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Autosomal Genes of Autosomal/X-Linked Duplicated Gene Pairs and Germ-Line Proliferation in *Caenorhabditis elegans*

John Maciejowski,* James Hyungsoo Ahn,* Patricia Giselle Cipriani,* Darrell J. Killian,* Aisha L. Chaudhary,* Ji Inn Lee,* Roumen Voutev,* Robert C. Johnsen,† David L. Baillie,† Kristin C. Gunsalus,*,‡ David H. A. Fitch* and E. Jane Albert Hubbard*,[1]

**Department of Biology, New York University, New York, New York 10003, †Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada and ‡Center for Comparative Functional Genomics, New York University, New York, New York 10003*

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ABSTRACT

We report molecular genetic studies of three genes involved in early germ-line proliferation in *Caenorhabditis elegans* that lend unexpected insight into a germ-line/soma functional separation of autosomal/X-linked duplicated gene pairs. In a genetic screen for germ-line proliferation-defective mutants, we identified mutations in *rpl-11.1* (L11 protein of the large ribosomal subunit), *pab-1* [a poly(A)-binding protein], and *glp-3/eft-3* (an elongation factor 1-α homolog). All three are members of autosome/X gene pairs. Consistent with a germ-line-restricted function of *rpl-11.1* and *pab-1*, mutations in these genes extend life span and cause gigantism. We further examined the RNAi phenotypes of the three sets of *rpl* genes (*rpl-11*, *rpl-24*, and *rpl-25*) and found that for the two *rpl* genes with autosomal/X-linked pairs (*rpl-11* and *rpl-25*), zygotic germ-line function is carried by the autosomal copy. Available RNAi results for highly conserved autosomal/X-linked gene pairs suggest that other duplicated genes may follow a similar trend. The three *rpl* and the *pab-1/2* duplications predate the divergence between *C. elegans* and *C. briggsae*, while the *eft-3/4* duplication appears to have occurred in the lineage to *C. elegans* after it diverged from *C. briggsae*. The duplicated *C. briggsae* orthologs of the three *C. elegans* autosomal/X-linked gene pairs also display functional differences between paralogs. We present hypotheses for evolutionary mechanisms that may underlie germ-line/soma subfunctionalization of duplicated genes, taking into account the role of X chromosome silencing in the germ line and analogous mammalian phenomena.

GENOMES are shaped over evolution by a variety of forces, including gene duplication. The functions of duplicated genes can change over evolutionary time as well. One copy of a duplicated gene pair may acquire deleterious mutations, ultimately rendering it useless to the organism. Alternatively, both genes may remain functional. In the latter case, the duplicates may remain fully redundant or they may take on nonredundant roles. If the ancestral gene fulfilled multiple roles (*e.g.*, was expressed in multiple regions of an organism), subfunctionalization can follow gene duplication such that the duplicated genes acquire different functional roles (Thomas 1993; Hughes 1994; Lynch and Conery 2000).

One potential factor postulated to influence the fate of duplicated genes is germ-line-specific X chromosome inactivation (McKee and Handel 1993). This phenomenon occurs in the genomes of many organisms, including the nematodes *Caenorhabditis elegans* and *C. briggsae* and male mammals. In mammals, the XY body forms during spermatogenesis (see Handel 2004 and references therein). In the nematodes, repressing histone modifications have been observed on the X throughout much of hermaphrodite and all of male germ-line development (Fong *et al.* 2002; Kelly *et al.* 2002).

Silencing of the X chromosome during germ-line development could conceivably place constraints on genome organization. Taking cell-essential or housekeeping genes into account, this phenomenon suggests that (1) cell-essential genes are not located on the X; (2) if located on the X, these genes are not subject to inactivation; or (3) germ-line-active copies of these genes must be located in at least one additional place in the genome (*i.e.*, on an autosome). The first possibility is suggested by functional-genomic and microarray studies that have demonstrated a dearth of essential genes (Piano *et al.* 2002; Kamath *et al.* 2003) and germ-line-enriched genes (Reinke *et al.* 2000) on the X of *C. elegans* and of late spermatogenesis-acting genes in mammals (Khil *et al.* 2004). The second possibility is still unresolved. The last possibility was suggested by McKee and Handel (1993) and may be a determining factor for the exis-

[1]*Corresponding author:* Department of Biology, New York University, 100 Washington Square East, 1009 Silver Center, New York, NY 10003. E-mail: jane.hubbard@nyu.edu

tence of several autosomal intronless genes that function late in mouse spermatogenesis and that are closely related to intron-containing genes on the X (Handel 2004).

Here, we provide genetic evidence suggesting that the activity of certain X-linked housekeeping genes in *C. elegans* is restricted to the soma and that the presence of an autosomal duplicate in the genome ensures that this activity is maintained in the germ line. In a forward genetic screen for genes required for early germ-line proliferation, we identified three alleles representing three different germ-line-active genes (*rpl-11.1*, *pab-1*, and *glp-3/eft-3*), all of which have a paralog on the X chromosome. Further functional analysis identified a fourth gene with these properties, *rpl-25.2*, and suggests that all four of these gene pairs are subfunctionalized with respect to the germ line and soma. Additional analysis of the *C. elegans* genome and results of large-scale RNAi screens suggests that members of other autosome/X gene pairs may be similarly subfunctionalized. An additional *rpl* gene, *rpl-24*, is an autosome/autosome duplicate pair and does not display the same degree of soma/germ-line subfunctionalization. Of the five duplications we examined in the *rpl*, *pab*, and *eft* genes, *eft-3/eft-4* occurred recently over the course of *C. elegans* evolution, while the others preceded the divergence of *C. elegans* and *C. briggsae* lineages. Functional analysis of the orthologous pairs of the four duplicated *C. briggsae* genes suggests that the members of the gene pairs are functionally distinct, but may exhibit a lesser degree of germline/soma distinction. We present several hypotheses for evolutionary mechanisms that may underlie germline/soma subfunctionalization of duplicated genes, X chromosome silencing, and genome organization.

