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The *Caenorhabditis elegans* Vitellogenin Gene Family Includes a Gene Encoding a Distantly Related Protein

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While the nematode *Caenorhabditis elegans* is more primitive than most egg-laying organisms, it's vitellogenins, or yolk protein precursors, appear to be more complex. *C. elegans* oocytes accumulate two major classes of yolk proteins. The first consists of two polypeptides with an M_r of about 170,000 (yp170A and yp170B) encoded by a family of five closely related genes called *vit-1* through *vit-5*. The second class consists of two smaller proteins with M_r values of 115,000 (yp115) and 88,000 (yp88) which are cut from a single precursor. Here we report the cloning and analysis of a single-copy gene (*vit-6*) that encodes this precursor. The lengths of the gene and its mRNA are about 5×10^3 base pairs. Like *vit-1* through *vit-5*, *vit-6* is expressed exclusively in adult hermaphrodites. Comparison of portions of the coding sequence indicates that *vit-6* is distantly related to the *vit-1* through *vit-5* gene family. Thus, even though the two classes of yolk proteins are antigenically and physically distinct, they are encoded by a single highly diverged gene family.

Organisms as diverse as insects and vertebrates have been shown to contain a single class of closely related vitellogenins, or yolk protein precursors. These vitellogenins are encoded by small families of closely related genes (1, 3, 17, 26–28). In contrast the yolk proteins of *Caenorhabditis elegans* can be subdivided into two classes. The first class consists of two polypeptides with an M_r of 170,000 (yp170A and yp170B) (21). We have recently cloned and characterized a family of five closely related genes that code for those proteins (5). yp170A is specified by three genes: *vit-3* and *vit-4*, which are tightly linked on the X chromosome, and *vit-5*, which is not closely linked to *vit-3* and *vit-4*. (The *vit* terminology (for vitellogenin) is used in place of YP to conform with standard *C. elegans* genetic nomenclature.) *vit-5* is the only one of the three genes known to be expressed. yp170B is encoded by *vit-2*. The remaining gene, *vit-1*, is closely related to *vit-2* and has recently been shown to be a pseudogene (23a).

The second class of yolk proteins is composed of two smaller polypeptides with M_r values of 115,000 and 88,000 (yp115 and yp88). These are two immunologically distinct proteins that are cleaved from a short-lived vitellogenin, or yolk protein precursor, with an M_r of about 180,000 (22), hereafter called VIT180. We show here that a single gene, *vit-6*, encodes VIT180 and that this gene is distantly related to the five-gene family that specifies that two larger yolk proteins. Thus, like previously studied organisms, *C. elegans* contains a small family of closely related vitel-

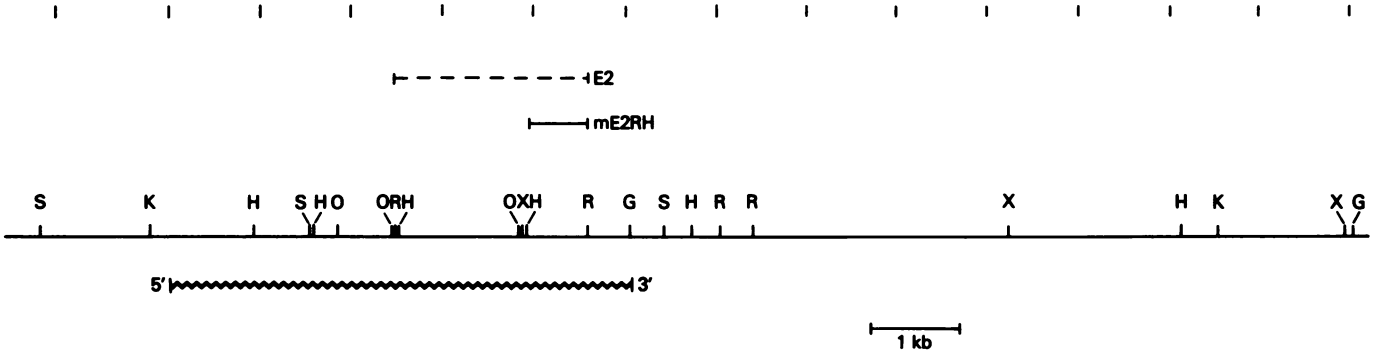

FIG. 1. Restriction map of genomic clone F9, containing *vit-6*. The positions that the restriction endonucleases *Bgl*II (G), *Eco*RI (R), *Hin*dIII (H), *Kpn*I (K), *Sal*I (S), *Xba*I (X), and *Xho*I (O) cleave the clone are shown. Above the map the dashed line represents the extent of the cDNA clone E2, and the solid line indicates the boundaries of the M13 clones used in the experiments shown in Fig. 6. The wavy line below the map represents the approximate extent of the *vit-6* gene as determined by hybridization of random- and oligo(dT)-primed cDNAs to fragments of the genomic clone. The coordinate scale is in kilobases.

