Altered Expression of An L1-Specific, O-Linked Cuticle Surface Glycoprotein in Mutants of the Nematode *Caenorhabditis Elegans*

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Abstract. Mouse mAb M38 was used in indirect immunofluorescence experiments to detect a stage-specific antigen on the surface of the first larval stage (L1) of the free-living nematode *Caenorhabditis elegans*, and to detect alterations in the apparent expression of this antigen in two distinct classes of *C. elegans* mutants. In previously described *srf-2* and *srf-3* mutants (Politz S. M., M. T. Philipp, M. Estevez, P. J. O'Brien, and K. J. Chin. 1990. *Proc. Natl. Acad. Sci. USA*. 87:2901–2905), the antigen is not detected on the surface of any stage. Conversely, in *srf-(yj43)* and other similar mutants, the antigen is expressed on the surface of the first through the fourth (L4) larval stages.

To understand the molecular basis of these alterations, the antigen was characterized in gel immunoblotting experiments. After SDS-PAGE separation and transfer to nitrocellulose, M38 detected a protein antigen in extracts of wild-type L1 populations. The antigen was sensitive to digestion by Pronase and O-glycanase (endo-α-*N*-acetylgalactosaminidase), suggesting that it is an O-linked glycoprotein.

This antigen was not detected in corresponding extracts of wild-type L4s or *srf-2* or *srf-3* L1s, but was detected in extracts of *srf-(yj43)* L4s. The antigen-defective phenotype of *srf-3* was epistatic to the heterochronic mutant phenotype of *srf-(yj43)* in immunofluorescence tests of the *srf-3 srf-(yj43)* double mutant, suggesting that *srf-(yj43)* causes incorrect regulation of a pathway of antigen formation that requires wild-type *srf-3* activity.

Nematode cuticles contain at least two characteristic but dissimilar classes of proteins, the cuticle collagens and surface-associated proteins. The former have been analyzed extensively by classical and molecular genetics in the free-living species *Caenorhabditis elegans* by taking advantage of partial structural homologies with vertebrate collagens. Mutations in *C. elegans* collagen genes sometimes produce striking body shape changes such as Dumpy, Roller, and Squat (Kusch and Edgar 1986; Kramer et al., 1988; von Mende et al., 1988). There are ∼100 collagen genes in *C. elegans* (Cox et al., 1984), with extensive regulation of collagen gene expression occurring in postembryonic development (Politz and Edgar 1984; Cox and Hirsh 1985, Kramer et al., 1985).

In contrast, relatively little is known either of the genes controlling expression of nematode surface molecules nor the effects of mutations in such genes. Nematode surface proteins are a structurally diverse group of proteins and glycoproteins defined by antibody recognition on the surface of live nematodes, radiolabeling by non-penetrating procedures, and ability to be solubilized from the cuticle by mild treatments that do not solubilize the cuticle collagens (Philipp and Rumjaneck, 1984). In nematode parasites of vertebrate animals and humans, these surface molecules are antigenic in the infected host, and expression of surface antigens is dynamic. Changes in surface antigenicity can occur at molts, when a new cuticle is synthesized and an old one shed, so that some surface antigens are stage specific (Philipp and Rumjaneck, 1984). Surface antigens can be a source of and target of protective immunity (Grencis et al., 1986).

We reported previously an adult-specific surface antigenic polymorphism in *C. elegans* varietal strains that mapped to a specific genetic locus, designated *srf-1* (Politz et al., 1987). More recently, we have described a set of *C. elegans* mutations in two genes, *srf-2* and *srf-3*, that cause alterations in surface composition (Politz et al., 1990). In contrast to collagen mutations known to cause alterations in body shape, none of these mutations causes striking alterations in overall morphology. The *srf* mutants were isolated in indirect immunofluorescence positive screening experiments using polyclonal rabbit antisera. Using a mouse mAb probe, we describe here the detection and biochemical characterization of a surface glycoprotein displayed specifically on the first larval stage (L1) of *C. elegans*.

Alterations in the expression of this antigen have been de-

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tected in two classes of *srf* mutants. One class appears defective in its expression, while the other appears to express the L1-specific antigen at inappropriate developmental stages. The implications for understanding control of expression of extracellular molecules in general and surface antigenicity in nematode parasites in particular are discussed.

Materials and Methods

Monoclonal Antibody Production

Mouse mAbs directed against the L1 cuticle were elicited by in vitro immunization. Wild-type (strain N2) embryos prepared by alkaline hypochlorite treatment of mixed stage cultures (Emmons et al., 1979) were incubated with BALB/c-derived mouse primary splenocytes using the media and protocols supplied by the manufacturer (In Vitro Immunization System, Hana Media, Berkeley, CA). Immunized splenocytes were fused with Pal-O-P3 myeloma cells and hybridomas were selected using standard methods (Morgan, 1984).

Hybridoma Screening by Indirect Immunofluorescence of Freeze-fractured Nematodes

Slides were subbed with 0.1% BSA as described (Sulston and Hodgkin, 1988). Mixed stages of wild-type *C. elegans* (var. Bristol, strain N2) were grown as described (Brenner, 1974) and harvested and fixed onto BSA-subbed slides by squashing and freeze fracturing (Sulston and Hodgkin, 1988). The slides were then immediately fixed sequentially for 2 min in methanol and 4 min in acetone, blotted on edge, and air dried. For assay of hybridoma supernatants, 10 μl of hybridoma supernatant from a separate microtiter well was spread onto each nematode sample spot. The slide was placed in a humidifying chamber for 1 h, and then washed by two sequential immersions in PBS (0.01 M sodium phosphate, pH 7.0, 0.15 M NaCl). The back of the slide was wiped dry and 20 μl of a 1:30 dilution of FITC-conjugated goat anti-mouse immunoglobulins (no. 1211-0231; Organon-Teknika-Cappel, West Chester, PA) was applied to each nematode sample spot. The slide was incubated and washed as for the primary incubation with hybridoma supernatant, covered with a drop of 90% glycerol, 10% PBS, then a cover slip, and viewed under FITC epifluorescence optics at 1,000× magnification (oil).

Monoclonal Antibody Purification and Characterization

L1-cuticle-specific mAbs M37 and M38 were purified from hybridoma supernatants. Hybridoma cells were grown using standard methods (Morgan, 1984). Immunoglobulins were recovered from cell supernatants by 0-50% ammonium sulfate precipitation, dialysis of redissolved precipitates against PBS, and were stored frozen at −20°C.

Antibody class of immunoglobulins purified by ammonium sulfate precipitation was determined by double immunodiffusion in agar against anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM antisera using materials and protocols supplied by the manufacturer (no. 64-690-1; ICN Immunobiologicals, Irvine, CA).