MATERIALS AND METHODS

Worm handling and strains: Wild types were the *C. elegans* var. Bristol N2 strain and Hawaiian CB4856 strain. The following mutations were used [from Brenner (1974) unless indicated]: LGI—*lin-11(n566)* (Trent *et al.* 1983), *unc-75(e950)* (Anderson and Brenner 1984), and *rrf-1(pk1417)* (Sijen *et al.* 2001); LGIII—*unc-32(e189)*, *glp-1(q175)* (Austin and Kimble 1987), *glp-3(q145)* (Kadyk *et al.* 1997), *sma-3(e491)*, and *unc-36(e251)*; and LGV—*dpy-11(e224)* and *unc-34(e566)*. Deficiencies used were *qDf7* (Ellis and Kimble 1995), *sDf70* (Stewart *et al.* 1991), *sDf50* (Johnsen and Baillie 1988), and *Df(e2173)* (Johnsen 1990). The following rearrangements were used: *eT1* (III;V) (Rosenbluth and Baillie 1981); *hT2[let-? qIs48]* (I;III), hereafter referred to as *hT2[let GFP]*; *nT1[unc-?(n754) let-? qIs50]*(IV;V) (Mathies *et al.* 2003); and the latter *nT1* without *qIs50* (Ferguson and Horvitz 1985). The *C. briggsae* strain was PB800.

Phenotypic analyses: For time course analysis of *ar228* and *ar232* germ-line proliferation, worms (homozygous mutants and sibling heterozygotes expressing a dominant GFP marker) were synchronized by hatch-off at 25° and cultured at either 15° and 25° (*ar228*) or 25° (*ar232*) before being examined live under Nomarski optics and/or individually fixed and stained with DAPI [synchronization and staining as described in Pepper *et al.* (2003a)] at three time points corresponding to early L1 (within 2 hr of hatching at 25°), L3 (48 hr at 15° or 24–26 hr at 25°), and early adult (96 hr at 15° or 48 hr at 25°).

All *C. elegans* RNAi experiments were conducted by feeding at 25° as described (Timmons *et al.* 2001). L4 animals were fed RNAi-inducing or control [L4440 vector in HT115(DE3)] bacteria and removed as adults after 24 hr. Progeny from these animals were maintained on the same bacteria until being scored as adults, 48 hr later. All animals were scored first under low magnification and sterile animals were inspected under higher magnification—both live and after fixation and staining with DAPI. The RNAi feeding plasmid was pGC10 for *rpl-11.1*: a PCR product was amplified from N2 worms (using sjj_T22F3.4 primers; Kamath *et al.* 2003), digested with *Sac*I, and cloned into *Sac*I/*Hin*cII of L4440 (Timmons *et al.* 2001). Feeding constructs were used for all additional experiments (MRC gene service; Fraser *et al.* 2000; Kamath *et al.* 2003).

Genomic DNA templates for *C. briggsae* RNAi were PCR amplified from the following primers (each forward primer sequence was preceded by gggaaggtacc and each reverse primer was preceded by caaaacggccg, where the underlined sequence is the *Kpn*I or *Eag*I recognition site in the case of each forward and reverse primer, respectively, that was subsequently used to clone each fragment into the same sites in the L4440 vector):

CBG01314—F:CAAGATTCAAAAGCTCTGCC, R:CACCTGAAAAAAGTCCGAAC;
CBG14053—F:GAGATAGACTTACCCGTGCC, R:TCATACTTCTGTTGGAACCAC;
CBG22273—F:ATCAGATGGACCGTCCTTTACAG, R:TTTATCGCTTTCCTCCGACGC;
CBG03702—F:CTGCTCTTCTCCGATCTACCC, R:TCTTCTTCTCCTTTGTTGCTG;
CBG14529—F:AGAAGGTCGCCAAGGGAC, R:GTTGGAGTGGGGTGATGAG;
CBG04080—F:CCTCGGTTCATTTCAGACG, R:TAGTCGGAAGCCAATCGCAC;
CBG02207—F:CAAGATGGTCTGCTCGAAGC, R:GTCCACCGATGACGATTCC;
CBG07431—F:GGATTCGGATTCGTTGCC, R:GGATAGATTGGAGCCATTCC.

The identity of each construct was verified by sequencing with M13F(-21) primer. Prior to *in vitro* transcription (Ribomax; Promega, Madison, WI), each construct or the "empty" L4440 (negative control) was linearized with appropriate (blunt or 5′-overhang) enzymes. Transcribed RNA products were checked by gel electrophoresis prior to injection. dsRNA was injected into both arms of the germ line as described by Guo and Kemphues (1995) and Fire *et al.* (1998). Injected animals were raised at 20°, transferred to a new plate after overnight recovery from the injection, and subsequently transferred as necessary to score for maternal sterility (injected worm) and progeny survivorship. The negative control was the L4440 plasmid carried through the entire protocol in parallel. A positive control dsRNA (Cb PAR-1) was also injected and a highly penetrant maternal embryonic lethality phenotype was observed.

The *glp-1; rpl-11.1* double-mutant analysis was carried out at 15° and 25° by picking Unc-32 Dpy progeny of *unc-32 glp-1/eT1; dpy-11 rpl-11.1(ar228)/eT1* mutant animals as L4 larvae and examining the germ line after fixation and DAPI staining 24 hr later. Control animals were Unc-32 Dpy progeny from the *unc-32/eT1; dpy-11 rpl-11.1(ar228)/eT1*.

Molecular identification of *rpl-11.1(ar228)*, *pab-1(ar232)*, and *glp-3/eft-3(ar229)*: *rpl-11.1(ar228):* Two- and three-factor mapping and deficiency complementation tests narrowed the *ar228* mutation to the area deleted by the deficiency

e2173 (Johnsen 1990), previously identified as an allele of *lin-40* (WormBase). In the course of mapping *ar228*, we positioned the left breakpoint of the *e2173* deletion between ORFs F35F10.10 and T20D4.5, while the right breakpoint was located near *egr-1*, between T27C4.4 and T27C4.1 (Figure 1A). This deletion is homozygous viable yet sterile, suggesting that no genes causing zygotic embryonic or larval lethality are located in this region. We determined the left endpoint of *e2173*, by amplifying PCR fragments from homozygous sterile *e2173* animals (with a positive control amplification outside the region). On the basis of these data, we determined that the left breakpoint of *Df(e2173)* is within the ~100-kb interval between T20D4.5 and F35F10.10 (Figure 1).

SNP mapping using the Hawaiian strain CB4856 further reduced the interval containing *ar228* by taking advantage of marked recombinants that lost *ar228* and thereby produced progeny. From the right side, 86 Dpy nonsterile self-progeny of *ar228 dpy-11/Hawaiian* mothers were selected, animals homozygous for the recombinant chromosome were identified in the next generation, and the strain was tested for the N2 or Hawaiian sequence of snp_T27C4[2]. Two recombinants that had undergone a crossover event to the left of snp_T27C4[2] (N2 DNA) both contained Hawaiian sequence at snp_T22F3[3]. From the left side, 35 Unc nonsterile self-progeny were picked from *unc-34 ar228/Hawaiian* mothers. Two of the 35 recombinant chromosomes had crossed over to the right of snp_F27E11[1] and were further tested: in one of two recombinants, the crossover occurred between snp_T24A6[3] and snp_T22F3[3].