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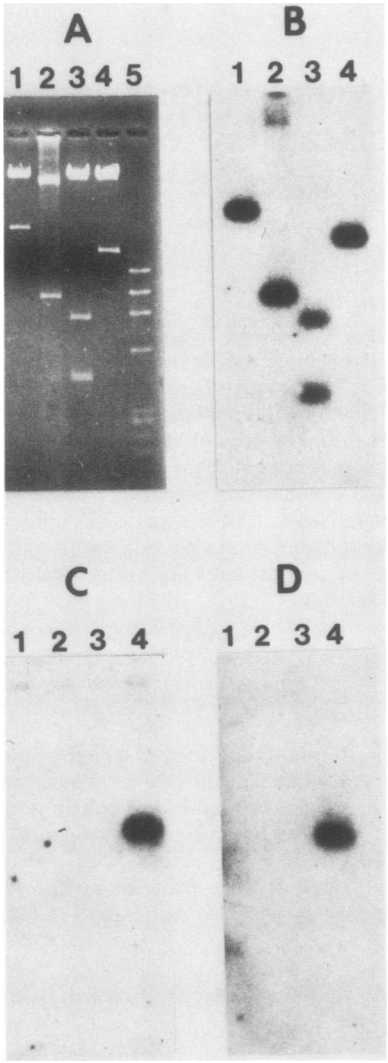

FIG. 2. Hybridization of adult, larval, and male cDNA to vitellogenin and actin cDNA clones. ^{32}P-labeled DNA was made to total RNA isolated from populations of worms enriched for either adult hermaphrodites (B), males (C), or larvae (D). Lanes 1, the 2.2-kb cDNA clone E2 from *vit-6*; lanes 2, the 1.0-kb cDNA clone 53 from the 3′ end of *vit-2*; lanes 3, the 1.6-kb cDNA clone 153 from the 3′ end of *vit-5* (Fig. 4 [5]); lanes 4, a cDNA clone containing most of the coding region of the *C. elegans* actin gene (gift of B. Meyer). The clones were digested with *Eco*RI, run on a 1.8% agarose gel, stained with ethidium bromide (A), and hybridized with the three ^{32}P-labeled cDNAs. φX174RF DNA digested with *Hae*III served as markers (panel A, lane 5).

logenin genes, but unlike the other species, it also contains a highly diverged member of the gene family that encodes a distantly related vitellogenin.

MATERIALS AND METHODS

General. Maintenance and methods for handling *C. elegans* have been described previously (7, 24). Polyacrylamide gel electrophoresis of protein was performed by the method of Laemmli (12), with modifications as described by Sharrock (21). Fluorography was performed by the method of Bonner and Laskey (6). Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass. T4 DNA Ligase and DNA polymerase Large Fragment were purchased from New England Biolabs, Inc., Beverly, Mass. Restriction endonucleases were obtained from New England Biolabs, Beverly, Mass., or Bethesda Research Laboratories, Gaithersburg, Md. 2′,3′-Dideoxynucleoside triphosphates were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Isolation of poly(A) RNA and cDNA library construction. Detailed procedures for the isolation of poly(A) RNA and the construction of the cDNA library have been described previously (5). RNA was isolated from liquid cultures of *C. elegans* growing on *Escherichia coli* NA22 (24). Worms were disrupted in guanidinium hydrochloride with a Stansted cell disruptor, and the RNA was purified as described by MacLeod et al. (13). Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography, denatured with methyl mercury, and fractionated on a 5 to 20% sucrose gradient. Double-stranded cDNA was made to high-molecular-weight RNA primed with oligo(dT). This cDNA was digested with the restriction endonuclease *Sau*3AI, ligated into *Bam*HI-digested plasmid pACYC184 (10), and transformed into *E. coli* HB101.