Indirect Immunofluorescence Assay of Monoclonal Antibody Binding to Live Nematodes

Live nematodes grown on agar plates supplied with *E. coli* OP50 spots (Brenner, 1974) were washed off plates with PBS, washed by repeated centrifugation and resuspension in PBS to remove *E. coli*, and transferred in ~100 μl of PBS to 2-ml flat-bottomed glass vials. Such samples contained 50-1,000 nematodes. By trial, it was determined that 1 μl of ammonium sulfate-purified immunoglobulins from the cell line that produced M38 (hereafter referred to as "M38") was sufficient to saturate binding to 1,000 L1s. Subsequently this was used as the standard volume of mAb solution per sample. Samples were incubated with M38 for 1.5 h in an ice-water bath with shaking (150 rpm). After washing three times by repeated centrifugation at 0°C and resuspension in ice-cold PBS in 3-ml conical centrifuge tubes, nematodes were transferred back to fresh vials and incubated for one hour in an ice-water bath with 10 μl of FITC-conjugated goat anti-mouse IgM (no. A-9259; Sigma Chemical Co., St. Louis, MO). The sample was washed six times as before, transferred to a microscope slide in a minimal volume PBS, and viewed under FITC optics in an Axioskop fluorescent microscope (Zeiss, Oberkochen, Germany). Antibody binding to live wild-type L1s or *srf-(yj43)* L1-L4 stage worms was uniform only when antibody incubations were performed at 0°C. Incubation at room temperature or warming of samples on the microscope stage produced patchy surface immunofluorescence. Therefore, when multiple samples were prepared, each sample was kept on ice until just before viewing. Before photomicroscopy of immunofluorescence, nematodes were anesthetized. After the last PBS wash, samples were resuspended in ice-cold 0.1 M sodium azide. Nematodes were wet mounted and examined microscopically. As soon as visible movement had ceased, fluorescence was photographed at 5 or 10× magnification using TMax 400 film (Eastman-Kodak Co., Rochester, NY) and 1-min exposures or TMax P3200 film and 7-30 exposures.

Mutant Screening by Indirect Immunofluorescence

Wild-type *C. elegans* (strain N2) nematodes were mutagenized with methanesulfonate ethyl ester as described (Brenner, 1974) and were then transferred to several 100-mm plates containing Nematode Growth Medium agar (see Brenner, 1974) supplemented with 4 g nutrient broth/liter and supplied with *E. coli* OP50 lawns (enriched plates). After recovery overnight at 20°C, eight L4 hermaphrodites were picked onto each of six 100-mm enriched plates. These nematode samples were allowed to grow and reproduce to the F2 generation (ca. 14,000 animals/plate) at 20°C. F2 nematodes were washed off plates and incubated with monoclonal and secondary antibody as described above, except that sample volumes were 500-1,000 μl. Worms from separate plates were incubated and examined separately, i.e., samples were not pooled. Surface immunofluorescence was observed under an Axioskop (Zeiss) at 5 or 10× magnification. L1s fluoresced uniformly, providing an internal control for antibody activity and specificity. Rare larger animals exhibiting surface fluorescence were picked by pipetting with a drawn-out micropipet and were transferred onto a 60-mm agar plate. Fluorescence of transferred animals was checked microscopically to verify that the correct animal had been transferred. Clonal stocks of putative mutants were established by self-fertilization and progeny were retested by immunofluorescence with M38 to eliminate false positives. Before further study of mutant phenotypes, mutants were back-crossed twice to wild-type (Brenner, 1974), using indirect immunofluorescence with M38 as a genetic marker as described previously (Politz et al., 1987; Politz et al., 1990).

Strain Construction

Wild-type (strain N2, Brenner 1974) and mutant strains of *C. elegans* var. Bristol were obtained from the *Caenorhabditis* Genetics Center (University of Missouri, Columbia, MO). Construction of *srf-3(yj10) unc-22(e66)* was described previously by Politz et al. (1990). *srf-(yj43) unc-4(e120)* was obtained in a three-factor cross between *dpy-10 unc-4*/++ males and *srf-(yj43)* hermaphrodites. Unc non-Dpy recombinant segregants of F1 non-Unc hermaphrodites were picked; the presence of the surface antigen marker was identified by antibody screening using mAb M38, and an antigen-positive Unc clone of the desired genotype was established. *srf-3(yj10) unc-22(e66); srf-(yj43) unc-4(e120)* was obtained by mating *srf-(yj43) unc-4*/++ males with *srf-3(yj10) unc-22* hermaphrodites. Unc-4 (kinker) Unc-22 (twitcher) segregants of F1 non-Unc-22 parents were picked and clones of these were established. The presence of *srf-3(yj10)* and *srf-(yj43)* in these putative double mutants was confirmed via heterozygous complementation tests with *srf-3(yj10)* and *srf-(yj43)* single mutants. In these tests, the *srf-3(yj10)* phenotype was assessed by immunofluorescence staining with adult-adsorbed anti-cuticle rabbit serum as described (Politz et al., 1990); *srf-(yj43)* phenotype was assessed by immunofluorescence staining with mAb M38.

Preparation of Nematode Extracts

For extraction, large numbers of *C. elegans*, either wild-type (strain N2; Brenner, 1974) or *srf-2(yj262)* mutants, were grown from dauer larvae. Dauer larvae were prepared as described (Cox et al., 1981*a*). Dauer larvae were plated onto 100-mm enriched plates supplied with *E. coli* OP50 lawns and incubated at 20°C for 45-50 h, at which time a large number of eggs were present. Worms and eggs were washed off plates with M9 buffer (Brenner, 1974) and washed several times to remove *E. coli*. Worms were lysed by alkaline hypochlorite treatment (Emmons et al., 1979). Eggs were pelleted by centrifugation and quickly washed three times with sterile M9 buffer. Washed eggs were placed on a 25-μm mesh nylon screen (Tetko, Inc.,

Elmsford, NY) immersed in M9 buffer in a Pyrex petri dish. Under these conditions, as L1s hatch, their small diameter allows them to slip through the screen into the buffer below. After 12-h incubation at 20°C, the screen was removed and L1s in the filtrate were harvested by centrifugation. The yield from 25 plates was 0.5–1 × 10^6 L1s.

L1s from *srf-3(yj10)* mutant stocks were obtained similarly, except that eggs were obtained after growth of mixed stage worms rather than dauer larvae, because *srf-3* dauer larvae are difficult to obtain in large numbers (Politz et al., 1990).

L1 pellets were transferred to 1.5-ml microcentrifuge tubes and resuspended in 0.125 M Tris-Cl, pH 6.8, containing 5% by volume of a 6 mg/ml solution of PMSF. Some samples contained 1% SDS in addition to the above solution components. Worm concentrations were 200,000/100 µl. Samples were heated in a boiling water bath for 2 min, rocked for 5 min at 21°C, and pelleted for 5 min in a microcentrifuge. Supernatants were transferred to fresh microcentrifuge tubes. Pellets were resuspended in the same buffer as above without PMSF, ± 1% SDS as appropriate, containing 10% 2-mercaptoethanol (2-ME)[1]. Samples were boiled and pelleted as before; supernatants were transferred to fresh tubes. All extracts were frozen rapidly and stored at −20°C.

L1 populations were also subjected to disruption by sonication, cuticle isolation and extraction of cuticles with SDS-containing buffers as described (Cox et al., 1981*b*).

Mutant *srf-(yj43)* L4s were grown from eggs hatched over a 8-h period on enriched plates containing OP50 lawns for 35 h at 20°C, at which time a synchronous population of L4s was present. These animals were harvested and extracted as described above. For direct comparison of antigenicity in *srf-(yj43)* and wild-type L4 extracts, N2 L4s were grown similarly.

Gel Electrophoresis

Protein concentration in nematode extracts was determined by standard methods (Lowry et al., 1951). Aliquots of protein extracts were prepared for separation by SDS–polyacrylamide gel electrophoresis by the addition of 9 vol of cold acetone, precipitation overnight at −20°C, recovery of proteins by centrifugation, and redissolution in SDS gel sample buffer (0.125 M Tris-Cl, pH 6.8, 1% SDS, 5% 2-ME, and 10% glycerol). All samples were boiled for 2 min. 1.5-mm-thick 12% polyacrylamide slab gels with 4% stacking gels were poured and run according to Laemmli (1970), using bromphenol blue tracking dye. Prestained low molecular weight protein size markers (no. 161-0305 Bio-Rad Laboratories, Richmond, CA) were run in separate lanes.