DNA was amplified by PCR from the *rpl-11.1* locus (ATG to stop, including introns) from animals homozygous for *ar228* and our laboratory stock of the wild-type N2 strain. Comparison of the sequence indicated a single base pair change (verified on both strands) in the coding region of the *rpl-11.1* gene as indicated in results.

pab-1(ar232): After linkage testing placed *ar232* on LGI, three-factor analysis was performed: from the self-progeny of *lin-11 unc-75/ar232*, 1/10 Lin non-Unc recombinants segregated sterile worms and 6/9 Unc non-Lin segregated sterile worms (Figure 1B). The deficiency *qDf7* failed to complement *ar232*: all (~50) non-GFP progeny of *qDf7/hT2[let GFP]* × *ar232/hT2[let GFP]* males were sterile. RNAi feeding was performed on 8 of 16 candidate genes among the ~270 genes in the region (WormBase version 110) that were previously identified as sterile (Ste or Stp) by RNAi (Fraser *et al.* 2000; Maeda *et al.* 2001). Among this set, only *pab-1(RNAi)* (clone Y106G6H.2) conferred a highly penetrant low-proliferation sterile phenotype. DNA was amplified by PCR from the *pab-1* locus and analyzed as noted above for *rpl-11.1(ar228).*

glp-3/eft-3(ar229): After linkage testing placed *ar229* on chromosome III, three-factor crosses narrowed *ar229* to the left of *dpy-18* (16/16 Unc-25 non-Dpy-18 and 0/13 Dpy non-Unc segregated *ar229*) and between *dpy-17* and *unc-32* (22/42 Unc non-Dpy and 25/53 Dpy non-Unc segregated *ar229*) (Figure 1C). Complementation between *glp-3(q145)* and *ar229* was tested as follows: (1) of 60 F_1 non-Unc cross-progeny of *glp-3/sma-3 unc-36* hermaphrodites crossed to *ar229/eT1* males, 48 were fertile and segregated Unc or Sma Unc and 12 were sterile (presumed genotype *glp-3/ar229*) and (2) from the reciprocal cross, of 36 F_1 non-Unc cross-progeny, 27 were fertile and segregated Unc or Sma Unc and 12 were sterile (presumed genotype *glp-3/ar229*).

Life-span and size assays: Life-span and size assays were conducted at 20°. Individual L4 animals were transferred every other day until the end of the reproductive period, after which they were maintained on the same plate (sterile animals were maintained on the same plates throughout). Missing animals were not included in life-span calculations. Worms were considered dead when they no longer responded to prodding. Body length and width at midbody were measured at ×50 magnification using a KR-851 1 mm/100 divisions stage micrometer, at ages indicated, after immobilization with 0.1 M NaN_3. For volume calculations, worms were treated as cylinders: $V = \pi(1/2D)^2L$, where D is the width and L is the length (McCulloch and Gems 2003).

Analysis of RNAi results for X autosome gene pairs: Reciprocal whole proteome BLASTP analysis (Altschul *et al.* 1997) and comparative analysis of RNAi results were performed as described (Fernandez *et al.* 2005). Homologous gene pairs were defined on the basis of BLASTP matches between *C. elegans* and human or between *C. elegans* protein pairs that met a reciprocal best hit criterion and had an expectation value of $\leq 10^{-50}$ and ≥75% identity over the length of both proteins.

Phylogenetic analysis: BLASTP was used with WormBase (release WS128, http://www.WormBase.org) to identify predicted proteins in *C. briggsae* that are homologous to the *pab-1/2*, *rpl-11.1/11.2*, *rpl-24.1/24.2*, *rpl-25.1/25.2*, and *eft-3/4* genes of *C. elegans.* Corresponding gene sequences (minus inferred introns) were aligned using Clustal X version 1.81 (Thompson *et al.* 1997) using a gap-open penalty of 90.0, a gap-extension penalty of 0.1, and default parameters otherwise. These alignments were imported into MacClade version 4.05 (Maddison and Maddison 2002) and refined by hand relative to the encoded amino acids. These final alignments were phylogenetically analyzed with maximum likelihood (GTR + SS, general time-reversible model, allowing site-specific rates for the three different codon positions, with all parameters estimated by likelihood from the data), implemented in PAUP* 4.0b10 (Swofford 2002). For each set of homologs, jackknife analyses (using 50% deletion) and log-likelihood tree comparisons were performed using maximum likelihood with the GTR + I + G model (Swofford 2002), with parameters estimated from the data. In all cases, the root was assumed to occur in the longest branch, which was always the internal branch of each set of homologs. Orthologs were inferred to be the closest gene relatives occurring in different species.

RESULTS

To investigate zygotic requirements for the reestablishment and maintenance of early germ-line proliferation, we performed an EMS mutagenesis screen and looked for adult worms that, as judged by Nomarski optics, had a normal somatic gonad but displayed little or no germ line (Pepper *et al.* 2003a; E. J. A. Hubbard, unpublished data). To identify mutants with an early germ-line proliferation defect (as opposed to germ-line specification defects), we concentrated on mutants in which Z2 and Z3 were present in the L1 gonad primordium but subsequently underwent few or no additional rounds of division and did not differentiate. This phenotype is distinct from the *glp-1(loss-of-function)* mutant phenotype, in which Z2 and Z3 undergo several rounds of division, complete meiosis, and mature as sperm (Kimble and Hirsh 1979), and is more similar to that of *glp-3* (Kadyk *et al.* 1997) and *glp-4* (Beanan and Strome 1992). A severe germ-line proliferation defect without subsequent maturation is also observed when RNAi is directed against the chromodomain-containing protein *mrg-1*, although the requirement for this gene

