Proteomic Changes during Disturbance of Cholesterol Metabolism by Azacoprostane Treatment in *Caenorhabditis elegans**

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Although nematodes like *Caenorhabditis elegans* are incapable of *de novo* cholesterol biosynthesis, they can utilize nonfunctional sterols by converting them into cholesterol and other sterols for cellular function. The results reported previously and presented here suggest that blocking of sterol conversion to cholesterol in *C. elegans* by 25-azacoprostane-HCl (azacoprostane) treatment causes a serious defect in germ cell development, growth, cuticle development, and motility behavior. To establish a biochemical basis for these physiological abnormalities, we performed proteomic analysis of mixed stage worms that had been treated with the drug. Our results from a differential display proteomic analysis revealed significant decreases in the levels of proteins involved in collagen and cytoskeleton organization such as protein disulfide isomerase (6.7-fold), β-tubulin (5.41-fold), and NEX-1 protein (>30-fold). Also reduced were enzymes involved in energy production such as phosphoglycerate kinase (4.8-fold) and phosphoenolpyruvate carboxykinase (8.5-fold), a target for antifilarial drugs such as azacoprostane. In particular, reductions in the expression of lipoprotein families such as vitellogenin-2 (7.7-fold) and vitellogenin-6 (5.4-fold) were prominent in the drug-treated worms, indicating that sterol metabolism disturbance caused by azacoprostane treatment is tightly coupled with suppression of the lipid transfer-related proteins at the protein level. However, competitive quantitative reverse transcriptase polymerase chain reaction showed that the transcriptional levels of *vit-2*, *vit-6*, and their receptors (*e.g. rme-2* and *lrp-1*) in drug-treated worms were 3- to 5-fold higher than those in the untreated group, suggesting a presence of a sterol regulatory element-binding protein (SREBP)-like pathway in these genes. In fact, multiple predicted sterol regulatory elements or related regulatory sequences responding to sterols were found to be located at the 5′-flanking regions in *vit-2* and *lrp-1* genes, and their transcriptional activities fluctuated highly in response to changes in sterol concentration. Thus, many physiological abnormalities caused by azacoprostane-mediated sterol metabolism disturbance appear to be exerted at least in part through SREBP pathway in *C. elegans*. *Molecular & Cellular Proteomics 2:1086–1095, 2003.*

Cholesterol is an essential molecule in most animals as it has diverse functions in membrane organization, production of bile salts and steroid hormones, signaling pathways (*e.g.* sonic hedgehog), and reproduction (*e.g.* sperm and oocyte development and egg laying) (1–4). Although nematodes like *Caenorhabditis elegans* are incapable of *de novo* cholesterol biosynthesis (5, 6), they can utilize a variety of nonpermissible (to nematode) sterol analogues (*e.g.* sitosterol, stigmasterol, and other phytosterols) taken from culture media or the environment, which are then converted by nematodes into so-called permissible sterol analogues such as 7-dehydrocholesterol (7-DHC)[1] following dealkylation and C-24 reduction (7). This sequential reaction seems possible in *C. elegans* because it appears to have an uncharacterized 7-cholesterol desaturase by which cholesterol is readily converted to 7-DHC, a major sterol in *C. elegans* for cellular function (7, 8).

During sterol-mediated reproduction, sterols are transported by vitellogenins to receptors such as RME proteins in the oocytes; this transfer process is very crucial (9). When exogenous sterol supply is restricted, many physiological abnormalities including growth inhibition, brood size reduction, egg-laying defects, and endomitotic (*emo*) phenocopy are easily observed (8, 10). Most of these phenomena also occur when worms are grown in the presence of sitosterol as a sterol nutrient and 25-azacoprostane-HCl (azacoprostane) (6, 11), an inhibitor of the sterol Δ^{24}-reductase (24-SR) that catalyzes conversion of desmosterol to cholesterol (12). The fact that poorly developed *C. elegans* grown in the presence of

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[1] The abbreviations used are: 7-DHC, 7-dehydrocholesterol; 25-azacoprostane-HCl, 25-aza-5β-cholestane hydrochloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; 2DE, two-dimensional electrophoresis; FBS, fetal bovine serum; LPDS, lipoprotein-deficient serum; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; 24-SR, sterol Δ^{24}-reductase; IPG, immobilized pH gradient; RT, reverse transcriptase; PEPCK, phosphoenolpyruvate carboxykinase; RME, receptor-mediated endocytosis.

azacoprostane plus sitosterol accumulated desmosterol (13, 14) suggests that sitosterol and desmosterol are nonpermissible sterol analogues that cannot substitute for cholesterol in cellular functions. Azacoprostane has also been known to inhibit viability and microfilarial production in the nematode *Brugia pahangi* (15). There was a significant reduction in growth, reproductive capability, and the percent development of the embryo to the adult in azacoprostane-treated *Caenorhabditis briggsae* (11).

These results have led to two important concepts. First, disturbance of sterol metabolism by blocking conversion of nonpermissible sterol analogues (*i.e.* sitosterol and desmosterol) to cholesterol (or 7-DHC) by azacoprostane may have resulted in a serious defect in growth and development. Second, the cause of these defects in germ cells may result from a direct effect of azacoprostane on common lipid transport proteins (*e.g.* vitellogenins) and their receptors (LRP-1 and RME-2) involved in a sterol-mediated reproductive system.

Two remaining issues relate to direct evidence for azacoprostane-induced sterol metabolism disturbance and the relationships between accumulation of nonpermissible sterols by azacoprostane treatment and expression of lipid transfer proteins or sterol receptor proteins. To address these issues, first, we have analyzed the proteomic profile resulting from disturbance in cholesterol metabolism by azacoprostane treatment in *C. elegans*. Second, we analyzed the gene expression of selected proteins that were differentially expressed by drug treatment. Despite the importance of sterol transport in *C. elegans* development, no detailed studies on sterol metabolism at both the proteomic and molecular levels have been reported. Here we show that azacoprostane treatment caused a substantial reduction in levels of both lipoproteins (*e.g.* VIT-2 and VIT-6) and their receptor proteins (*e.g.* LRP-1 and RME-2), while their gene expression levels *in vivo* were paradoxically induced. Consequently, our *in vitro* transcriptional assays provide evidence that the transcriptional activation of *vit-2* and *lrp-1* genes containing sterol regulatory element (SRE) (16) in their 5′-flanking regions was due to their sensitive response to sterol concentration in media.

EXPERIMENTAL PROCEDURES

Nematode Culture—The wild-type *C. elegans* (N2) were grown at 20 °C in S medium (50 mM phosphate buffer, pH 6.0, 0.1 M NaCl, 10 mM potassium citrate, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 0.05 mM EDTA, 0.025 mM $FeSO_4$, 0.01 mM $MnCl_2$, 0.01 mM $ZnSO_4$, and 0.001 mM $CuSO_4$) with 5 μg/ml β-sitosterol (Steraloids Inc., Newport, RI) using the OP50 strain of *Escherichia coli* as a food source. The worms were grown for 8 days during which they were supplied with both 25-azacoprostane hydrochloride that was synthesized previously (13) and β-sitosterol at the concentration of 5 μg/ml (14). Following this period, worms were harvested by centrifugation and separated from remaining bacteria by flotation on 35% sucrose. After being washed with M9 buffer (44 mM KH_2PO_4, 21 mM Na_2HPO_4, 86 mM NaCl, pH 7.0, and 1 mM $MgSO_4$), mixed stages of worms were stored at −70 °C until use.