Selection of cDNA clones. Putative yolk protein clones have been previously selected on the basis of their intense hybridization with ^{32}P-labeled cDNA made from high-molecular-weight, poly(A)-containing RNA (5). Sixteen clones that were not previously known to be members of the *vit-1* through *vit-5* gene family were electrophoresed in a

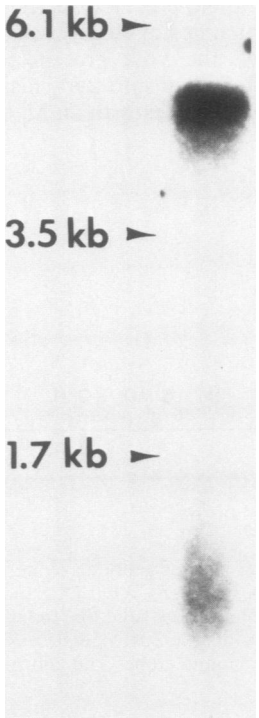

FIG. 3. Hybridization of the *vit-6* cDNA clone B7 to *C. elegans* RNA. Approximately 2 μg of total RNA isolated from adult worms was electrophoresed on a 1.0% agarose gel containing 6% formaldehyde and transferred to nitrocellulose by the method of Bruskin et al. (9). Hybridization was to nick-translated cDNA plasmid B7 DNA. The 6.1-kb marker is based on the band that hybridized to nick-translated *unc*-54 myosin gene DNA (8). The 3.5- and 1.7-kb markers are based on the rRNA bands visible after staining with ethidium bromide.

1.0% agarose gel and transferred to nitrocellulose (23). The blots were hybridized to four ^{32}P-labeled probes. cDNA probes were made by oligo(dT)-primed reverse transcription of either adult or larval RNA preparations (provided by W. Sharrock) with avian myeloblastosis virus RNA-dependent DNA polymerase (Life Sciences. Inc., St. Petersburg, Fla.). An 8.0-kilobase (kb) *Sal*I fragment containing *vit-5* was isolated from clone 2017, and 4.5-kb *Hin*dIII fragment containing *vit-1* was isolated from clone 23 (see Fig. 4 [5]). The two fragments were nick translated individually. The cDNA and nick-translated probes were hybridized to the cDNA clone blots at 65°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–Denhardt solution (14)–herring sperm DNA (250 μ/ml). The filters were washed in SSC containing 0.1% sodium dodecyl sulfate at 65°C. One clone (B7) that hybridized only to the adult cDNA probe was chosen for further analysis.

Selection of phage clones. An amplified lambda 1059 library (gift of J. Karn) containing partial *Sau*3AI digests of *C. elegans* genomic DNA (SG_{25}) or a library (gift of B. Meyer) of oligo(dT)-primed cDNA clones from *C. elegans* in the vector lambda gt10 was plated on *E. coli* Q358 (11). Plaques were transferred to nitrocellulose (4) and hybridized to nick-translated, gel-purified restriction fragments from plasmid DNA.

Hybrid-arrest translation. Hybrid-arrest translation was performed as described by Paterson et al. (16) and as modified by Blumenthal et al. (5). Translations in wheat germ extracts were as described by Roberts and Paterson (18).

DNA sequence analysis. DNA nucleotide sequence was determined by the dideoxynucleotide method of Sanger et al. (19, 20). Shotgun libraries were prepared in bacteriophage M13 (15). A 1.2-kb *Sal*I-*Sst*I fragment from the *vit-2* genomic clone 15, a 1.3-kb *Sal*I-*Hin*dIII fragment from the *vit-5* genomic clone 2017 (5), and a 1.1-kb *Kpn*I-*Hin*dIII fragment from the *vit-6* genomic clone F9 (Fig. 1), each containing the 5′ end of their respective genes, were electroeluted from agarose. Each was digested with *Sau*3AI and ligated into the *Bam*HI site of M13. The end fragments were cloned into the appropriate doubly digested M13 vector. The sequences of the clones were aligned by fitting restriction sites derived from the sequence to a detailed restriction map of the region sequenced. The alignments were confirmed by sequencing fragments that crossed the boundaries of the aligned sequences and that were cloned from restriction sites predicted in the sequence. Sequence data were compiled and analyzed with DNA/Protein Sequence Analysis Software (International Biotechnologies Inc., New Haven, Conn).

RESULTS

Clone selection. The *vit-1* through *vit-5* genes were cloned on the basis of the abundance of their mRNAs in a poly(A)-containing fraction of *C. elegans* high-molecular-weight RNA (5). A cDNA library in pACYC184 was made from this RNA and then screened by colony hybridization with ^{32}P-labeled cDNA made from the same RNA preparation. Most clones which gave intense hybridization signals were shown to be from *vit-1* through *vit-5* mRNA. All of the inserts were small since the cDNA was cut with *Sau*3AI prior to cloning. These small cDNA fragments were then used to select larger cDNA and genomic clones from lambda phage libraries (5).