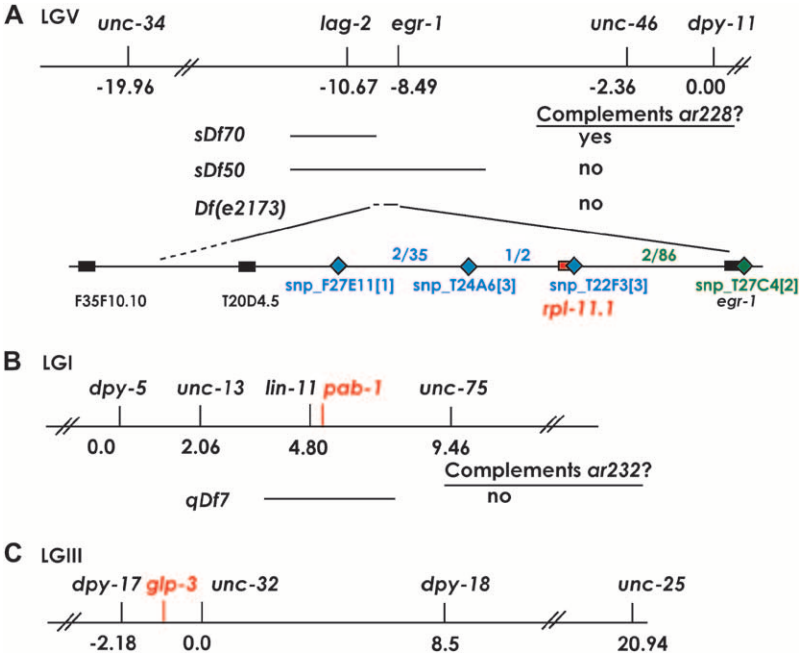

FIGURE 1.—Mapping of (A) *rpl-11.1(ar228)*, (B) *pab-1(ar232)*, and (C) *glp-3/eft-3(ar229)*. Relevant sections of linkage groups V, I, and III are depicted with genetic map positions indicated for markers used in the analysis. Complementation information is given for deficiencies tested in each region. For A, SNPs used to map *ar228* from the left (blue) and right (green) are given with the relevant intervals and recombinants observed in each interval [see MATERIALS AND METHODS for details and further information regarding *Df(e2173)*].

in early germ-line proliferation is maternal (FUJITA *et al.* 2002). Other genes required for germ-line proliferation have been identified using RNAi (HANAZAWA *et al.* 2001; MAEDA *et al.* 2001; COLAIACOVO *et al.* 2002), and a large-scale analysis of RNAi-mediated germ-line proliferation defects is in progress (E. J. A. HUBBARD, unpublished data).

Phenotypic analysis of *ar228*: *ar228* is recessive and causes sterility at all temperatures, although the most severe and most penetrant proliferation defect (>80% of adults with 10 or fewer germ cells per animal) is seen at a lower temperature (Figure 2). Further phenotypic analysis of the *ar228* mutant revealed that Z2 and Z3 are present in the early L1 gonad ($n > 40$), but they do not appear to proliferate well in subsequent larval stages. On the basis of the distribution of germ cell counts at two time points (L2/L3 and adult; Figure 2), germ cells in animals with very few germ cells in the L2/L3 did not appear to proliferate further, while germ cells that underwent some early proliferation did appear to proliferate further over time (Figure 2). In some cases, germ cells appeared to be in various stages of disintegration. Because Z2 and Z3 were visible in the L1 primordium of all animals examined, we assume that Z2 and Z3 or their early descendents disintegrated completely in those animals with no visible germ cells in the L3 or adult.

To assess whether the few germ cells produced in *ar228* animals were capable of entering meiosis, we examined the phenotype of *glp-1(q175); ar228* double-mutant animals under restrictive (15°) and less restrictive (25°) conditions for the *ar228* proliferation defect (see MATERIALS AND METHODS). In *glp-1* loss-of-function mutants, all germ cells enter meiosis early and differentiate as sperm. At 15°, the majority of the double-mutant animals (26/28), like the *ar228* single mutants ($n = 28$), did not produce any sperm and were morphologically similar to *ar228* single mutants. At 25°, the germ lines of 4/19 animals contained a total of 0–2 germ cells that did not differentiate (similar to *ar228* single mutants), while in 5/19 animals, the germ line contained a total of 5–30 sperm [similar to *glp-1(q175)* single mutants]. Genetically matched control animals produced 0–9 ($n =$ 28 animals) and 0–17 ($n = 19$) total undifferentiated germ cells per animal at 15° and 25°, respectively. Surprisingly, the germ lines of the remaining 10 double-mutant animals at 25° produced 46–200 sperm, far more than are normally seen in *glp-1(q175)* mutants alone. One possibility is that the germ cells produced by *ar228* mutants are resistant to differentiation and proliferated abnormally in the *glp-1* mutant background prior to differentiating *en masse*. Nevertheless, the results at the restrictive temperature suggest that the *ar228* proliferation defect precludes differentiation.

Molecular identification of *rpl-11.1(ar228)*: *ar228* was mapped to a region of the left arm of LGV containing ~37 ORFs (MATERIALS AND METHODS; Figure 1). One gene in the region, *rpl-11.1*, was reported to confer sterile (Ste), sterile progeny (Stp), or embryonic-lethal (Emb) and growth defective (Gro) phenotypes in previous RNAi experiments (PIANO *et al.* 2000, 2002; SIMMER *et al.* 2003). This gene also displayed a strong germ-line expression pattern (NEXTDB http://nematode.lab.nig.ac.jp; Y. KOHARA, personal communication). Sequence analysis of DNA from the *rpl-11.1* gene amplified from *ar228* homozygotes revealed a single C-to-T change that causes a premature termination at position Q175 (MATERIALS AND METHODS). Therefore, we refer to this allele as *rpl-11.1(ar228)*.