Morphological Visualization of C. elegans following Treatment with Azacoprostane—After the wild-type N2 worms were grown for 8 days at 20 °C in S medium containing azacoprostane and sitosterol at the concentration of 5 μg/ml (14) as described above, mixed stages of worms were collected with M9 buffer, anesthetized by 0.2 mM levamisole, and transferred to a glass slide to observe the morphological changes. To visualize worm nuclei, the worms were transferred to spots containing 1 μl of water on a polylysine-coated slide. The slide was flamed briefly to evaporate the water. The dried worms were visualized in a drop of 10 μg/ml Hoechst 33343 (Sigma) in M9 buffer under a coverslip. Each sample on the slide was examined and photographed using a Zeiss Axioskop (Carl Zeiss) microscope.

Sample Preparation for Two-dimensional Electrophoresis (2DE)—Worms were washed with distilled water and suspended with an appropriate volume of sample Buffer A containing 50 mM Tris, 5 mM EDTA, 7 M urea, 2 M thiourea, 4% CHAPS, and protease inhibitor. Suspensions were sonicated for ~30 s on ice, and the soluble fractions were collected by centrifugation at 36,000 × *g* for 40 min at 4 °C. Protein concentration of the soluble fraction was determined by the Bradford method (17) using bovine serum albumin as a standard. Aliquots were stored at −70 °C until use.

2DE—Protein samples (100 μg for analytical gels and 1 mg for preparative gels) were suspended in sample Buffer B containing 7 M urea, 2 M thiourea, 2% v/v of IPG buffer, pH 3–10 nonlinear (Amersham Biosciences), 2% CHAPS, 15 mM dithiothreitol, and a trace of bromphenol blue to obtain a final volume of 350 μl. Aliquots of *C. elegans* proteins in sample buffer were applied onto the IPG strip (Immobiline Dry strip, pH 3–10 nonlinear, 18 cm; Amersham Biosciences) that had been rehydrated with a sample protein solution at 20 °C for 14 h. Isoelectric focusing was performed at 20 °C under a current limit of 50 μA/strip as follows: 100 V for 2 h, 300 V for 2 h, 1000 V for 1 h, 2000 V for 1 h, and then continuous at 3500 V until reaching optimal voltage hour (Vh). Focusing was carried out for a total of 43,000 Vh. For preparative samples, focusing was achieved with a total of 67,000 Vh. IPG strips were equilibrated for 20 min by gently shaking in 375 mM Tris-HCl, pH 8.8, containing 6 M urea, 2% SDS, 5 mM tributyl phosphine, 2.5% acrylamide solution, and 20% glycerol. In the second dimension of electrophoresis, vertical SDS gradient slab gels (9–16%, dimensions 180 × 200 × 1.5 mm) were used. The equilibrated IPG strips were cut to size; then the second-dimensional gels were overlaid with a solution containing 0.5% agarose, 24.8 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS, and a trace of bromphenol blue. Electrophoresis was conducted at a constant 15 mA/gel. After protein fixation in 40% methanol and 5% phosphoric acid for at least 1 h, the gel was stained with Coomassie Brilliant Blue G-250 overnight. After destaining, the gel image was obtained using a GS-710 image scanner (Bio-Rad). The gel images were processed with Melanie 3 software (GeneBio).

Matrix-assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry Analysis—Spots on the gels were excised with end-removed pipette tips to accommodate various spot diameters. The gel slice in the microtube was destained and dehydrated with 50 μl of acetonitrile for 5 min at room temperature. The dried gels were rehydrated with 10 μl of trypsin solution (10 μg/ml in 25 mM ammonium bicarbonate, pH 8.0) for 45 min on ice. After removing the excess solution, proteins in the gels were digested at 37 °C for 24 h. The peptide mixtures thus obtained were treated with POROS R2 beads (18). Then the digested peptides were analyzed by Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA). About 0.5 μl of α-cyano-4-hydroxycinnamic acid was mixed with the same volume of sample. Time-of-flight measurement used these parameters: 20 kV of accelerating voltage, 75% grid voltage, 0% guide wire voltage, a 120-ns delay, and a low mass gate of 500 Da. Internal calibration was also performed using autodigestion peaks of porcine trypsin (M^+H^+, 842.5090 and 2211.1064). The peptide mass profiles produced by MALDI mass spectrometry were analyzed using search programs

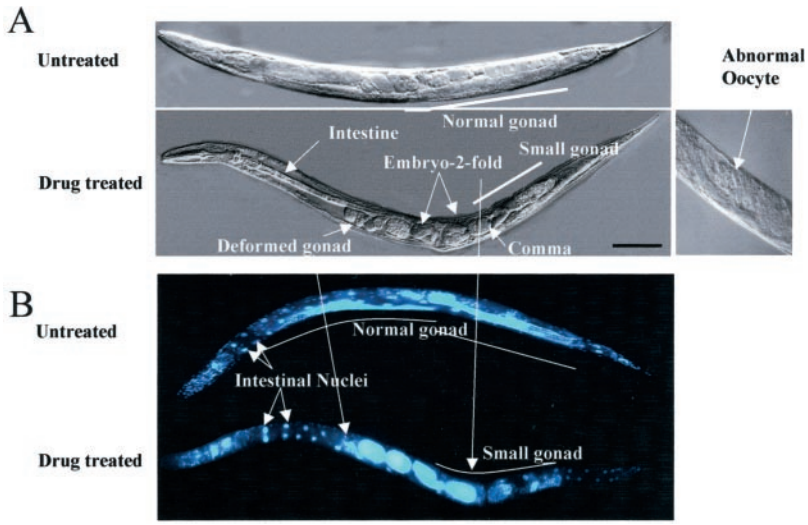

FIG. 1. **Morphological changes in *C. elegans* resulting from treatment with azacoprostane.** *A*, Nomarski view of untreated and drug-treated whole wild-type worms, revealing azacoprostane-induced abnormal gonad, normal and abnormal embryos, embryos hatched inside the body (egg-laying defect), and abnormal oocytes. *B*, micrographs of nuclei in the two groups of worms stained with Hoechst 33343, revealing a small gonad in the posterior region. *Scale bar*, 20 μm.

such as MS-Fit 3.2 provided by the University of California-San Francisco (prospector.ucsf.edu/) and ProFound (Version 4.7.0) provided by The Rockefeller University (129.85.19.192/profound_bin/WebProFound.exe) with the National Center for Biotechnology Information (NCBI) database. A mass tolerance of 20 ppm was used for masses measured in reflector mode.

Competitive Reverse Transcriptase Polymerase Chain Reaction—For quantitative analysis of mRNA of the proteins identified by 2DE, quantitative reverse transcriptase (RT) polymerase chain reaction was carried out using target template RNA plus mimic RNAs (which share the same primer-annealing sites but are different in length) as reported previously (19). In the current study, *C. elegans* genomic DNA of phosphoenolpyruvate carboxykinase (PEPCK), VIT-2, VIT-6 precursor, RME-2, and LRP-1 fragments containing introns of 103, 52, 91, 94, and 104 base pairs, respectively, were used as mimics. Mimic mRNAs were added onto the reaction at the reverse transcription stage to control for cDNA synthesis efficiency as well as for PCR. Each mimic DNA was prepared from genomic DNA of *C. elegans* by PCR and was used for preparing cRNA by *in vitro* transcription. After the cRNAs were mixed with total RNA from each condition (with or without azacoprostane), first stranded cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase. Then PCR amplification with the same primer sets was performed. After the PCR reaction, the resulting PCR amplification products were visualized by ethidium bromide in a 3% agarose gel and quantified using an ImageMaster program (Amersham Biosciences).