Some samples were analyzed in a second SDS-PAGE separation after proteolysis of proteins in situ in gel slices from an initial electrophoretic separation (Cleveland et al., 1977). A lane from the first SDS-PAGE separation containing proteins from a +SDS extract of N2 L1s was cut into slices horizontally; each slice was incubated with 0.125 M Tris-Cl, pH 6.8, 0.1% SDS, 1 mM EDTA (gel slice buffer) for 30 min. A second slab gel was poured as usual, except that both stacking and separating gels contained 1 mM EDTA and the stacking gel length was increased to 5 cm. Second dimension wells were filled with gel slice buffer and a gel slice from the first dimension was pushed to the bottom of each well. Slices were overlaid with 10 µl of gel slice buffer containing 10% glycerol and 20 µg of Pronase (Calbiochem-Behring Corp., San Diego, CA). Electrophoresis was performed as usual, except that the power supply was turned off for 30 min when the molecular weight markers neared the bottom of the stacking gel.

Immunoblotting Analysis

Proteins in SDS slab gels were transferred electrophoretically to BA-85 nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) for immunoblot analysis (Towbin et al., 1979) using transfer buffer (0.025 M Tris-Cl, 0.192M glycine pH 8.3 containing 20% methanol vol/vol and 0.02% SDS). Some nitrocellulose strips were stained for total protein with colloidal gold immediately after electrotransfer, using the manufacturer's directions (Bio-Rad Laboratories). Binding of L1-specific mAb M38 to antigens on nitrocellulose was enhanced by either of two protocols, low pH incubation or glutaraldehyde fixation. For low pH incubation, the membrane was incubated overnight at 37°C in 1% BSA (Fraction V ICN Immunobiologicals) in 0.01 M sodium succinate, pH 5.5, 0.15 M NaCl (sodium succinate buffer). The membrane was then transferred to 50-ml fresh sodium succinate buffer containing 0.05% polyoxyethylene sorbitan monolaurate (Tween-20) and incubated with 100 µl of M38 hybridoma supernatant for 3 h at 4°C. Antibody solution was removed and the membrane washed twice with sodium succinate buffer containing 0.05% Tween-20. The membrane was incubated for 2 h at 4°C with HRP-conjugated goat anti-mouse IgM (no. 31440; Pierce Chemical Co., Rockford, IL) at 1:5,000 final dilution or alkaline phosphatase-conjugated goat anti-mouse IgM (A-7784; Sigma Chemical Co.) at 1:1,000 final dilution in 50 ml sodium succinate buffer. Buffer was removed and the membrane was washed once with sodium succinate buffer. Bound enzyme-conjugated antibody was detected using the appropriate substrate (4-chloronapthol for HRP, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium for alkaline phosphatase) using the conditions described by the manufacturer (Pierce Chemical Co. or Bio-Rad Laboratories respectively).

For glutaraldehyde fixation (Ikegaki and Kennett, 1989), the membrane was washed in PBS for 5 min at 4°C. The membrane was then incubated in PBS containing 0.25% glutaraldehyde for 15 min at 4°C. After a 5-min wash in PBS, the membrane was incubated in 2% BSA, 2% polyvinylpyrrolidone, 2% ficoll in 50 mM Tris-Cl, pH 8.0 for 1 h at 37°C. The membrane was then incubated in PBS containing 0.05% Tween-20 and 100 µl M38 hybridoma supernatant overnight at 4°C. After washing three times in PBS containing 0.05% Tween-20, the membrane was again treated with 0.25% glutaraldehyde in PBS for 15 min at 4°C. The membrane was washed once with PBS at 4°C and reblocked with 0.1% BSA in PBS, pH 8.5, for 20 min at 4°C. After discarding this solution, 50 ml of PBS was added and the membrane was incubated with secondary antibody as described above. This incubation was followed by three washes with PBS containing 0.05% Tween-20. Detection of bound antibody with the appropriate substrate was as described above.

Enzyme Digestions

Some samples were pre-digested with enzymes before analysis by immunoblotting. Protein samples were precipitated by acetone as described above. For Pronase digestion, precipitates were redissolved in 18 µl Pronase buffer (0.1 M Tris-Cl, pH 8.0, 1.0 mM $CaCl_2$). A 2-µl aliquot of varying concentrations Pronase (Calbiochem-Behring Corp.) dissolved in Pronase buffer was added. Samples were incubated overnight at 37°C. Reaction conditions for digestion with proteinase K (Type XI fungal protease, Sigma Chemical Co.) were similar. After protease incubation, 10 µl SDS sample buffer was added and the samples were prepared for SDS-PAGE.

For O-glycanase digestion, acetone precipitates containing 20 µg protein were redissolved in 50 µl of 20 mM sodium cacodylate, pH 6.8, 10 mM calcium acetate. 4 µl of 1 U/ml neuraminidase (Genzyme Corp., Boston, MA) was added and the sample was incubated for 1 h at 37°C. Then 2 µl (4 µU) of endo-a-*N*-acetylgalactosaminidase (O-glycanase; Genzyme Corp.) was added and the incubation at 37°C continued for 18 h. Samples were then prepared for SDS-PAGE.

Results

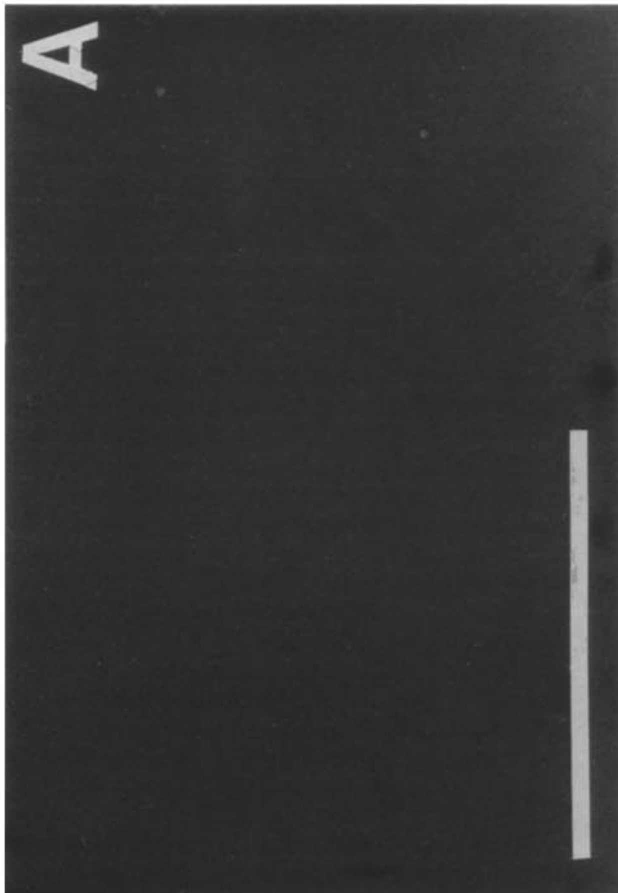
mAbs M37 and M38 were selected initially by indirect immunofluorescence testing against mixed stages of *C. elegans* wild-type (strain N2) in squashes of whole worms fixed with acetone–methanol. M37 and M38 hybridoma supernatants caused immunofluorescence of the cuticle of the first larval stage (L1). M38 was chosen for further study, purified from hybridoma supernatant by ammonium sulfate precipitation, and shown to be class IgM. In indirect immunofluorescence tests of binding to mixed stages of live wild-type worms, the surface of the L1 stage bound M38 specifically while the surface of other larval stages or adults showed no significant antibody binding (Fig. 1, *B* and *D*). Some L1-sized animals did not fluoresce. However, these probably were L2s indistinguishable in size from L1s, because L1-specific antigen detection by immunofluorescence is efficient. In a synchronous population of wild-type L1s stained with M38, 90/90 animals were immunofluorescent.