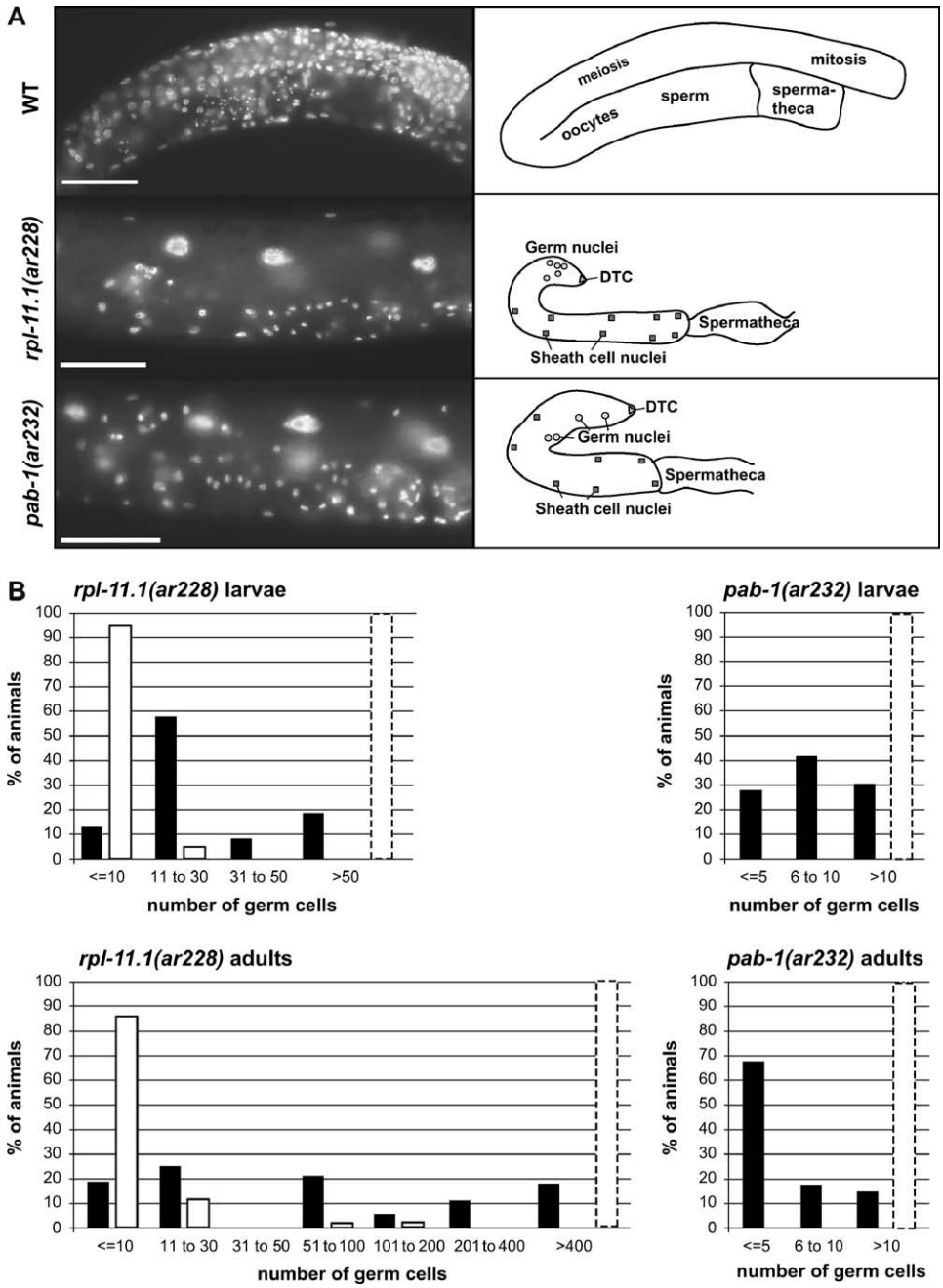

FIGURE 2.—Germ-line proliferation in *rpl-11.1(ar228)* and *pab-1 (ar232)*. (A) Images of DAPI-stained early adult animals: wild type (WT), *rpl-11.1(ar228)*, and *pab-1(ar232)* and corresponding cartoons. Bars, 50 μm. (B) Histograms depicting distributions of germ cell counts in *rpl-11.1(ar228)* and *pab-1(ar232)*. Solid bars represent total counts taken per animal at 25° and open bars represent counts taken from animals at 15°. Larval counts were obtained 24 hr post-hatch at 25° (early L3) or 48 hr post-hatch at 15° (late L2). Adult counts were taken 48 hr post-hatch at 25° and 96 hr post-hatch at 15° (both early adult). Dashed bar indicates the cell count category for the wild type at the equivalent time point (HIRSH *et al.* 1976; KIMBLE and HIRSH 1979; PEPPER *et al.* 2003b).

RPL-11.1 is a large ribosomal subunit L11 protein. These results suggest that robust translation is an absolute requirement for early germ-line proliferation.

Because the mutant phenotype of *rpl-11.1(ar228)* is zygotic sterility rather than embryonic lethality, L11 may be provided to the embryo maternally. To investigate this possibility we turned to RNA interference (RNAi) that degrades both the maternal and the zygotic mRNAs and found that the *rpl-11.1(RNAi)* phenotype is more severe than the *rpl-11.1(ar228)* phenotype. Early progeny of animals consuming *rpl-11.1(RNAi)* bacteria (see MATERIALS AND METHODS) exhibit a proliferation-defective sterility phenotype identical to that seen in *rpl-11.1(ar228)* homozygous animals. However, consistent with previous RNAi observations (PIANO *et al.* 2000, 2002; SIMMER *et al.* 2003), animals that continue to feed on RNAi-inducing bacteria produce dead embryos and, eventually, no embryos at all. These results suggest that the homozygous *rpl-11.1(ar228)* self-progeny of heterozygous mothers are rescued from embryonic lethality by the maternal contribution of *rpl-11.1*. The similarity of the RNAi-induced sterility defect to that of the mutant phenotype lends further credence to the supposition that the mutation at 15° confers an essentially null phenotype in the germ line despite the fact that the predicted protein product from *rpl-11.1(ar228)* is truncated by only 22 of 196 amino acids.

Germline-specific zygotic role of *rpl-11.1*: Among the

TABLE 1

RNAi analysis of duplicated *rpl* genes in wild-type (N2) and *rrf-1(pk1417)* mutant animals

RNAi target	LG	Strain	Phenotype[a]				
			Germ-line defect		Somatic defect		
			WT[b] (%)	Sterile[c] (%)	Unhealthy[d] (%)	Gro/Lva[e] (%)	*n*
(None)		N2	*76*	0	21	0.7	275
		rrf-1	24	25[f]	*40*	0	114
rpl-11.1	V	N2	0	*77*[f]	5	0.7	298
		rrf-1	0	*75*[f,g]	5	0	59
rpl-11.2	X	N2	3	12	3	*78*	295
		rrf-1	0	15[g]	*45*	10	80
rpl-25.1	X	N2	*73*	0	25	0	149
		rrf-1	*44*	*43*[f,g]	7	4	99
rpl-25.2	I	N2	8	*86*[f]	6	0	152
		rrf-1	10	*68*[f,g]	5	0	19
rpl-24.1	I	N2	1	22[f,g]	15	*62*	112
		rrf-1	0	*76*[f]	17	0.2	58
rpl-24.2	I	N2	24	10[g]	18	*47*	180
		rrf-1	0	*89*[f]	11	5	121

[a] Data are reported here for one parallel experiment; several experiments gave similar results (see MATERIALS AND METHODS). Percentages for phenotypes that were most penetrant for each treatment are in italic type. Remaining animals fell into "other" phenotypic categories that were not scoreable, such as lethal (Let) or ruptured (Rup).

[b] Normal-sized fertile adults.

[c] Sterile adults (both normal sized and reduced size).

[d] Fertile adults but small or sick looking.