Genomic DNA and Mimic Construction—Genomic DNA fragments used as mimics in the quantitative RT-PCR were initially amplified from genomic DNA of *C. elegans* extracted from wild-type worms using a genomic DNA isolation kit (Nucleogen, Incheon, Korea) according to the manufacturer's instructions. The PCR mixture contained genomic DNA template, TAKARA Ex *Taq*DNA polymerase (TAKARA, Otsu, Shiga, Japan) in the presence of a 1.5 mM Mg^{2+} buffer (pH 7.9), 100 μM dNTPs, and 0.4 μM each primer: PEPCK (forward, 5′-GGAGAGCCAGGAGTTGCTGCTCA-3′; reverse, 5′-CGGAACCAATTGACGTGGTAG-3′), VIT-2 (forward, 5′-GAGAAGGACACCGAGCTCATCC-3′; reverse, 5′-TCTCGACTTCTTGGATTTGCTC-3′), VIT-6 precursor (forward, 5′-CTCTCTTGGGAGCGGCACTCG-3′; reverse, 5′-CTCTTGGTGCTCACGGTTCATGC-3′), RME-2 (forward, 5′-GCAACAACAAAATGTTCATGTC-3′; reverse, 5′-GTTGTCGAGAAGGATTCTGAC-3′), and LRP-1 (forward, 5′-ACTGCAGTTCCCAAAATGTATT-3′; reverse, 5′-CACAACGGAATTTTCGAGGTTG-3′). The PCR was carried out in the GeneAmp 2400 PCR thermal cycler (PerkinElmer Life Sciences) using 30 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min. The resulting PCR fragments were ligated into pGEM-T Easy vector (Promega). The nucleotide sequence of each plasmid was confirmed by DNA sequencing analysis using the Big Dye Terminator sequencing method (PerkinElmer Life Sciences).

cDNA Synthesis—Total RNA was extracted from 0.2 g of frozen worms using TRI reagent (Molecular Research Center) after crushing by mortar and pestle in liquid nitrogen. The RNAs were treated with DNase I (TAKARA) and extracted with phenol/chloroform. Absence of genomic DNA was confirmed by PCR with a sample of total RNA using the primers described above. The first strand of cDNA was synthesized using 1 μg of total RNA, Moloney murine leukemia virus reverse transcriptase (Invitrogen), 1 mM dNTPs, 0.5 μg of random hexamers (Promega), and 20 units of RNasin (Promega) in a 20-μl volume. The mixture was incubated at 37 °C for 1 h, and the reactions were terminated by heating at 95 °C for 5 min. PCR was carried out as described above using 1 μl of the cDNA. The nucleotide sequences of amplified fragments were confirmed by DNA sequencing analyses as described above.

cRNA Preparation—One μg of the template plasmid containing genomic DNA mimics was transcribed using the Riboprobe *in vitro* transcription system (Stratagene) according to manufacturer's instructions. DNA templates were removed by DNase I treatment at 37 °C for 30 min. The cRNA was subsequently purified by phenol/chloroform extraction and stored at −80 °C. The absence of DNA contamination was established by performing PCR on the cRNA using a 30-cycle reaction.

mRNA Expression—cDNA synthesis was achieved using 2 μg of total RNA from either the azacoprostane-treated or the untreated control worms and using the cRNAs of PEPCK, VIT-2, VIT-6 precursor, RME-2, and LRP-1 as internal standards in the reaction. Initially, 1.83 fmol of PEPCK, 2.04 fmol of VIT-2, 2.5 fmol of VIT-6 precursor, 1.95 fmol of RME-2, 2.15 fmol of LRP-1 mimic cRNA, and 0.2 μg of random hexamers (Promega) were incubated at 70 °C for a 5-min period. This was followed by the addition of 40 units of Moloney murine leukemia virus reverse transcriptase and its buffer (Invitrogen) and 30 units of ribonuclease inhibitor (Amersham Biosciences). Incubation was conducted at 25 °C for 15 min, 37 °C for 1 h, and 95 °C for 5 min. At the end of incubation, reaction products were diluted to 50 μl. PCR was performed in a 50-μl volume containing 10 μl of the diluted cDNA synthesis reaction, TAKARA Ex *Taq*DNA polymerase and its buffer (1.5 mM Mg^{2+}, pH 7.9), 1 mM dNTPs, and 1 μM each primer set. The thermal cycling program was run for 23 cycles at 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 45 s. The resulting PCR amplification products were visualized by ethidium bromide in a 3%