Previously characterized *C. elegans* mutants carrying EMS-induced mutations in genes *srf-2* and *srf-3* appear to have a component or components missing from the surface

1. *Abbreviations used in this paper*: 2-ME, 2-mercaptoethanol; Unc, uncoordinated movement.

B

D


A

C

Table I. Epistasis Testing of srf-3(yj10) and srf-(yj43)

Test	Partial genotype	Number positive/total* M38	Adult-adsorbed serum
1	*srf-3(yj10)*	0/95	138/138
2	*srf-(yj43)*	90/95	0/86
3	*srf-3(yj10); srf-(yj43)*‡	0/85	68/68
4	*srf-3(yj10)/+; srf-(yj43)*‡§	20/37	NA‖
5	*srf-3(yj10); srf-(yj43)/+*‡§	NA‖	17/56

* All entries indicate L2–L4 animals scored.
‡ These genotypes also included unc-22(e66); unc-4(e120) (test 3) or unc-22(e66)/+unc-4(e120)/+ (tests 4 and 5).
§ It is assumed that approximately 50% of the non-Twitcher non-Unc progeny in this test had the genotype shown.
‖ Not applicable.

of all stages, causing exposure of normally hidden antigens (Politz et al., 1990). When mixed stage populations of *srf-2* or *srf-3* were incubated with M38 followed by FITC-conjugated secondary antibody, immunofluorescent worms were not detected (e.g., Fig. 1, *A* and *C*). In one experiment, 0/127 *srf-2* animals of mixed stages ranging from L1 to adult showed immunofluorescence. Similar results with *srf-3* L2-L4-sized larvae are presented in Table I (line 1); *srf-3* L1s and adults also showed no immunofluorescence (data not shown). These results provide independent evidence for the above model for the mutant lesions, as they now include the L1-specific antigen recognized by M38 among the molecules missing from the surface of *srf-2* and *srf-3* mutants.

srf-2 and *srf-3* mutant phenotypes might represent specific defects in biosynthetic steps involved in expression of the L1-specific antigen. We were also interested in the regulation of stage specificity of the M38 antigen. Rather than failing to express the antigen, mutants altered in genes controlling timing of antigenic expression might express the antigen at later developmental stages than the L1. To search for such mutants, wild-type parents were mutagenized with EMS and their F2 progeny screened for binding of M38 by immunofluorescence at stages L2 adult. Nine such apparent heterochronic mutants were isolated. Animals in these strains bound M38 at larval stages later than the L1, but not as adults, when mixed stages were tested by immunofluorescence. Fig. 2 shows larval and adult animals carrying the homozygous mutation *srf-(yj43)* after staining with M38 and FITC-conjugated secondary antibody; in contrast to wild type, larval animals clearly larger than L1s, but not adults, showed positive immunofluorescence. In one sample in which immunofluorescent L2–L4 sized larvae were counted, the penetrance of the mutant phenotype was 95% (Table I, line 2).

To explore the biochemical basis for the L1-specific antigenicity of the wild-type, and to begin to understand the basis for altered expression of this antigen in the mutant strains, the L1-specific antigen recognized by M38 was characterized in wild-type L1 extracts by gel immunoblotting (Western blotting). Synchronous populations of up to 10^6 wild-type

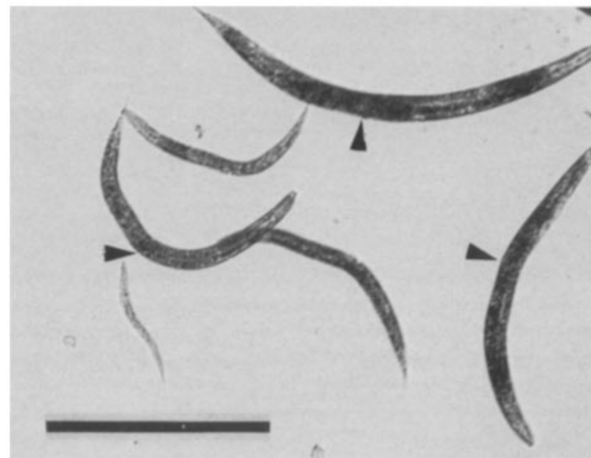


Figure 2. Light micrographs of a field of mixed stages of *srf-(yj43)* stained with mAb M38 and FITC-conjugated goat anti-mouse IgM. (*Top*) Bright field optics, arrows indicate the position of the vulva in adult animals. (*Bottom*) FITC optics (same field as top). Bar, 500 μm.

L1s were extracted by heating at 100°C briefly in protease inhibitor–containing buffer in the presence or absence of SDS. These extracts are hereafter referred to as +SDS or −SDS extracts. All samples, whether +SDS or −SDS, were denatured by heating in SDS-2ME before electrophoresis. Extracts were separated on 12% SDS-PAGE slabs, transferred to nitrocellulose, and incubated with M38 followed by HRP- or alkaline phosphatase-conjugated anti-mouse IgM and substrate. Antigenic bands were readily detected only when the antibody incubations were performed at pH 5.5 or when the binding of primary antibody was stabilized by the glutaraldehyde fixation procedure of Ikegaki and Kennett (1989). The patterns of antigens detected by these two procedures were very similar. When more typical conditions of neutral pH and no fixation were used, no antibody binding was observed. These results suggest that M38 is a low-affinity antibody whose dissociation from antigen on nitro-

Figure 1. Light micrographs of mixed stages of *C. elegans* mutant and wild-type strains stained with mAb M38 and FITC-conjugated goat anti-mouse IgM. *A*, *srf-2(yj262)*, FITC optics; *B*, wild-type (strain N2), FITC optics; *C*, *srf-2(yj262)*, bright-field optics, same field as *A*; *D*, wild-type (strain N2), bright-field optics, same field as *B*. Bar, 500 μm.

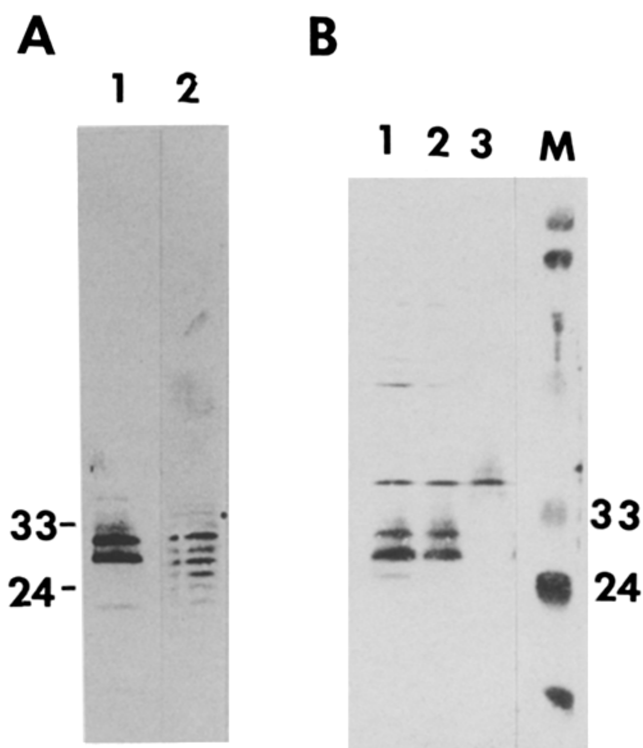


Figure 3. Immunoblot analysis of antigens recognized by monoclonal antibody M38 in extracts of synchronous L1 wild-type *C. elegans* populations, separated by SDS-PAGE on 12% acrylamide slabs. (*A*) Lane *1*, 20 μg +SDS extract; lane 2, 20 μg −SDS extract. Molecular weight markers are indicated in kD at the left. Blot was developed using low pH antibody incubation and alkaline-phosphatase–conjugated goat anti–mouse IgM (see Materials and Methods). (*B*) O-glycanase sensitivity of the M38 antigen. Lane *1*, 20 μg untreated +SDS extract; lane *2*, 20 μg +SDS extract mock-digested under O-glycanase conditions; lane *3*, 20 μg +SDS extract predigested with O-glycanase; lane *M*, molecular weight markers in Kd. Blot was developed using glutaraldehyde fixation and HRP-conjugated goat anti–mouse IgM (see Materials and Methods).