We used this same pACYC184 library to obtain clones corresponding to the gene for VIT180. Based on the amounts of yp115 and yp88 made in vivo and the amount of VIT180 synthesized in vitro, the mRNA for VIT180 would be expected to be as abundant as the mRNA for the two larger yolk proteins. Therefore, 16 additional clones were selected on the basis of strong hybridization to ^{32}P-labeled cDNA made against either size-selected, poly(A)-containing RNA or total poly(A)-containing RNA. These 16 clones all hybridized to cDNA made against total RNA from a population of *C. elegans* enriched for adults and failed to hybridized to cDNA made against total RNA from larvae (both RNA preparations were generously provided by W. Sharrock). Of the 16 clones, 15 were determined to be from members of the *vit-1* through *vit-5* gene family based on intense hybridization to nick-translated fragments from *vit-1* or *vit-5*. The remaining clone (B7) contained a 75-base-pair *Sau*3AI fragment that did not hybridize to *vit-1* or *vit-5*. This clone was used to isolate related clones from lambda libraries.

B7 was used to screen a library containing oligo(dT)-primed cDNA cloned into lambda gt10. One of the lambda gt10 recombinants (E2) was used to screen a library containing a *Sau*3AI partial digest of *C. elegans* genomic DNA

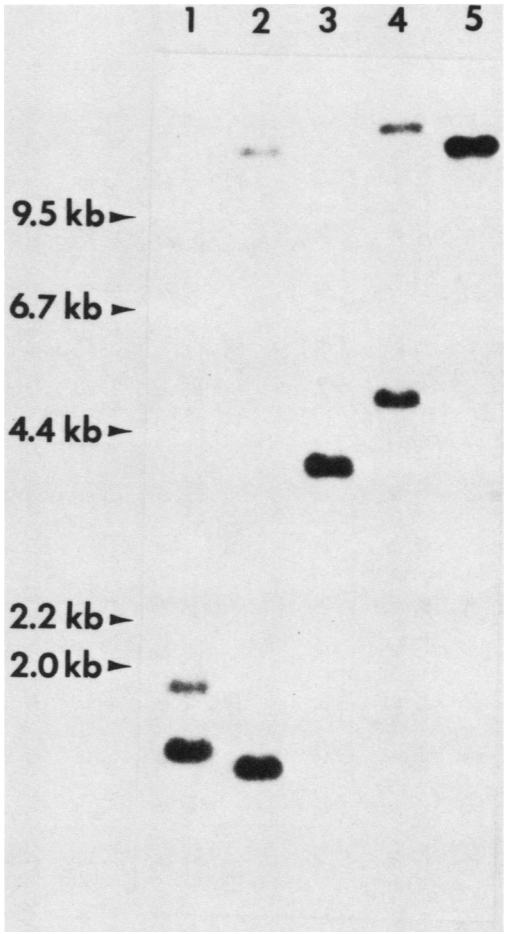

FIG. 4. Hybridization of the *vit-6* cDNA clone E2 to *C. elegans* genomic DNA. Genomic DNA (approximately 4.5 μg per lane) was digested with restriction endonucleases *Hin*dIII (lane 1), *Xho*I (lane 2), *Sal*I (lane 3), *Xba*I (lane 4), and *Bgl*II (lane 5); electrophoresed on a 0.6% agarose gel; and transferred to nitrocellulose by the method of Southern (23). The 2.1-kb *Eco*RI insert from the cDNA clone E2 was electroeluted from an agarose gel, nick translated, and hybridized to the nitrocellulose filter containing the bound DNA. Lambda DNA digested with the restriction endonuclease *Hin*dIII served as markers.