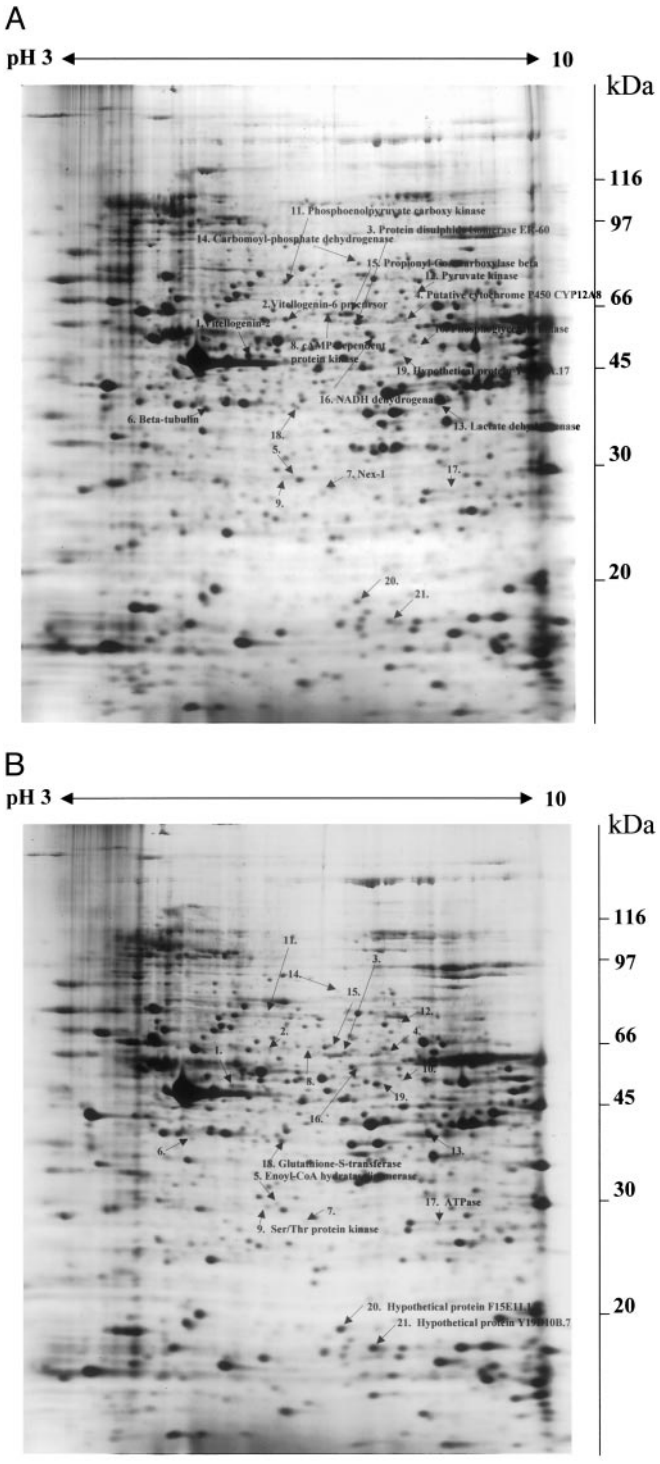

FIG. 2. **2DE gel pattern of protein extracted from mixed stages of *C. elegans*.** *A*, 2DE gel image of untreated worms. Proteins extracted from wild-type worms were separated on a nonlinear pH 3–10 IPG strip followed by a 9–16% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Each labeled numeral indicates a protein for which the expression level was up- or down-regulated. *B*, 2DE gel image of drug-treated worms. Each numeral indicates a protein for which the expression level was up- or down-regulated by treatment of azacoprostane.

agarose gel and quantified using an ImageMaster program (Amersham Biosciences). The amount of unknown template RNA was calculated from the ratio of template/mimic band intensities as the amount of mRNA in amol/μg of total RNA.

Construction of Luciferase Reporter Vector for Promoter Assay of vit-2 and lrp-1 Genes—To analyze *vit-2* (20, 21) and *lrp-1* (GenBank™) gene promoters, the 2.65 and 2.36 kb of the 5′-flanking region of each gene were ligated into the luciferase reporter vector as follows. At the initial stage of this work, the 5′-flanking region of each gene was isolated from *C. elegans* genomic DNA by PCR. The PCR mixture contained genomic DNA template, *pfu* DNA polymerase (Stratagene), 1.5 mM Mg^{2+} buffer (pH 7.9), 100 μM dNTPs, and 0.4 μM each primer: the 5′-flanking region of *vit-2* (forward, 5′-GTGGACAGGTACCAAACGGAACATACTGGA-3′; reverse, 5′-AGGAAGATCTGGCTGAACCGTGATTGGACTGTTT-3′) and the 5′-flanking region of *lrp-1* (forward, 5′-CCGGGGTACCTATCTCTGACCGATGGACACG-3′; reverse, 5′-AGGAAGATCTTCGAAGCATTTGATGGTGGTGA-3′). PCR was carried out in the GeneAmp 2400 PCR thermal cycler (PerkinElmer Life Sciences) using 30 cycles of 94 °C for 45 s, 55 °C for 5 min, and 72 °C for 1 min. PCR products were digested with *Kpn*I and *Bgl*II, and the DNA fragments were ligated into the *Kpn*I and *Bgl*II sites of the luciferase vector pGL3-basic (Promega). All plasmids were verified by DNA sequencing.

Cell Culture and Transient Transfection of Luciferase Reporter Genes—H4IIE cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 5% (v/v) FBS, 1 mM glutamine, and 10 μg/ml gentamycin in a 5% CO_2 incubator at 37 °C (22). Cells plated onto 12-well plates were grown to 50–80% confluence before transfection. One μg of test constructs was co-transfected into H4IIE cells with 0.2 μg of *Renilla* luciferase control vector, pRL-SV40 (Promega), using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. H4IIE cells were transferred to serum-free medium and grown for 3 h and then were switched to a medium containing either 10% FBS (sterol-supplied) or 5% LPDS (sterol-depleted). After incubation for 24 h with the appropriate medium, cells were harvested, and extracts were assayed in triplicate for luciferase activity.

Preparation of Cell Extracts and Luciferase Enzyme Assays—Following transfection, the cells were washed with phosphate-buffered saline and lysed in 0.2 ml of 1× passive lysis buffer (Promega). Cell extracts were assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase® Reporter assay system according to the manufacturer's instructions (Promega). Amounts of lysates employed for the firefly luciferase activity assays of test constructs were normalized to the *Renilla* luciferase activities and the amount (mg) of proteins of cell lysates.

RESULTS

Morphological Changes in C. elegans by Treatment of Azacoprostane—To investigate the effects of sterol metabolism disturbance on the morphology of *C. elegans*, mixed stages of worms were grown in 5 μg/ml azacoprostane and examined under Nomarski optics with and without 4′,6-diamidino-2-phenylindole staining. As anticipated from the previously reported case of *C. briggsae* (11), there was a significant reduction in brood size (average 30% decrease, $n = 20$) and an increase in embryonic lethality (average 80% increase, $n = 20$) and growth retardation in the azacoprostane-treated group (data not shown). Furthermore, serious defects in gonads (*i.e.* decrease in germ nuclei of the gonad arm) and oocytes (unfertilized oocyte) were clearly observed (Fig. 1*A*).

TABLE I

Identified proteins that exhibited significant differential expression in C. elegans following treatment with 25-azacoprostane-HCl

Identification no. in gel[a]	Name of protein	Changed -fold[b] ($n = 3$)	GenBank™ accession no.	Molecular mass (Da)/pI	Coverage	Matched peak
					%	
Lipid metabolism/transport-related proteins						
1	Vitellogenin-2 (M10105)	−7.78 ± 0.77	156493	11,060/6.3	62	5
2	Vitellogenin-6 precursor	−5.45 ± 0.54	552073	30,164/6.0	42	8
3	Protein disulphide isomerase (NM_059594)	−6.72 ± 0.20	17507915 M	54,921/6.0	29	12
4	Putative cytochrome P450, CYP13A8	−2.54 ± 0.11	Q27516	58,566/8.4	20	6
5	Enoyl-CoA hydratase/isomerase (NM_062047)	+3.1 ± 0.22	17536985 M	32,976/6.9	45	12
Cytoskeletal structure/membrane trafficking						
6	β-tubulin (NM_066966)	−5.41 ± 0.16	17553980 M	50,239/4.8	40	20
7	NEX-1, ZC155.1.p (NM_065708)	−30.14 ± 0.89	17554342 M	35,696/6.1	53	12
Cell-signalling proteins						
8	cAMP-dependent protein kinase (NM_069057)	−2.53 ± 0.12	17538604 M	66,772/6.0	20	8
9	Ser/Thr protein kinase (NM_067738)	+5.67 ± 0.16	17539480 M	39,125/6.0	14	5
Carbohydrate metabolism- and energy metabolism-related proteins						
10	Phosphoglycerate kinase (NM_058844)	−4.83 ± 0.13	17508823 M	44,113/6.5	33	12
11	Phosphoenolpyruvate carboxykinase (NM_059773)	−8.47 ± 0.84	17508605	73,432/5.8	57	12
12	Pyruvate kinase (NM_060058)	−2.94 ± 0.09	17506831 M	65,080/7.0	34	16
13	Lactate dehydrogenase (NM_072255)	−3.12 ± 0.09	17561064 M	35,806/6.6	44	11
14	Carbamoyl-phosphate carboxylase (NM_069376)	−4.82 ± 0.25	17540106 M	75,660/6.0	35	24
15	Propionyl-CoA carboxylase β (NM_076127)	−6.58 ± 0.20	17568007 M	58,495/6.2	39	16
16	NADH dehydrogenase (NM_066163)	−4.63 ± 0.13	17554064 M	50,862/6.0	39	19
17	ATPase (NM_068639)	+3.95 ± 0.16	17538698 M	25,587/6.8	43	7
Other proteins						
18	Glutathione *S*-transferase (NM_064716)	+2.67 ± 0.16	17537489 M	23,603/5.5	39	10
19	Hypothetical protein Y47D3A.17	−2.87 ± 0.09	7509907	51,265/7.3	19	6
20	Hypothetical protein F15E11.1 (AF039045)	+2.19 ± 0.18	14625150	17,428/6.0	53	6
21	Hypothetical protein Y19D10B.7 (AC006723)	+3.98 ± 0.15	N7322367	17,184/6.3	50	7

[a] The identification number indicates the protein spot in the 2DE master gel.