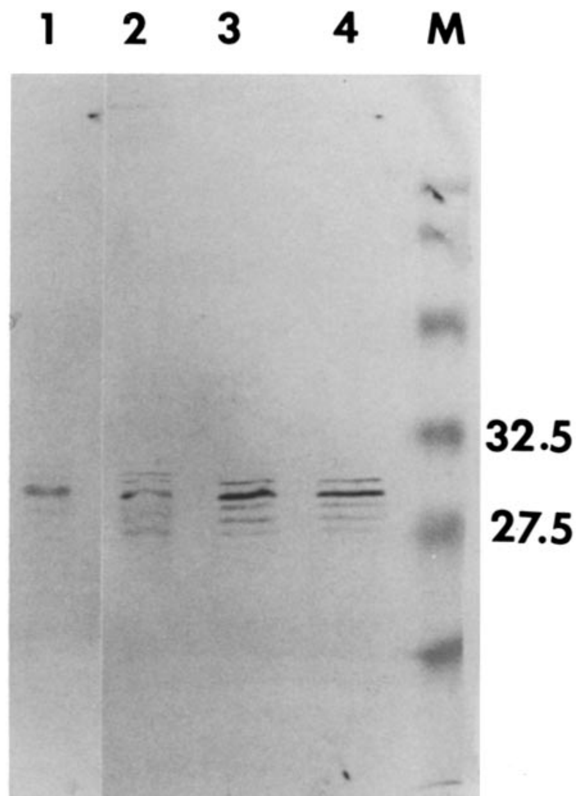


Figure 4. Immunoblot analysis of products of Pronase digestion of +SDS extracts of synchronous wild-type L1 populations, separated by SDS-PAGE on 12% slabs and probed with mAb M38. Lane *1*, 20 μg untreated +SDS extract; lanes *2–4*, 20 μg +SDS extract pretreated with Pronase. Pronase levels: lane *2*, 1 μg/μg total +SDS protein; lane *3*, 2.5 μg/μg total +SDS protein; lane *4*, 5 μg/μg total +SDS protein. Lane *M*, molecular weight markers indicated in kD. Blot was developed using glutaraldehyde fixation and HRP-conjugated goat anti–mouse IgM.

cellulose can be inhibited by low pH or prevented by covalent glutaraldehyde fixation at neutral pH.

The pattern of antigenic bands was different in +SDS and −SDS extracts (Fig. 3 *A*). In +SDS extracts, two major bands of M_r 30,000 and 27,500 and a minor band of variable intensity, M_r 36,000, were detected (Fig. 3 *A*, lane *1*). The width of the major bands suggested heterogeneity of the antigenic material, as would be predicted for a posttranslationally modified protein such as a glycoprotein (see below). In some +SDS preparations, only one band at M_r 30,000 was observed (e.g., compare Fig. 3 *A*, lane *1*, to Fig. 4, lane *1*). In contrast, −SDS extracts showed a different antigenic banding pattern (Fig. 3 *A*, lane 2). A series of up to 11 evenly spaced sharp bands was observed in the M_r range 20,000 to 30,000. This pattern will be referred to as the antigenic ladder. The overall appearance of these bands was reproducible from one blot or preparation to another. The main limitation on the number of bands detected seemed to be sensitivity, as the center bands of the ladder were always more intense, and the number of less intense bands observed at the ends of the ladder varied.

The antibody binding detected in these L1 extracts did not appear to be caused by nonspecific binding to major proteins represented in the extracts. When similar lanes transferred to nitrocellulose were stained for total protein with colloidal gold, numerous bands were detected in the entire molecular weight range represented, but no especially prominent bands were detected at the mobilities corresponding to the relative molecular masses of the +SDS antigenic bands, and no protein ladder was detected in the −SDS extract (data not shown).

The experiment of Fig. 3 *B* provides evidence that the two major antigenic bands observed in +SDS extracts are O-linked glycoproteins. +SDS extracts were incubated either under mock digestion conditions or in the presence of the enzyme O-glycanase, separated by SDS-PAGE, and probed by Western blotting with M38. Fig. 3 *B*, lane *2*, shows that incubation under mock digestion conditions did not significantly change the antigenic pattern compared with the non-incubated control (lane *1*). In contrast to the mock-digested control, the O-glycanase–digested sample showed a significant alteration in the pattern of antigenic bands (Fig. 3 *B*, lane *3*); the two major antigenic bands at M_r 30,000 and 27,000 were completely absent, but the upper minor band M_r 36,000 appeared unaffected. Based on the substrate specificity of O-glycanase, it can be concluded that the two major antigenic bands contain, minimally, a Gal(β1-3)GalNAc