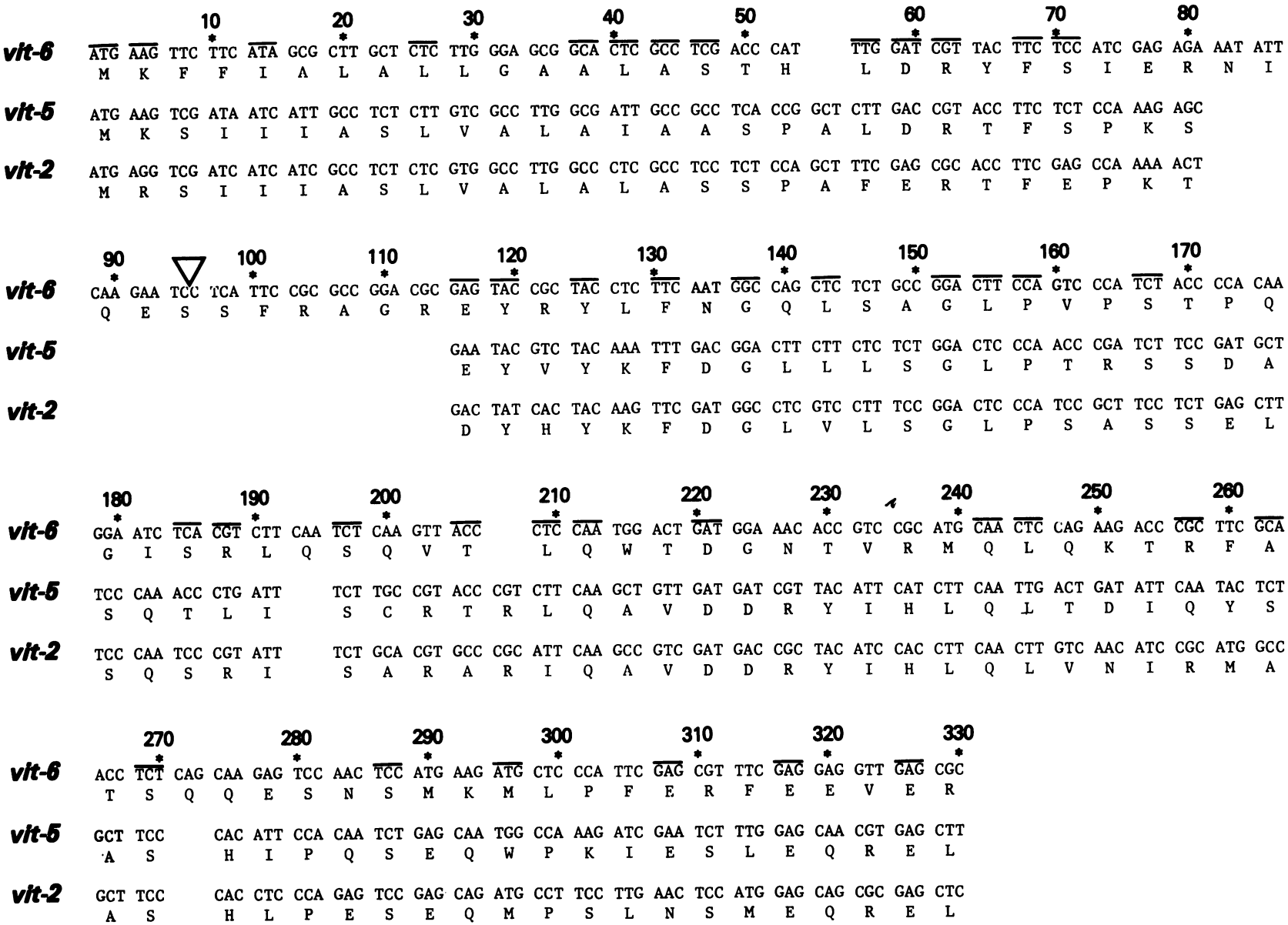

FIG. 5. Comparison of DNA sequences and predicted amino acid sequences ot *C. elegans* vitellogenin genes. The sequences form the first AUG codon in each mRNA are shown (Spieth et al., submitted). The derived amino acid sequence was aligned to give the greatest degree of homology with the fewest insertions and deletions. A solid line over a codon indicates that *vit-6* shares an identical amino acid with at least one of the other proteins at that position. The position of a 90-base-pair intron in *vit-6* is indicated by a triangle.

cloned into lambda 1059. A restriction map of one of the clones (F9) containing the entire gene (*vit-6*) is shown in Fig. 1. The approximate limits of the coding region were determined by hybridizing blots of the restriction endonuclease-digested clone F9 to ^{32}P-labeled cDNA made by reverse transcription of total *C. elegans* RNA with either small fragments of calf thymus DNA (25) or oligo(dT) as primers for the reaction. The 3′ end of the gene was tentatively identified on the basis of its more intense hybridization to the oligo(dT)-primed cDNA probe. This assignment has since been confirmed by DNA sequencing and hybrid-arrest translation experiments (see below). The precise location of the 5′ end of the gene has been determined by primer extenxion analysis (23a). The size of the gene is approximately 5 kb. Thus, *vit-6* is very similar in size to *vit-1* through *vit-5*, but there are no similarities between the restriction map of *vit-6* and those of *vit-1* through *vit-5*.