[b] The value represents the mean ± S.D. ($n = 3$) that was obtained from the three different gels.

In particular, embryos hatched inside the body were evidence of an egg-laying defect (Fig. 1*A*, *bottom*). Although gonad size was relatively smaller in the drug-treated worms than in the untreated group, sperm production seemed normal in treated worms (data not shown). The cuticle appeared poorly developed, indicating a possible suppression of cuticular proteins (Fig. 1*B*). This abnormal cuticle development coincided with reduced motility in the drug-treated group (data not shown). Therefore, azacoprostane treatment seemed to cause an insufficient sterol supply leading to serious defects in growth, development, and the sterol-mediated reproductive system in *C. elegans*, as consistent with previous reports (8, 11).

Proteomic Changes in Expression of Proteins Involved in Morphology and Motility by Azacoprostane Treatment in C. elegans—To explore a biochemical basis for the defects in morphological, structural, and functional defects that were believed to be caused by sterol metabolism disturbance with azacoprostane treatment, proteomic analysis was performed using mixed stage worms. Fig. 2 is a typical 2DE gel image showing separation of proteins from untreated worms (Fig. 2*A*) and azacoprostane-treated worms (Fig. 2*B*). More than 1000 protein spots were detected on 2DE gels, and each spot was localized in the ranges of pI 3–10 and M_r 10,000–200,000. The spots in 2DE were isolated, digested with trypsin, and then analyzed by MALDI-TOF. Protein identifications were validated by mass fingerprinting of selected peptide peaks by applying low tolerance (<20 ppm) with recalibration. The location of the corresponding peak for each protein and its expected mass and pI were also confirmed (Table I).

Based on the protein spots that were differentially changed more than 2-fold by drug treatment as identified with Melanie 3 software analysis, we focused on proteins with these criteria: 1) proteins involved in synthesis of structural components (*e.g.* collagen of cuticle, cytoskeleton) and cell signaling, 2) proteins involved in energy production, and 3) proteins involved in the development and reproduction of *C. elegans*, which might have caused reduced motility, morphological abnormality, and growth retardation in azacoprostane-treated *C. elegans* (Table I). First, for the structural and morphological defects that might be caused by sterol metabolism disturbances in *C. elegans*, we found protein disulfide isomerase (6.7 ± 0.2-fold down, $n = 3$), β-tubulin (5.41 ± 0.16-fold down, $n = 3$), and NEX-1 (30.14 ± 0.89-fold down, $n = 3$) in the azacoprostane-treated worms. To determine the cause of motility reduction in azacoprostane-treated groups, enzymes involved in energy production were examined. Significant re-

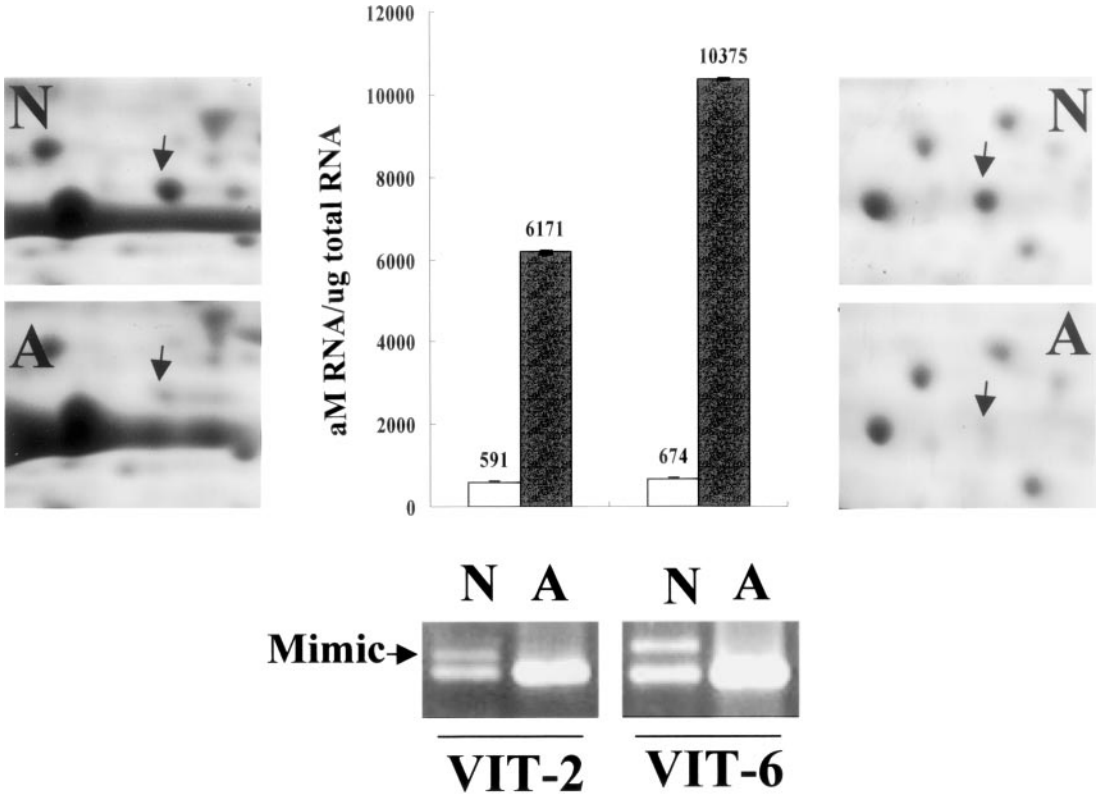

FIG. 3. **Protein and mRNA expression of VIT-2 and VIT-6 proteins following treatment of *C. elegans* with azacoprostane.** VIT-2 and VIT-6 protein levels are visible on the gels on the *left* and *right* sides of the figure. Shown at the *center* are mean mRNA levels that were calculated as described under "Experimental Procedures." VIT-2 and VIT-6 mRNA levels were 591 ± 9.77 and 674 ± 27.7 amol of mRNA/μg of total RNA (n = 3) at the normal (*N*) condition but were 6171 ± 23.79 and 10375 ± 26.25 amol of mRNA/μg of total RNA (n = 3) after azacoprostane (*A*) treatment. The data is expressed as amol (10^{-18} mol) of mRNA/μg of total RNA.

ductions in glycolytic enzymes such as phosphoglycerate kinase (4.8-fold down, the first ATP-forming step in glycolysis), lactate dehydrogenase (3.1-fold down), and PEPCK (8.5-fold down), an important regulatory enzyme in gluconeogenesis and a target of the antifilarial centperazine, were found (23). Compared with the decrease in glycolytic enzymes, ATPase was increased by 3.95-fold. Other enzymes that were decreased by azacoprostane treatment include a propionyl-CoA carboxylase β (6.6-fold down), a mitochondrial, biotin-dependent enzyme involved in the catabolism of amino acids, odd chain fatty acids, and other metabolites, and cytochrome P450 (2.54-fold down). In contrast, azacoprostane treatment increased glutathione *S*-transferase, a major defense against reactive oxygen species, adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase), ATP-dependent RNA helicase, tRNA processing protein (SEN3), 40 S ribosomal protein, elongation factor Tu family, carbamoyl-phosphate carboxylase, and glucosamine-6-phosphate deaminase.