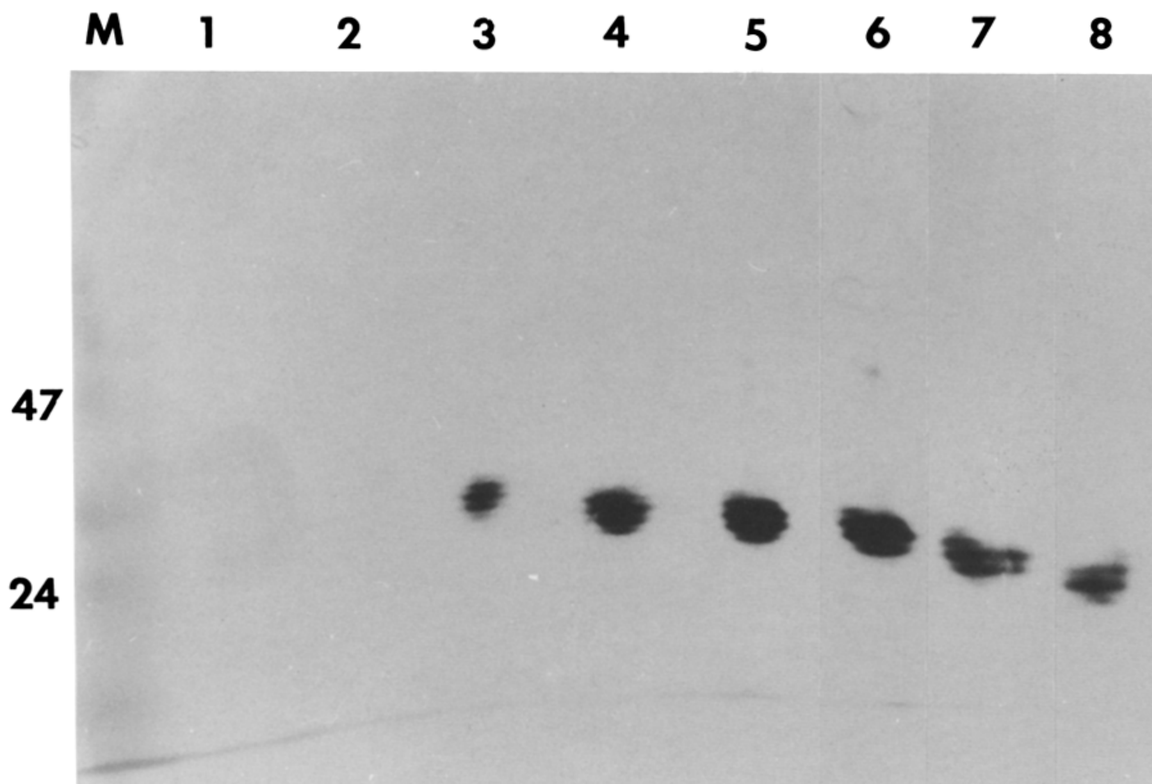


Figure 5. Immunoblot analysis of Pronase digestion products of proteins from 21,000 to 46,000 M_r region of SDS-PAGE separated +SDS extract. This M_r region of a lane containing 20 μg of separated +SDS extract proteins (e.g., Fig. *3*, lane *1*) was cut into eight gel slices of equal width; proteins were digested in situ with Pronase and electrophoresed in a second SDS-PAGE separation (see Materials and Methods). Lanes *1–8* correspond to products of digestion of successive slices from the highest (lane *1*) to the lowest (lane *8*) M_r region. Lane *M*, molecular weight markers in kD. Blot was developed using glutaraldehyde fixation and alkaline phosphatase–conjugated secondary antibody.

core disaccharide linked either to a serine or threonine residue by an O-glycosidic bond (Kobata, 1979).

The relationship between the two major antigenic bands detected in the +SDS extract and the antigenic ladder detected in the −SDS extract was explored in Western blotting experiments shown in Fig. 4, which shows the antigenic bands detected after aliquots of a +SDS extract were predigested with varying concentrations of the relatively non-selective proteolytic enzyme Pronase. An undigested control was also analyzed (lane *1*). Amounts of Pronase ranging from 1 μg/μg total sample protein to 5 μg/μg total sample protein (lanes *2–4*) showed similar results; i.e., an antigenic ladder similar to that observed in Western blots of −SDS extracts was detected. A similar antigenic ladder was also obtained after digestion of +SDS extract with protease K at 1 μg enzyme/μg total sample protein (data not shown).

To determine the molecular weight range of the substrate that produced the ladder, the portion of an SDS-PAGE lane containing separated components of a +SDS extract in the M_r range 21,000 to 46,000 was cut into eight slices; each gel slice was incubated with Pronase in the wells of a second SDS-PAGE slab and then electrophoresed. Only the six slices corresponding to the M_r region of the first gel from 21,000 to 38,000 produced the antigenic ladder pattern (Fig. 5); it appeared that digestion of the higher or lower relative molecular weight portion of this region produced the corresponding portion of the antigenic ladder. In similar experiments, digestion of slices from the complete range of relative molecular weights separated on the first gel yielded no antigenic bands except from this same region (data not shown). Thus the 21,000 to 38,000 M_r region of the first gel is both necessary and sufficient to produce the antigenic ladder upon digestion by Pronase.

In addition to whole-worm extractions, crude cuticle fragments were prepared for extraction by sonication of live L1s and differential centrifugation (Cox et al., 1981*a*,*b*). In contrast to +SDS extracts prepared by boiling whole worms, +SDS extracts of such crude cuticle preparations exhibited the antigenic ladder on Western blots (Fig. 6, lane *1*), not the two major antigenic bands represented in the whole worm +SDS extract. The ladder detected in crude cuticle +SDS extracts was insensitive to protease K digestion (Fig. 6, lane *2*).

Extracts of *srf-2* (Fig. 7 *A*) and *srf-3* (Fig. 7 *B*) L1 populations were also analyzed by Western blotting. Neither +SDS extracts (Fig. 7 *A*, lane *2*, and Fig. 7 *B*, lane *1*) nor −SDS extracts (Fig. 7 *A*, lane *3*, and Fig. 7 *B*, lane *2*), showed detectable levels of the antigen when amounts of sample were analyzed that were equivalent to those in which wild-type antigen was readily detected. Moreover, when material insoluble after +SDS or −SDS extraction was subjected to more extensive extraction by heating in the presence of SDS plus 2-mercaptoethanol, the solubilized extracts showed no detectable antigenicity (Fig. 7 *A*, lanes *4* and *5*, respectively, and Fig. 7 *B*, lanes *3* and *4*, respectively). Finally, residual material insoluble after *srf-2* or *srf-3* L1s had been boiled in SDS plus 2-ME showed no detectable antigenicity when

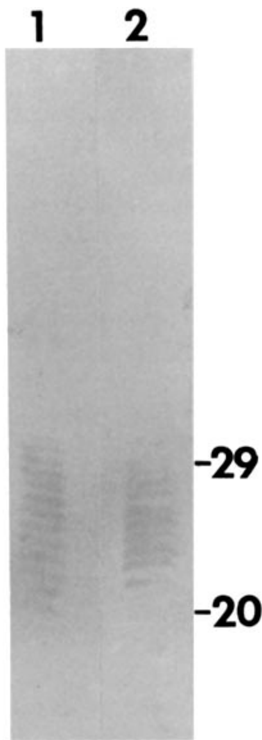


Figure 6. Immunoblot analysis of +SDS extract of crude cuticle fragments isolated from synchronous populations of wild-type L1s and probed with mAb M38. Worms were disrupted by sonication and cuticle fragments were isolated by low-speed centrifugation (see Materials and Methods). Extracts were separated by 12% SDS-PAGE. Lane *1*, 40 μg of untreated +SDS extract. Lane *2*, 40 μg of +SDS extract pretreated with proteinase K (1.2 μg enzyme/μg total sample protein). Blot was developed using low pH antibody incubation and alkaline phosphatase–conjugated secondary antibody. Molecular weight markers in kD are indicated at the right.

tested for M38 binding by immunofluorescence (not shown). Because proteins solubilized in these extracts plus the 2-ME insoluble residue should comprise very nearly all the protein recoverable from L1s (Cox et al., 1981*b*), it is concluded that the antigenicity characteristic of wild-type L1s is not only missing from the cuticle surface, but is not sequestered internally in *srf-2* and *srf-3* L1s.

When +SDS and −SDS extracts of synchronous wild-type L4s were analyzed by Western blotting, no antigenicity was observed (Fig. 8, lanes *1* and *2*). Similarly, when wild-type L4s or adults were extracted by heating worms in SDS plus 2-ME, no antigenicity was detected in solubilized material by Western blotting or in residual insoluble material by immunofluorescence (data not shown), suggesting that the stage specificity results from failure of the antigen to be expressed rather than internal sequestration in the L4 and adult stages.

In contrast to the results obtained for wild-type L4 extracts, +SDS and −SDS extracts of synchronous *srf-(yj43)* L4s showed patterns of antigenicity very similar to those of wild-type L1 extracts, with the two major bands and upper minor band apparent in the +SDS sample, and the antigenic ladder apparent in the −SDS sample (Fig. 8, lanes *3* and *4*, respectively). Thus the L1-specific antigen appears to be expressed at the L4 stage in this mutant in a form similar to that observed in wild-type L1s.