Adult hermaphrodite specificity and size of the mRNA. The sex and stage-specific expression of *vit-6* was investigated by hybridizing ^{32}P-labeled cDNA to blots of the restriction enzyme-digested cDNA clone E2. The cDNA was made by reverse transcription of total RNA isolated from a population of worms enriched for either adult hermaphrodites (Fig. 2B), males (Fig. 2C), or larvae (Fig. 2D) (male RNA was provided by U. Heine). Each of the cDNA probes hybridized to a control cDNA clone containing the *C. elegans* actin gene (Fig. 2, lanes 4). However, only the cDNA probe made from adult hermaphrodite RNA hybridized to the *vit-6* cDNA clone (Fig. 2, lanes 1). Subclones of the *vit-1*; *vit-2* (Fig. 2, lanes 2); and the *vit-3*, *vit-4*, *vit-5* (Fig. 2, lanes 3) subfamilies also only hybridized to the cDNA probe from adult hermaphrodite RNA. The relative hybridization of adult hermaphrodite cDNA to *vit-6* is at about the same intensity as it is to the other two subfamilies, indicating that the RNAs are approximately equal in abundance in the adult hermaphrodite. [Hybridization between *vit-6* and the other vitellogenin genes is not observed unless the stringency is reduced (unpublished data) so the hybridization to *vit-6* cannot be from *vit-1* through *vit-5* mRNA under the conditions used.]

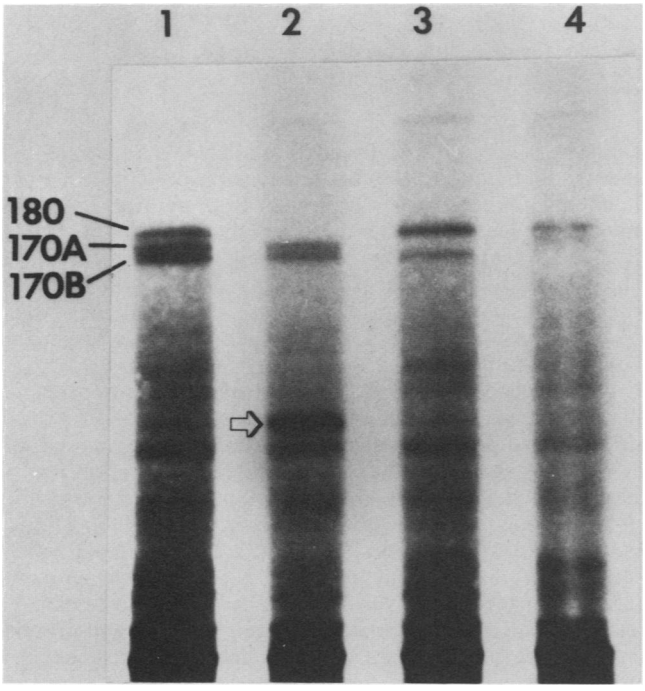

FIG. 6. Translation of *C. elegans* RNA after hybridization to M13 clones. Single-stranded DNA isolated from M13 clones was hybridized to total *C. elegans* RNA isolated from a population of worms enriched for adults. The hybridization reactions were translated in wheat germ extracts and electrophoresed on a 5% polyacrylamide-sodium dodecyl sulfate gel. Lane 1, no DNA; lane 2, clone E2RH, a *vit-6* subclone; lane 3, clone 828, a *vit-5* subclone; lane 4, clone 933, a *vit-2* subclone (Fig. 4, [5]). The arrow indicates the position of a polypeptide possibly resulting from premature termination of translation of the *vit-6* mRNA. The truncated products form the reactions in lanes 3 and 4 are visible on 10% polyacrylamide-sodium dodecyl sulfate gels (data not shown).

To determine the size of the mRNA, the cDNA clone B7 was nick translated and hybridized to blots of total RNA isolated from a population enriched for adults. B7 hybridized to a single 5.1-kb band (Fig. 3). The RNA homologous to B7 is indistinguishable in size from RNA homologous to the *vit-1* through *vit-5* family, but it cannot be *vit-1* through *vit-5* RNA, since B7 does not hybridize detectably to these genes, even under conditions of reduced stringency (data not shown). A size of 5.1 kb is very close to that of both the estimated length of the gene and the length of mRNA required to encode a 180-kilodalton polypeptide. Thus, like *vit-1* through *vit-5*, *vit-6* contains little room for introns or untranslated regions.