[e] Larval arrest (Lva) or larval-sized animals.

[f] Majority of sterility due to strong Glp (germ-line proliferation defective) phenotype; this phenotype is observed variably in the *rrf-1* background and may be temperature sensitive. In the particular experiment reported here, the penetrance is on the high side of the range of variability compared to other experiments.

[g] Sterility largely due to mild germ-line proliferation defect often accompanied by signs of gametogenesis (*f,g* indicates that a large number of worms exhibited both types of sterility defect).

genes encoding proteins highly similar to RPL-11.1 from other phyla is a second *C. elegans* L11-encoding gene, *rpl-11.2*, located on the X chromosome (WormBase). Given previous reports of a dearth of germ-line-enriched genes on the X chromosome (REINKE *et al.* 2000, 2004), underrepresentation of expression of essential X-linked genes (PIANO *et al.* 2002; REINKE *et al.* 2004), and reports of X chromosome silencing in the germ line (FONG *et al.* 2002; KELLY *et al.* 2002), we wondered if there might be a clear functional separation of the L11-encoding genes in the germ line and soma, with the autosome-linked *rpl-11.1* functioning in the germ line and the X-linked *rpl-11.2* functioning in the soma. We used several assays to assess the germ-line *vs.* soma activities of these two genes.

First we examined carefully the RNAi phenotypes of both *rpl-11* genes. Consistent with previous reports, we found that *rpl-11.2* confers a penetrant growth defect (Gro) that is not observed in parallel *rpl-11.1* RNAi experiments (Table 1). We also investigated the RNAi phenotypes of the two genes in a genetic background that reduces the efficacy of RNAi in the soma while retaining it in the germ line, *rrf-1(pk1417)* (SIJEN *et al.* 2001). We found that in an *rrf-1* mutant background, *rpl-11.1* still conferred a penetrant germ-line proliferation defect, while the somatic growth defect of *rpl-11.2* was largely suppressed. Other adult defects were observed in association with *rpl-11.2(RNAi)* in the *rrf-1* mutant background, including sterility (Table 1).

Second, we compared the available expression patterns of *rpl-11.1* and *rpl-11.2* images from the NEXTDB expression pattern database (http://nematode.lab.nig.ac.jp; Y. KOHARA, personal communication). Weakly detectable levels of *rpl-11.1* mRNA are observed in a limited number of cells in the embryo. Strong levels are detected, however, in a small number of cells located

centrally in the larvae (likely Z2 and Z3 and their descendants) and thereafter in the germ line, primarily in the distal proliferating regions. In contrast, *rpl-11.2* mRNA is detectable in a subset of cells in the embryo, with high levels in what appear to be intestinal cells in later embryonic stages. During larval development, *rpl-11.2* mRNA is weakly detectable, with somewhat higher levels in the adult. These observations suggest that vastly differential levels of mRNAs encoding ribosome subunits could be synthesized in different tissues, a phenomenon observed in other organisms as well (Agarwal *et al.* 1999). Given the presumably cell-essential function of the L11 protein, we found the dynamic and nonubiquitous levels of expression surprising. These results likely reflect tissue-specific requirements for high translational capacity. Previously reported microarray analysis comparing wild type with the germ-line-deficient *glp-4(bn2)* mutant also firmly places *rpl-11.1* in the germ-line-enriched class ($\log_2$ ratio of +4.1; Reinke *et al.* 2004) while *rpl-11.2* is not enriched in the germ line ($\log_2$ ratio of −1.3; Reinke *et al.* 2004).

Third, previous studies documented two dramatic germ-line-associated phenotypes after removal of Z2 and Z3 by laser microsurgery: life-span extension (Hsin and Kenyon 1999) and gigantism (Patel *et al.* 2002). We reasoned that if *only* the germ line is affected by *rpl-11.1(ar228),* we should see the same phenotypes previously reported for germ-line-ablated animals. If, on the other hand, *rpl-11.1* also plays a role in somatic development that would compromise the somatic contribution to life-span or size determination, these phenotypes may be less severe in our mutant than those observed in the cell-ablation studies. We found that in *rpl-11.1(ar228)* animals, both life-span extension and overall increase in the volume of the worms is similar to that observed in germ-line-ablation experiments (Figure 3). Life span is extended in *ar228* sterile homozygotes to 27 days (starting from the L4 stage) from a mean life span of 14 days in control animals, an increase of 1.9-fold. These results are comparable to the increase in mean life span observed after ablation of Z2 and Z3 (an extension from 19 to 32 days or 1.7-fold starting from day of birth; Hsin and Kenyon 1999). We also observed a positive correlation between *rpl-11.1(ar228)* mutant body length, width, and volume. Homozygous mutant and wild-type L4 larvae and early adults were similar in size, but adult size increased more in the mutant than in the wild type as the animals aged (Figure 3). The overall volume increase in adult sterile *rpl-11.1(ar228)* animals (as compared to genetically matched controls) is from 8.7 μm^3 to 11.9 μm^3 8 days post-L4 (or 7 days after reaching adulthood), an increase of 1.4-fold. These results are similar to the previously reported increase in volume from 5.8 to 8.4 μm^3, also 1.4-fold, 7 days postadulthood after Z2/Z3 cell ablations (Patel *et al.* 2002).

Taken together, the results of RNAi experiments, expression data, and phenotypic analysis suggest that the autosomal copy of the gene encoding the L11 protein of the large ribosome subunit is dedicated to germ-line function and affects early embryogenesis as a consequence of its germline role.

***rpl-25* genes are duplicated and are organized similarly to *rpl-11*:** We next asked if other *rpl* genes were duplicated in the *C. elegans* genome and if their genome distribution correlated with a separation of germ-line/soma function. Of the 43 *rpl* genes in the database (WormBase Release WS130), we found 2 additional duplicated genes: *rpl-24* (likely encoding the L24 protein of the large ribosome subunit), with both duplicates (*rpl-24.1* and *rpl-24.2*) on LGI, and *rpl-25* (likely encoding the L23a protein of the large ribosome subunit) with an X-linked paralog, *rpl-25.1*, and an autosomal paralog, *rpl-25.2*, on LGI. Previous RNAi studies indicate that reduction of *rpl-24.1* and *rpl.25.2* function can cause embryonic lethality and sterility (Fraser *et al.* 2000; Simmer *et al.* 2003) and that *rpl-25.2* transcripts are germ-line enriched ($\log_2$ ratio for *+/glp-4(bn2)* of +1.6 *vs.* −1.2 for *rpl-25.1*; Reinke *et al.* 2004) while *rpl-24.1* and *rpl-24.2* are not ($\log_2$ ratios of 0.174 and 0.5, respectively; Reinke *et al.* 2004).