The *srf-2* and *srf-3* mutant phenotypes evaluated by M38 binding represent a loss of antigenicity compared to wild-type; in contrast, the *srf-(yj43)* phenotype assessed biochemically represents the gain of antigenicity at the L4 stage compared to wild-type. To test whether a *srf-3* mutation prevents heterochronic expression of the L1-specific antigen in *srf-(yj43)* mutants, a double mutant strain containing *srf-3(yj10)* and *srf-(yj43)* was constructed and tested for M38 binding. Results are shown in Table I. Complementation testing confirmed that the putative double mutant contained both *srf-3(yj10)* and *srf-(yj43)* (Table I, lines 4 and 5). The

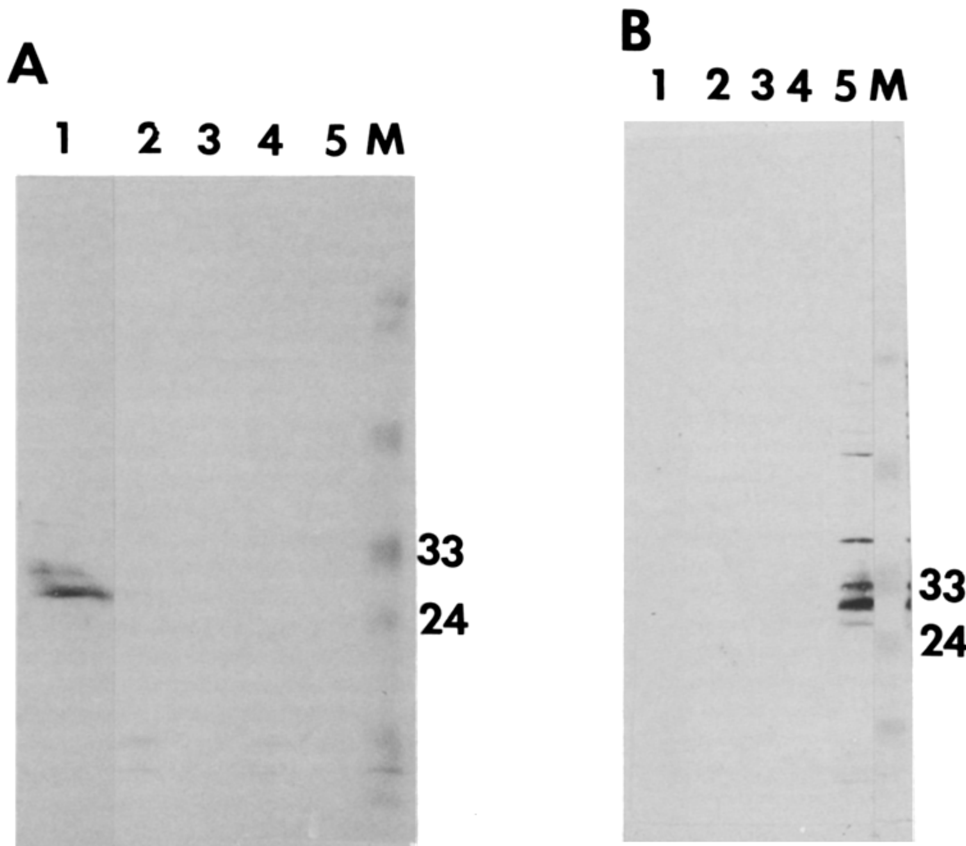


Figure 7. Immunoblot analysis of extracts of *srf-2(yj262)* (*A*) and *srf-3(yj10)* (*B*) mutants. All samples contained 20 μg of protein extracts of synchronous L1 populations. Proteins were separated by 12% SDS-PAGE. Blots were developed using glutaraldehyde fixation and HRP-conjugated secondary antibody. (*A*) Lane *1*, wild-type +SDS extract (positive control); lane *2*, *srf-2(yj262)* +SDS extract; lane *3*, *srf-2(yj262)* −SDS extract; lane *4*, 2-ME extract of +SDS extracted *srf-2(yj262)* L1s; lane *5*, 2-ME extract of −SDS extracted *srf-2(yj262)* L1s. (*B*) Lane *1*, *srf-3(yj10)* +SDS extract; lane *2*, *srf-3(yj10)* −SDS extract; lane *3*, 2-ME extract of +SDS extracted *srf-3(yj10)* L1s; lane *4*, 2-ME extract of −SDS extracted *srf-3(yj10)* L1s; lane *5*, wild-type +SDS extract (positive control).

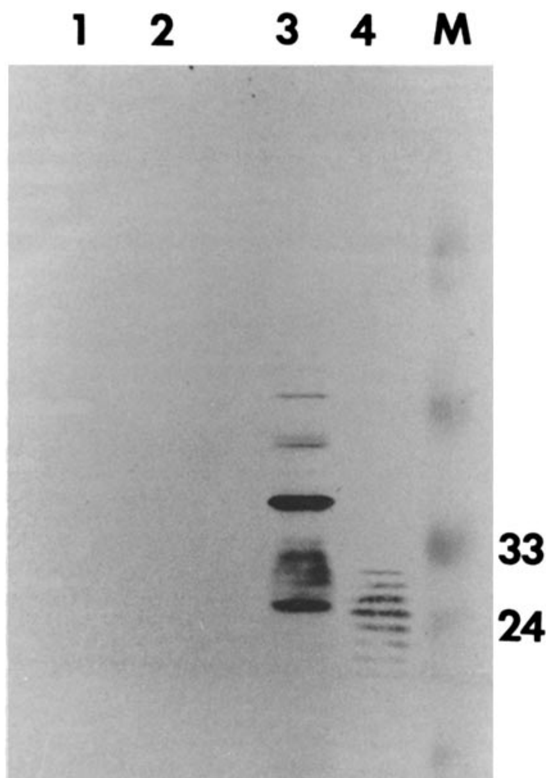


Figure 8. Immunoblot analysis of extracts of wild-type and *srf-(yj43)* synchronous L4 populations. Blot was developed using glutaraldehyde fixation and horseradish peroxidase-conjugated secondary antibody. Each sample contained 20 μg protein extract. Lane *1*, wild-type +SDS extract; lane *2*, wild-type −SDS extract; lane *3*, *srf-(yj43)* +SDS extract; lane *4*, *srf-(yj43)* −SDS extract.

homozygous double mutant was antigen-negative for M38 binding at stages L2-L4 (Table I, line 3), indicating that the heterochronic phenotype characteristic of *srf-(yj43)* was not expressed. However, L2-L4 double mutant animals did bind adult-adsorbed anti-cuticle rabbit antiserum that binds *srf-3* mutants but not wild-type animals (Politz et al., 1990), indicating that the *srf-3* phenotype was expressed (Table I, line 3). In control tests, M38 or adult-adsorbed serum staining of *srf-3* (Table I, line 1) or *srf-(yj43)* (Table I, line 2), produced the expected results. It can be concluded that *srf-3(yj10)* prevents heterochronic expression of the L1-specific antigen caused by *srf-(yj43)*.

Discussion

We have characterized biochemically an L1-specific surface glycoprotein molecule to understand the basis of its altered expression in *srf-2*, *srf-3*, and *srf-(yj43)* mutants of *C. elegans*. mAb M38 detects this marker in indirect immunofluorescence experiments in the wild-type strain only on the L1 surface, and in *srf* mutant strains either not at all or on inappropriate stages. These mutant phenotypes might arise in the former case by blocks in specific steps in the pathway of antigen synthesis, and in the latter case by altered regulation of timing of antigen expression. Such a phenotypic marker might be a valuable tool in the elucidation of the genetic mechanisms controlling expression of molecules at the nematode surface.