***vit-6* is a single gene distantly related to *vit-1* through *vit-5*.** To determine if *vit-6* is a single gene, the 2.1-kb *Eco*RI fragment from the cDNA clone E2 was hybridized to blots of *C. elegans* genomic DNA (Fig. 4). All of the restriction fragments which hybridized to the probe (at moderate stringency) were found on the restriction map of the cloned DNA, indicating that there are no other genes closely related to *vit-6*. We have not eliminated the possibility that there is more than one gene identical to *vit-6*, but this possibility is made highly unlikely by the fact that most of the restriction sites at which the DNA was cut in the experiment in Fig. 4 are outside the gene. Hence, a very large region of the DNA including *vit-6* would have to be duplicated. This situation would be without precedent in *C. elegans*.

Some fragments of *vit-6* hybridized to *vit-2* and somewhat less strongly to *vit-5* under conditions of reduced stringency (data not shown). Furthermore, we determined the nucleotide sequence of the region surrounding the 5′ end of *vit-6* and compared that with the same region of *vit-2* and *vit-5*. The data show that *vit-6* is related to *vit-1* through *vit-5* but has diverged substantially. The 330 bp of *vit-6* shown in Fig. 5 is 52% homologous to *vit-2* and 44% homologous to *vit-5* at the nucleotide sequence level. The polypeptide encoded by this region of *vit-6* is 30% homologous to those encoded by both *vit-2* and *vit-5*. The relationship between *vit-5* and *vit-6* was verified though a computer analysis of the first 259 amino acid residues by the mutation data matrix and ALIGN program of Barker et al. (2). In this region there are 70 of 228 possible matches between residues, with 12 breaks (gaps). The alignment score is 18.81 SD which is highly significant (2).

vit-6 contains two introns in this region: the one indicated by the triangle in Fig. 5 and another that is 483 base pairs further in the 3′ direction. *vit-5* does not contain introns at either of these positions (J. Spieth et al., unpublished data).

***vit-6* encodes VIT180.** The 2.1-kb *Eco*RI insert of E2 was isolated and cut with *Hin*dIII. A resulting 0.7-kb *Eco*RI-*Hin*dIII fragment was subcloned into M13mp8 and M13mp9. The single-stranded DNA from the subclones was used in