To assess the relative germ-line and somatic roles of these gene pairs, we examined the effects of RNAi targeted against these genes individually in the wild-type (N2) and the *rrf-1* mutant backgrounds (Sijen *et al.* 2001; Table 1; see materials and methods). In this experiment, *rpl-11* and *rpl-25*, the two gene pairs with an autosome/X chromosome duplication, behave more similarly to each other than does the autosome/autosome duplicated *rpl-24* gene pair. For the *rpl-11* and *rpl-25* pairs in the N2 background, the primary defect after RNAi of the autosome-linked genes of each pair (*rpl-11.1* and *rpl-25.2*) is a germ-line proliferation defect (77 and 86%). However, RNAi directed against the X-linked genes of each pair confers either growth/larval arrest (*rpl-11.2*, 78%) or a weak overall health defect (*rpl-25.1*, 25%) but not a significant germ-line proliferation defect (12 and 0%, respectively). As expected for genes acting primarily in the germ line, RNAi directed against the autosomal copies of these genes confers similar phenotypes in the *rrf-1* mutant background and in the wild type, while as expected for genes acting in the soma, the phenotypic profile changed significantly between N2 and *rrf-1* when the X-linked paralogs were targeted. Specifically, the percentage of growth defective/larval arrest animals in *rpl-11.2(RNAi)* is reduced from 78 to 10% in N2 *vs. rrf-1* and the unhealthy phenotype observed in *rpl-25.1(RNAi)* is reduced from 25 to 7%. In addition, *rpl-25.1(RNAi)* displayed a significant increase in sterility in the *rrf-1* background, but the germ-line proliferation defect was not as severe as that observed with *rpl-25.2* (Table 1).

Finally, the effect of RNAi-mediated depletion of *rpl-24.1* and *rpl-24.2* resulted in quite similar phenotypic profiles, but differed from that of *rpl-11* and *rpl-25* (Ta-