Differential Expression of Lipid Transfer Proteins and Their Genes—For the functional defects that might also be caused by sterol metabolism disturbance in *C. elegans*, we examined a basis for defects in the sterol-mediated reproductive system in which lipid transfer proteins are known to be involved (9, 24). The lipoproteins vitellogenin-2 (VIT-2) and vitellogenin-6 (VIT-6), two apo-B 100 homologues in *C. elegans*, were more than 7-fold (n = 3) and 5-fold (n = 3) reduced in azacoprostane-treated worms than in untreated worms (Table I). To detect any correlation between suppression of these proteins, VIT-2 and VIT-6, and corresponding gene expression, we performed competitive quantitative RT-PCR using mimic DNA of each protein as an internal standard. Fig. 3 shows the relative level of transcription and protein expressions of *vit-2* and *vit-6*. Surprisingly, in contrast to the suppression of proteins, transcriptional levels of *vit-2* and *vit-6* genes in azacoprostane-treated worms were at least 5-fold higher than those in the untreated group. The transcriptional up-regulation of these genes might be caused by deprivation of regulatory sterols that trigger their response elements in azacoprostane-treated worms. We further examined transcriptional levels of their receptors, RME-2 and LRP-1, which are in fact not detected in 2DE gels. As anticipated, transcripts of *rme-2* and *lrp-1* were also increased by treatment of azacoprostane, as seen in the cases of *vit-2* and *vit-6* genes (Fig. 4). This result strongly suggests that there may be a SREBP pathway regulating the genes of vitellogenins and their receptors, which might be activated by sterol depriva-

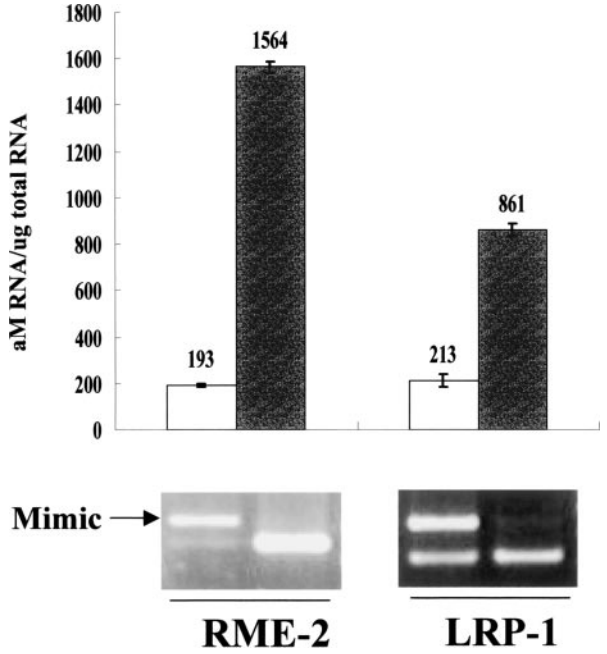

FIG. 4. **Altered patterns of protein and mRNA expression of RME-2 and LRP-1 proteins following treatment of *C. elegans* with azacoprostane.** Mean mRNA levels are shown at the top of each bar. RME-2 and LRP-1 mRNA levels were 193 ± 7.39 and 213 ± 21.9 amol of mRNA/μg of total RNA ($n = 3$) under normal (*N*) conditions but increased to 1564 ± 27.2 and 861 ± 26.5 amol of mRNA/μg of total RNA ($n = 3$) after azacoprostane (*A*) treatment. The data are expressed as amol (10^{-18} mol) of mRNA/μg of total RNA.

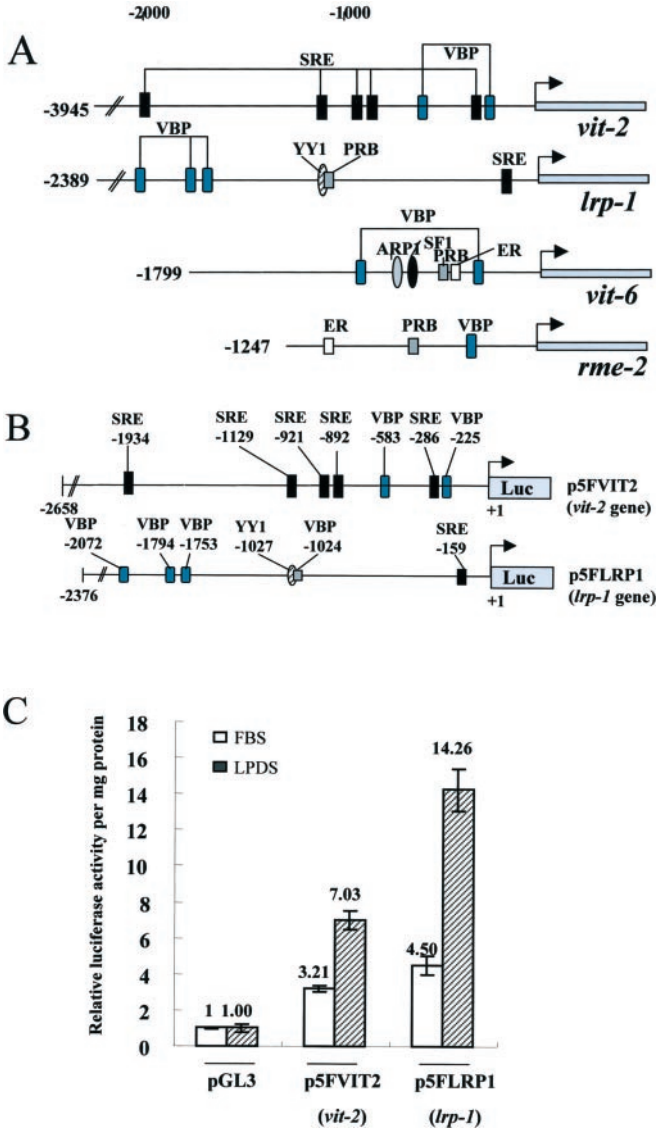

FIG. 5. **Putative transcriptional regulator-binding sites and sterol-mediated promoter activity of *vit-2* and *lrp-1* genes.** *A*, the numbers to each side of the *solid lines* indicate the nucleotide positions in base pairs relative to the translation start site. *SRE*, *VBP*, *ARP1*, *SF1*, *PRB*, *ER*, and *YY1* indicate sterol regulatory element-binding sites, retinoic acid receptor-type chicken vitellogenin promoter-binding protein sites, an apolipoprotein A1 regulatory protein 1-binding site, a steroidogenic factor 1-binding site, progesterone receptor-binding sites, estrogen receptor-binding sites, and a Yin and Yang 1-binding site, respectively. *B*, the 5′-flanking regions of the *vit-2* (−2658/+1) and *lrp-1* (−2376/+1) genes were ligated into a pGL3 vector, and their resulting chimeric reporter constructs (*e.g.* p5FVIT2 and p5FLRP1) were transfected into rat hepatoma cell lines (H4IIE cells). *C*, cells were grown in the presence (in FBS) or absence (in LPDS) of cholesterol in media. Luciferase assays ($n = 3$, each in triplicate) were performed, and their relative luciferase activities were measured.