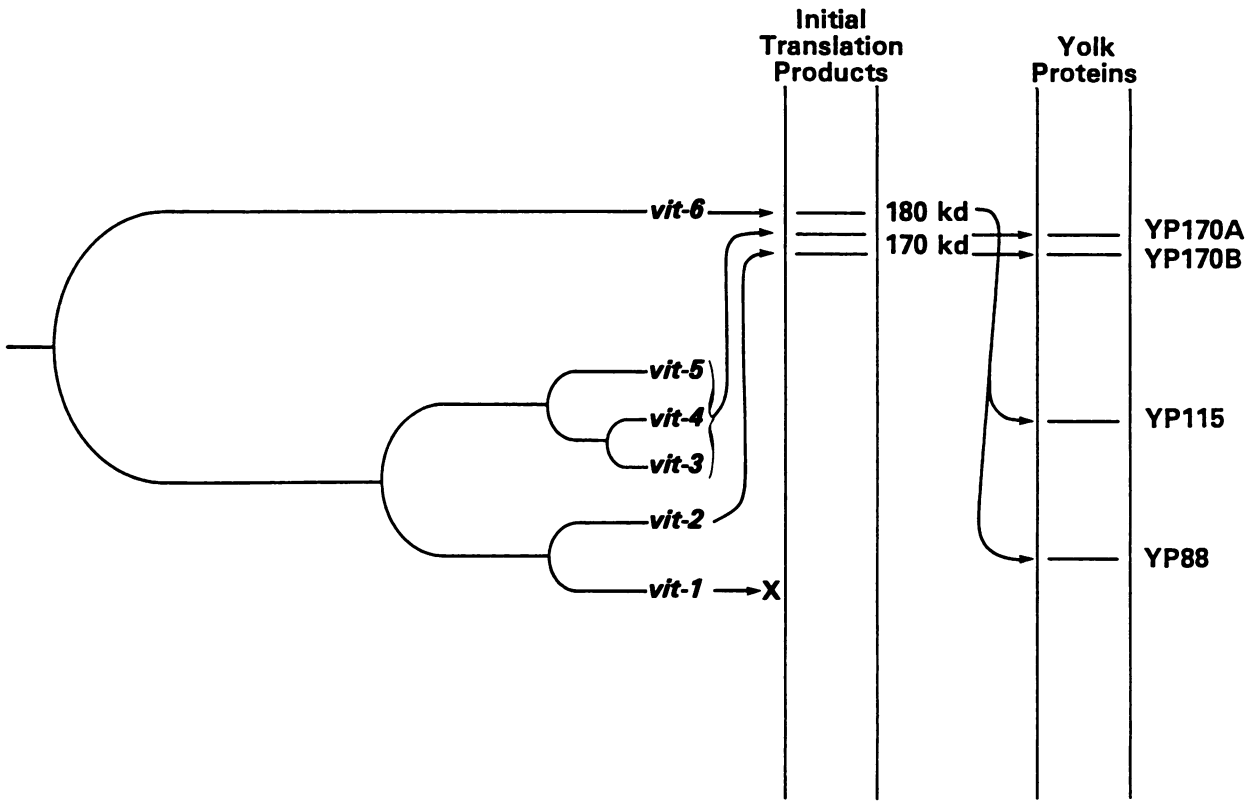

FIG. 7. Relationships of the members of the *C. elegans* vitellogenin gene family and the encoded proteins. kd, Kilodaltons.

hybrid-arrest translation experiments (Fig. 6). One of the M13 subclones (mE2RH, Fig. 1) arrested the translation of VIT180 and also caused the formation of a new polypeptide with an M_r of 140,000, which is the size predicted for a truncated polypeptide from premature termination of translation at the site of hybridization (Fig. 6, lane 2, arrow). A subclone containing the opposite strand (the same fragment cloned in reverse orientation) did not affect translation (data not shown). Neither subclone affected the translation of yp170A or yp170B. However, in control experiments M13 subclones containing fragments of either the *vit-1* and *vit-2* (Fig. 6, lane 4) or *vit-3* through *vit-5* (Fig. 6, lane 3) subfamilies did arrest translation of yp170B and yp170A, respectively, but did not affect the translation of VIT180. We conclude that *vit-6* encodes VIT180.

DISCUSSION

Vitellogenin gene families have previously been analyzed in insects and vertebrates. *Drosophilia* sp. has been shown to contain three closely related vitellogenin genes encoding polypeptides with very similar sizes and structures (3, 17). Frogs and chickens also each contain small gene families encoding major vitellogenins very similar to each other (1, 26–28). On the other hand *C. elegans* contains two quite divergent classes of yolk proteins (21). The first class consists of two large proteins (yp170A and yp170B) encoded by a five-gene family, *vit-1* through *vit-5* (5). The second class consists of two smaller proteins (yp115 and yp88) which are cut from a single precursor molecule, VIT180 (22). We have described here the cloning and characterization of a gene (*vit-6*) that codes for VIT180. *vit-6* is a single-copy gene that shares many characteristics with genes *vit-1* through *vit-5*. The gene is about 5.1 kb in length; its RNA is 5 kb in length and is abundant in the adult hermaphrodite but is not detectable in either males or larvae. Both hybridization and nucleotide sequence data indicate that *vit-6* is a highly diverged member of the *vit-1* through *vit-5* gene family. Thus, in *C. elegans* the single gene family that encodes vitellogenins is more complex than those in higher organisms. It produces polypeptides which are quite dissimilar from one another.

The six genes which we have cloned appear to represent all of the genes encoding the four major yolk proteins, since all of the bands seen on genomic blots can be accounted for by *vit-1* through *vit-6*. They encode three electrophoretically distinct vitellogenins (Fig. 7). *vit-5*, and possibly *vit-3* and *vit-4*, encode yp170A. *vit-2* encodes yp170B (*vit-1* is a pseudogene). *vit-6* encodes the precursor to yp115 and yp88. Although we have discovered no similarities between nematode and insect genes, vertebrate genes appear to be closely related to those from nematodes. Their mRNAs and protein products are similar in size; their 5′ untranslated sequences are exceptionally short; and, most convincingly, the encoded vitellogenins of nematodes are remarkably homologous to those of vertebrates, at least for the regions sequenced so far, including the 71 amino acids at the N-terminus (23a). Thus, it seems likely that the vitellogenin gene families of nematodes and vertebrates are of common origin. This extended gene family has evolved to produce dissimilar polypeptides. At least three strategies for production of the final yolk proteins are apparent. First, the products of *vit-1* through *vit-5* do not appear to be cleaved and are packaged into the yolk intact (21). Second, the products of the vertebrate genes are cleaved into lipovitellin

and phosvitin after uptake into the oocyte (26, 27). Finally, the product of *vit-6* is cleaved in the body cavity to form yp115 and yp88 (22).

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