However, the layered organization and overall structural complexity of the *C. elegans* cuticle (Cox et al., 1981*a*) do not permit an immediate interpretation of these mutant phenotypes as changes in the expression of the surface antigen per se. For example, mutant phenotypes such as those described here might as well result from masking a normally exposed antigen (in the case of *srf-2* and *srf-3*) or ectopic expression of an antigen that is normally hidden at a given stage (in the case of *srf-(yj43)*. To distinguish between these possibilities, the antigen recognized by M38 was characterized biochemically.

In Western blotting experiments, the O-glycanase–sensitive antigenic pattern characteristic of +SDS extracts was indicative of an O-linked glycoprotein or proteins, while the antigenic ladder apparent in −SDS extracts was protease insensitive and could also be obtained by predigestion of +SDS extracts with Pronase. These results make it seem likely that the components of the ladder are protease-resistant, antigenic glycopeptide fragments produced by digestion of the intact antigen. It may be that extraction in the presence of SDS denatures an endogenous protease activity that otherwise converts the intact antigenic glycoprotein to glycopeptides. The antigenic ladder, and not the intact glycoprotein, was also detected in +SDS extracts of crude L1 cuticle fragments obtained after sonication of live L1s. This is consistent with the above model; because SDS was not present during sonication, the putative protease-inactivating effect of SDS would not have occurred in this case. Finally, the fact that a form of the antigen (the antigenic ladder) was extracted from crude cuticle fragments as well as from whole worms suggests that the antigen is indeed associated with the cuticle, and is not an unrelated internal cellular component. Such cuticle fragments are purified free of most cellular tissues except for remnants of muscle and hypodermis (Cox et al., 1981*a*,*b*).

The heterogeneous glycopeptide products of protease digestion may reflect heterogeneity in the lengths of oligosaccharides containing the M38 epitope. This may explain the broadness of the two O-glycanase–sensitive major antigenic bands in the +SDS extract. However, we have not yet distinguished whether the epitope itself is carbohydrate, peptide, or a mixed structure; neither the protease digestion results nor the O-glycanase digestion results resolve this issue definitively.

The M38 antigen can be dissociated from the cuticle without disulfide reduction, suggesting that it is more readily removed than the major structural components of the cuticle, the cuticle collagens (Cox et al., 1981*a*). In these respects, the M38 antigen is typical of a class of nematode cuticular molecules that have been termed "surface-associated" proteins. Several of these easily solubilized proteins and glycoproteins have been shown to be actively released in vitro by living parasitic nematodes (e.g., Philipp et al., 1980, 1988; Smith et al., 1981; Maizels et al., 1984). Presently, there is no direct evidence for release of the M38 epitope from the wild-type *C. elegans* L1 surface. However, it is interesting that the temperature sensitivity of M38 binding described in Materials and Methods is observed with live worms, but not with the freeze-fractured worms treated with methanol-acetone that were used for hybridoma screening (S. Donkin and S. Politz, unpublished results); the latter fixation should immobilize molecules in situ.

Partial characterization of the M38 antigen in wild-type extracts has allowed the mutant expression of this antigen to be characterized. When extracts of *srf-2* and *srf-3* mutant L1s

were analyzed, the antigen was not detected, suggesting that corresponding failure to detect the antigen on the surface of live mutant L1s was not due to masking of an antigen that was present in the cuticle, but unavailable for antibody binding. This explanation was also supported by failure to detect the antigen in extracts of mutant L1s with SDS plus 2-ME, a procedure that solubilizes the cuticle collagens, or by immunofluorescence of residual cuticle material insoluble after such extractions.

Although the *srf-2* and *srf-3* phenotypes described here appear to be loss of a stage-specific antigenic determinant, independent evidence has indicated that the surface of other stages in these mutants is affected as well (Politz et al., 1990), suggesting that a precursor common to biosynthesis of more than one surface antigen may be missing. The dominance of the wild-type allele of these genes in heterozygotes of *srf-2* and *srf-3* mutations further suggests that the phenotypes result from a loss of gene activity (Politz et al., 1990).

By analogy with studies of N-linked glycoprotein secretion in yeast (Deshaies, 1989) and mammalian cells (Stanley, 1987), the expression of an extracellular cuticle surface glycoprotein would require the execution of a multistep pathway involving protein synthesis, glycosylation, secretion, and transport and assembly at the cuticle surface. Therefore, functionally distinct genes ranging from those encoding the polypeptide portion of the surface protein to the enzyme genes responsible for its posttranslational modification might be identified by studying mutations like those in *srf-2* and *srf-3* that appear to block antigen expression.

The apparent heterochronic expression of the L1-specific surface antigen recognized by M38 in *srf-(yj43)* mutants cannot readily be explained as ectopic expression of a normally hidden antigen, as was reported previously for *srf-2* and *srf-3* (Politz et al., 1990), because the antigen was not detected at the L4 stage in any extracts of wild-type worms. A simple explanation consistent with the evidence is that the *srf-(yj43)* phenotype results from failure to down-regulate L1-specific antigen expression at later stages. An alternative possibility, that the antigen formed at the L1 stage is persisting at later stages rather than being synthesized then, cannot be eliminated at present. However, in *srf-(yj43)* synchronous populations at the L1-L2 molt, as in wildtype, the entire cuticle appears to be lost during ecdysis (D. Grenache and S. Politz, unpublished observations). Moreover, the extracted material equivalent to approximately seven times as many wild-type L1s as *srf-(yj43)* L4s must be used to obtain an immunoblotting signal of equivalent intensity (R. M. Hemmer and S. Politz, unpublished results). It therefore seems unlikely that the amount of antigen per worm is constant through postembryonic development.

The results of testing epistasis between *srf-3(yj10)* and *srf-(yj43)* indicated that heterochronic expression of the antigen in *srf-(yj43)* requires wild-type *srf-3* activity. This suggests that the *srf-(yj43)* heterochronic phenotype represents incorrect regulation of a single pathway, rather than activation of an alternative pathway for antigen synthesis. Western blotting experiments also indicated that the antigen present in *srf-(yj43)* L4s is unaltered compared to the antigen present in wild-type L1s, suggesting that its biosynthesis is similar regardless of stage.

By analogy with previously characterized *lin* heterochronic mutants of *C. elegans*, *srf-(yj43)* and the other mutants that express the L1 antigen at later stages may have alterations in regulatory genes that control timing of expression of this molecule. The *lin* heterochronic mutants affect the timing of execution of the developmental transition termed the larval-adult switch that normally occurs at the last molt (Ambros, 1989). These mutations, which are thought to identify major regulatory genes controlling timing of postembryonic developmental events, have their effect by displacing certain cell lineage patterns relative to the time that they occur in wild type (Ambros and Horvitz, 1984). It will be interesting to see whether any cell lineage alterations occur in the *srf* mutants. Further investigation of *srf* mutant phenotypes should reveal whether the heterochronic defect is restricted to surface molecules or extends to other developmentally regulated markers.

Inheritable changes in the molecules displayed at the nematode surface may be of adaptive significance. In nematode parasites, the surface is dynamic, with changes occurring both at and between molts (Philipp and Rumjaneck, 1984). Differential recognition of genetically determined surface variants by host immune systems might play a role in selection of intraspecific variants, and thereby affect the course of speciation. Moreover, such genetically based changes in surface antigenicity may affect the choice of antigens for vaccine development; selection for parasites that fail to express a certain antigen may preclude the use of that antigen as a protective vaccine.

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