tion. A major question is whether *C. elegans* contains any SRE-like sequences within these genes that are subject to transcriptional regulation. After extensive searching through GenBank™ for SRE-like sequences in genes encoding lipid transfer proteins or their receptors, we found five predicted SREBP sites and one SREBP site located in the *vit-2* and *lrp-1* genes, respectively (Fig. 5*A*) (MatInspector, www.genomatix.de). However, there is no predicted SREBP site in *vit-6* and *rme-2*, which instead possess an SF-1 (steroidogenic factor) site (*vit-6*) and estrogen receptor-binding and progesterone receptor-binding sites (*rme-2*) (Fig. 5*A*). To examine a basis for sterol-mediated transcriptional activation of the SRE-containing genes *vit-2* and *lrp-1* as depicted in Fig. 5*B*, chimeric luciferase reporter genes containing their 5′-flanking regions (*i.e.* p5FVIT2 (−2658/+1) for *vit-2* gene and p5FLRP1 (−2376/+1) for *lrp-1* gene) were constructed and transfected into H4IIE cells grown in either LPDS (sterol-depleted) or FBS (sterol-supplied) medium for examining their sterol responsiveness (Fig. 5*C*). The promoters of both *vit-2* (2.19-fold induction, $n = 3$) and *lrp-1* (3.17-fold induction, $n = 3$) genes were activated by sterol depletion (in LPDS medium), suggesting a presence of the functional SREBP pathway in *C. elegans* (Fig. 5*C*). To determine whether this sterol depletion-mediated transcriptional activation of *vit-2* and *vit-6* genes can be reversed by supplying cholesterol back to the medium, worms that had been grown in the presence of azacoprostane for 5 days received 5 μg/ml cholesterol and were further incubated for an additional 3 days, while control worms were grown in the presence of the drug for 8

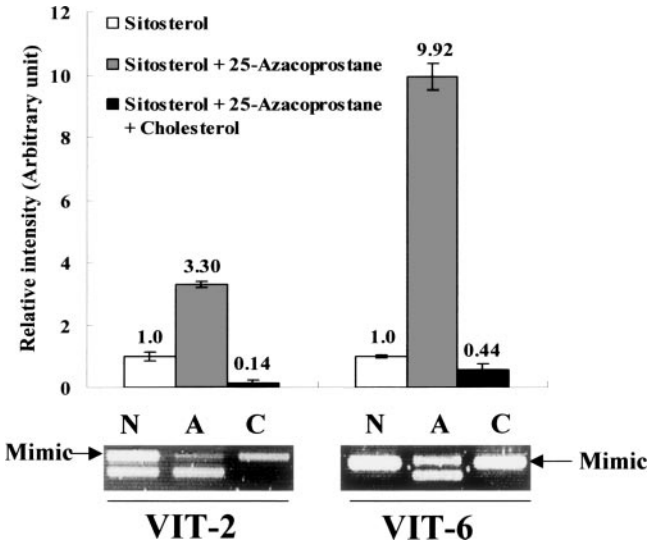

FIG. 6. **Reverse regulation of transcriptional levels by addition of permissive sterol (cholesterol) after treatment of 25-azacoprostane.** After worms were seeded in the S medium containing sitosterol with or without 25-azacoprostane-HCl at the concentration of 5 μg/ml, each group was incubated for 8 days at 20 °C. At the end of the 5th day, one of the groups received 5 μg/ml cholesterol and was incubated for 3 days. Total RNA was prepared from both groups at the 8th day of incubation and was analyzed for transcriptional activity using competitive RT-PCR as described under "Experimental Procedures." *N*, sitosterol; *A*, sitosterol + 25-azacoprostane-HCl; *C*, sitosterol + 25-azacoprostane-HCl + cholesterol.

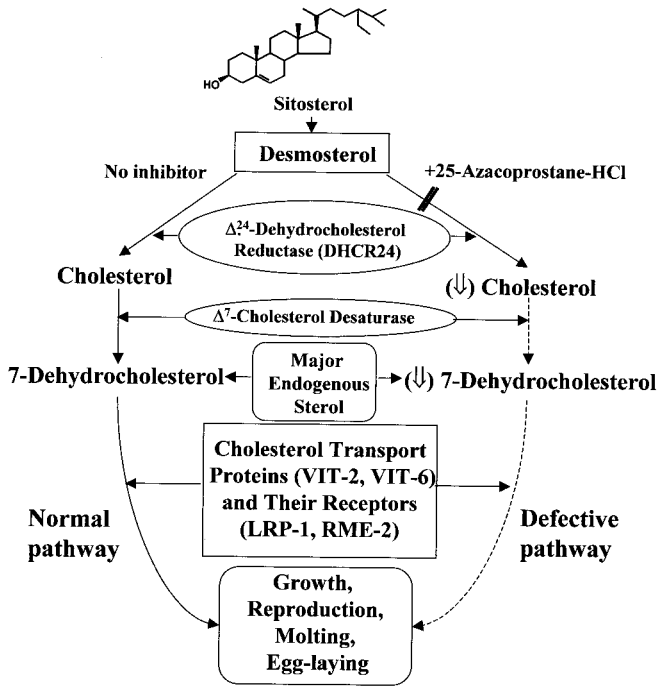

FIG. 7. **Schematic diagram of the relationship between cholesterol deprivation and defective phenotypes.**

days. In addition, the third group of worms was grown only in the presence of sitosterol. Competitive RT-PCR was performed using the total RNA isolated from three different groups of worms, and the relative transcriptional activities of *vit-2* and *vit-6* were analyzed. As shown in Fig. 6, the promoter activity of *vit-2* and *vit-6* was drastically suppressed (*e.g.* 23.6-fold decrease (from 3.3 to 0.14) for the *vit-2* promoter and 22.5-fold decrease (from 9.92 to 0.44) for the *vit-6* promoter), suggesting that there is a reversible response to change in sterol concentration in *C. elegans*.

DISCUSSION

C. elegans is routinely propagated on agar plates containing cholesterol, a structural and functional constituent of the plasma membrane in cells of other animals. Worms grown on cholesterol-depleted plates display defects in molting (24), growth, development, and egg laying (8), which are typical phenomena resulting from defects in a sterol-mediated reproductive system. The initial purpose of our study was to identify proteins uniquely or differentially expressed before and after sterol metabolism was disturbed by treatment of *C. elegans* with azacoprostane. Four major points can be addressed with our results.

