260} readings generally; *ii*) none of the microarray comparisons (which are normalized to total readout) revealed any sterol-dependent change in *ama-1* levels; and *iii*) the 4MS-dependent increases in expression of the two genes showing the most dramatic effects by microarray, *grl-20* and *col-36*, were confirmed by RT-PCR assays normalized to *ama-1*. A reexamination of the suitability of this gene as a reference may be warranted for future studies, however. Finally, it should be noted that although renormalization might affect the relative positions of the cholesterol and lophenol curves in Figs. 2–7, it would not alter their shapes, except perhaps for the 48 and 51 h lophenol values.

One may speculate that this versatile animal, evolved to adapt to rapid and dramatic alterations in the environment of its compost pile home, may be able to adopt a wide array of vastly different transcriptional regimes, affecting many or even most of its genes. Such a capability might also be suggested by the extraordinarily large size of so many of its gene families.

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