The first point relates to the structural importance of sterol analogues for cellular function in *C. elegans*. In previous research, azacoprostane-treated *C. elegans* accumulated desmosterol and other Δ^{24}-sterols and exhibited decreases in growth and reproduction (13, 14), suggesting that the only direct mode of inhibition is upon *C. elegans* 24-SR activity. That is, sitosterol or desmosterol cannot substitute for cholesterol or 7-DHC as a permissible sterol for cellular function in *C. elegans* under sterol deprivation conditions (Fig. 7). Similarly, human desmosterolosis, an autosomal recessive disorder characterized by multiple congenital anomalies and caused by mutation in the 24-SR gene (25–27), is characterized by an accumulation of desmosterol in plasma. Thus, critical functions of sterols depend on steroid ring structures in *C. elegans* as well as humans. However, it is curious that 7-DHC seems to be permissible as a cholesterol substitute in *C. elegans* but is not tolerable to humans in large quantities. For example, Smith-Lemli-Opitz syndrome results from defective 7-dehydrocholesterol reductase, which normally catalyzes the reduction of 7-DHC to cholesterol; the syndrome is characterized by accumulation of 7-DHC in plasma and tissue (28, 29). Some inhibitors of cholesterol biosynthesis enzymes (*e.g.* DHCR and sterol 8-isomerase) can also exhibit teratogenic effects (30–32).

The second point relates to the behavioral and morphological defects caused by azacoprostane treatment. Significant reductions in the levels of protein disulfide isomerase (6.7-fold down), β-tubulin (5.4-fold down), NEX-1 (>30-fold down), and proteins involved in energy production appear to relate to the observed behavioral and morphological abnormality in which motility was significantly decreased in the drug-treated group. For example, protein disulfide isomerase is a prolyl 4-hydroxylase, an enzyme that catalyzes the hydroxylation of prolines

in procollagen during the synthesis of collagen (33). Because the major nematode cuticle protein is collagen, suppression of this protein by azacoprostane treatment could also explain some of the structural abnormalities observed in the cuticle. Similarly, β-tubulin is a component of microtubules, which are essential for patterning cytoskeletal and extracellular structures (34). Actin filaments and microtubules affect the pattern of cuticle formation during postembryonic molts (35). The decrease in these proteins may explain why motility in azacoprostane-treated worms was significantly decreased. NEX-1 protein binds to membranes, aggregates vesicles in a calcium-dependent fashion, and contains a binding site for calcium and phospholipids through which a major pathway for communication between cellular membranes and their cytoplasmic environment can be achieved (36). Thus, reduction of NEX-1 might also have caused a down-regulation of RME-2 or LRP-1 and a subsequent decrease in the endocytosis-mediated transport of cholesterol-carrying vitellogenin into growing oocytes in *C. elegans* (see below). A negative change in glycolytic enzymes involved in energy production may be directly correlated to the suppression of growth rate in azacoprostane- or alkylamine-treated *C. elegans* and *C. briggsae* (11). Reduction in these enzymes may confirm abnormality in morphology and motility of drug-treated worms.

The third point is the important role of sterol transfer proteins coupled to sterol function in *C. elegans*. Besides cholesterol biosynthesis, cholesterol transport is also an essential process in the development and reproduction of nematodes, which require specialized cholesterol transport proteins such as vitellogenins and their receptors like RME-2 (9). Vitellogenins were first identified as interacting partners of cholesterol in *C. elegans* through use of *rme-2* worms lacking the vitellogenin receptor (37), which failed to accumulate the fluorescent probe dehydroergosterol in oocytes and embryos (9). Vitellogenins are usually synthesized outside the ovary and then transported to the growing oocyte, where they are selectively bound to the receptors RME-2 and LRP-1 through receptor-mediated endocytosis (9, 24, 37). Suppression of these critical proteins (*e.g.* VIT-2 (7.7-fold down) and VIT-6 (5.4-fold down)) involved in lipid transfer in azacoprostane-treated worms may be partly related to the observed reproductive defects (Fig. 1). The reduction of cAMP-dependent protein kinase and Ser/Thr protein kinase, which are involved in cell signaling in lipogenesis, was also noted. Taken together, these results indicate that starvation of cholesterol or its functional analogue (*e.g.* 7-DHC) by blocking the conversion of sitosterol to these sterols with azacoprostane treatment might have directly caused a significant reduction in the expression of lipid transfer proteins. Therefore, a reasonable speculation is that depletion of cholesterol or other permissible sterols (*e.g.* 7-DHC) in *C. elegans* by azacoprostane treatment may co-suppress these transport proteins and their receptors, which was indeed elucidated in our study (Figs. 3–6). Cholesterol uptake by *C. elegans* oocytes occurs via an endocytotic pathway involving yolk proteins; two major sites of cholesterol accumulation are oocytes and developing sperm (9). Thus, the function of these lipid transfer proteins and their receptors seems totally dependent on the availability of cholesterol or 7-DHC in the cell. For instance, if there is insufficient cholesterol or 7-DHC, as in the case of azacoprostane-treated worms, suppression of these lipid transport proteins and their receptors is unavoidable (Table I). Although sitosterol can be readily converted to cholesterol (or 7-DHC) via C-24 reduction of desmosterol by 24-SR, azacoprostane treatment blocks the conversion of desmosterol to cholesterol, thereby resulting in the accumulation of the nonpermissible sterol desmosterol, which leads to defects in development, growth, and the sterol-mediated reproductive system.

Finally, a curious induction of gene transcripts for these lipid transport proteins occurs despite significant suppression at the protein level. Perhaps some compensation reaction accommodates the protein depletion caused by azacoprostane treatment. For example, co-suppression of permissible sterol levels and their transport proteins may have caused activation of the SREBP pathway of these target genes (*i.e.* vitellogenin, *lrp-1*, and *rme-2*) (Figs. 3 and 4). In fact, the predicted SREBP site(s) in the *vit-2* and *lrp-1* genes were found to be functional *in vitro* (Figs. 5*C* and 6), suggesting that cholesterol metabolism in *C. elegans* can operate in a similar manner as seen in mammals (16). It was noted that the number of SREs present in a gene does not appear to be additive; instead, SRE position may be important in governing the response to sterol. That is, the proximal SRE of the *lrp-1* gene (-159) seems more effective than the distal multiple SREs of the *vit-2* gene (-1934, -1129, -921, -892, and -286) in sterol response, as observed previously (38). However, there is no predicted SREBP site in *vit-6*, which instead possesses an SF-1 (steroidogenic factor) site and estrogen receptor-binding and progesterone receptor-binding sites (Fig. 5*A*). As yet, there is not good evidence about whether these binding sites can equally match the function of an SREBP site for gene activation. Interestingly, the upstream region in *rme-2* also contains estrogen receptor- and progesterone receptor-binding sites, and a retinoic acid receptor-type chicken vitellogenin promoter-binding protein site occurs upstream in the *vit-2* gene (Fig. 5*A*). These sites deserve more investigation in the future.

In conclusion, we have provided the first proteomic investigation of the disturbance in sterol metabolism by azacoprostane treatment in *C. elegans.* Moreover, a direct link between functional sterol deficiency and lipid transfer-related proteins has likely resulted in defects in sterol-mediated reproduction. However, further studies are needed on whether the proteomic change of VIT-2 and LRP-1 due to sterol deficiency can be reversed by cholesterol, as seen at their mRNA level. Whether this is a typical type of regulatory mode for the nematode to overcome during sterol deprivation-related stress is also unclear.

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