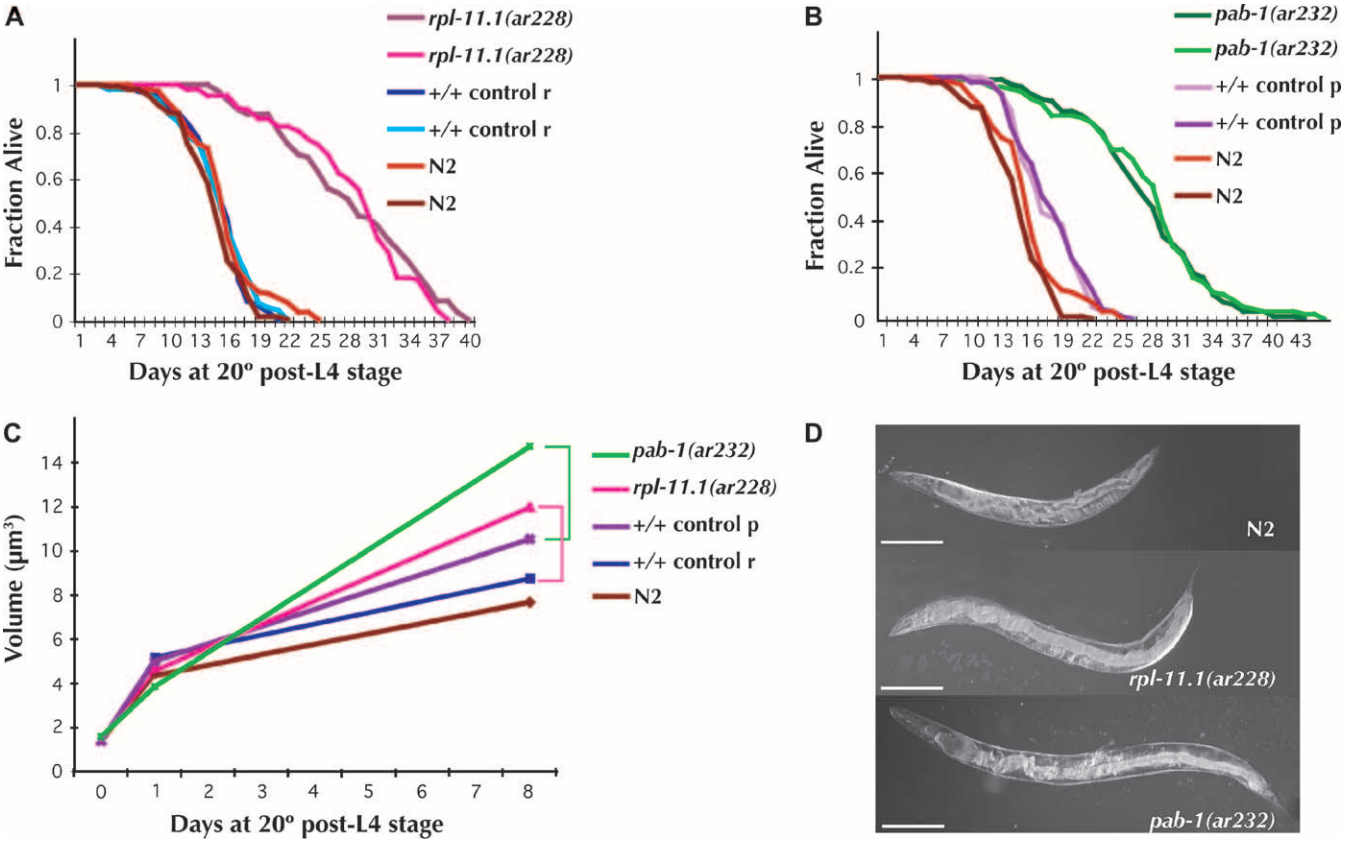

FIGURE 3.—Life span and gigantism in *rpl-11.1(ar228)* and *pab-1(ar232)*. Survival curves (A) for *rpl-11.1(ar228)* and (B) for *pab-1 (ar232)* and (C) worm volumes, with respective control strains and the wild-type strain N2, are shown. All non-N2 strains (mutant and respective controls) are homozygous progeny of heterozygous mothers balanced by *nT1[unc-?(n754) let-?]* for *rpl-11.1(ar228)* (control r) and by *hT2[let-? qIs48]* for *pab-1(ar232)* (control p). Survival curves are given for two independent experiments for each non-N2 strain. Taken together, for N2 wild type (brown and orange), mean life span (m) = 14.2 ± 3.5 days and number of animals observed (n) = 163; for +/+ control r, m = 14.2 ± 3.3 days and n = 127; for *rpl-11.1(ar228)*, m = 27.4 ± 6.9 days and n = 126; for +/+ control p, m = 16.9 ± 3.4 days, n = 126; and for *pab-1(ar232)*, m = 26.4 ± 6.9 days, n = 185. (C) Volume calculations are based on length and width measurements (see MATERIALS AND METHODS); n = 20 worms of each genotype per time point. Brackets link mutants with relevant genetically matched controls. Measurements were taken at the mid-L4 (day 0), as early adults (day 1), and a week later (day 8) as older adults. Actual volumes (in cubic micrometers) calculated for the three indicated time points (±1 SD) are as follows: for *pab-1(ar232)*, 1.55 ± 0.27, 3.82 ± 0.43, and 14.7 ± 1.12; for *rpl-11(ar228)*, 1.37 ± 0.31, 4.55 ± 0.90, and 11.94 ± 5.84; for control p, 1.37 ± 0.19, 4.98 ± 0.77, and 10.50 ± 1.67; for control r, 1.37 ± 0.23, 5.14 ± 1.00, and 8.72 ± 2.12; and for N2, 1.37 ± 0.27, 4.43 ± 1.27, and 7.64 ± 1.21. (D) Digital images of wild-type (N2), *rpl-11.1(ar228)*, and *pab-1(ar232)* animals 10 days after the L4 stage at 20°. Bars, 200 µm.

ble 1). After RNAi depletion of each gene in the *rpl-24* pair in wild type and *rrf-1*, each displayed a similar decrease in severe growth/larval arrest phenotypes and concomitant increase in sterility in the *rrf-1* background as compared to the wild-type background. Thus, in contrast to the germ-line-specific roles of the autosomal copies of *rpl-11* and *rpl-25*, it is likely that both *rpl-24* genes (both autosomal) are redundant in the soma and the germ line.

In summary (Table 2), our functional analysis of the three *rpl* genes (and the *pab* genes, see below) is consistent with the hypothesis that the two X-linked members of the X/A gene pairs act in the soma while the autosome-linked members of each pair act either in the germ line alone (*e.g.*, *rpl-11.1*) or in the germ line and soma (*e.g.*, *rpl-25.2*). In contrast, the pair of *rpl-24* genes (both autosomal) appears to act in both the soma and the germ line.

Identification and characterization of *pab-1(ar232)*: We positionally cloned *pab-1(ar232)*, another mutation identified in our screen (Figure 1; MATERIALS AND METHODS). DNA sequence revealed a C-to-T transition, resulting in a conceptual translation of Q408 in Y106G6H2.a and Q428 in Y106G6H2.b and Y106G6H2.c to an amber termination. This mutation causes a recessive fully penetrant severe germ-line proliferation defect (n = 120) and an incompletely penetrant protruding vulva (Pvl) phenotype. *pab-1* encodes a cytoplasmic poly(A)-binding protein (PABP) and is the autosomal copy of an autosome/X-linked gene pair: *pab-1* and *pab-2*.

Further analysis suggested that *pab-1* has an essential germ-line function whereas *pab-2* does not [consistent

TABLE 2

Functional comparison of *C. elegans* and *C. briggsae* orthologs

C. elegans ortholog	A/X[a]	Phenotype[b]	*C. briggsae* ortholog	Phenotype[c] (*n*)
rpl-11.1	A	Sterile	CBG01314	Sterile (7/7)
rpl-11.2	X	Fertile (Gro)	CBG14053	Sterile (1/8), sub-Fertile[d] (7/8) Progeny: Lva/Gro
rpl-24.1	A	Fertile (Gro)	CBG22273	Sterile (2/8), sub-Fertile[d] (6/8) Progeny: Lva/Gro
rpl-24.2	A	Fertile (Gro)	CBG03702	sub-Fertile[d] (7/7) Progeny: Lva/Gro
rpl-25.1	X	Fertile (WT)	CBG14529	Fertile[e] (5/5) Progeny: WT
rpl-25.2	A	Sterile	CBG04080	Sterile (3/3)
pab-1	A	Sterile	CBG02207	Sterile (8/8)
pab-2	X	Fertile (WT)	CBG07431	sub-Fertile[d] (7/7) Progeny: WT/Gro
			L4440 (−)ctrl	Fertile (5/5)[f] Progeny: WT

Lva, larval arrest.

[a] Autosome or X-linked locus in *C. elegans*; Gro, growth defect.

[b] Summarized from Table 1 and from the text. Phenotype refers to progeny phenotype from RNAi feeding experiments (see MATERIALS AND METHODS).

[c] Phenotype of injected animals (*n*, number of animals displaying phenotype per number of surviving injected animals) as assessed after overnight transfer to deplete animals of embryos produced prior to the injection (see MATERIALS AND METHODS). In addition, few of the adult progeny produced by these fertile injected animals exhibited sterility compared to a high penetrance of sterility observed in the progeny (first 12 hr) from injected animals that quickly became sterile.

[d] These animals were subfertile, producing few embryos, some embryonic lethality, and few live progeny: CBG14053, average <4 surviving progeny per injected animal; CBG22273 and CBG03702, average <2 surviving progeny per injected animal; CBG07431, average <5 surviving progeny per injected animal.

[e] These animals were fertile, producing many embryos but few live progeny due to embryonic lethality: CBG14529, average <2 surviving progeny per injected animal.

[f] Negative control (see MATERIALS AND METHODS), ≥20 progeny per injected animal over 2–3 days scored.

with recent findings of CIOSK *et al.* (2004)]. RNAi directed against *pab-1* resulted in several phenotypes, including a high penetrance of sterility (43%, $n = 84$) and Pvl (32%, $n = 84$) and, at lower penetrance, a ruptured vulva (Rup) and flaccid-looking body morphology. In the *rrf-1* mutant background all *pab-1(RNAi)* animals were sterile ($n = 54$) and 25% also displayed the Pvl phenotype. These results suggest that the sterility is a result of germ-line function of *pab-1*. In contrast, RNAi directed against the X-linked *pab-2* in N2 resulted in 94% wild-type animals with low-penetrance sterility, Rup, and Pvl phenotypes (2%, 4%, and 0.3%, respectively; $n = 325$). Very similar observations were made in the *rrf-1* mutant background where *pab-2(RNAi)* produced 90% wild-type, 7% sterile, and 4% Rup animals ($n = 119$). These results indicate that *pab-1* and *pab-2* act redundantly in the soma but that *pab-1* is required in the germ line.

Consistent with a role for *pab-1* in the germ line and similar to *rpl-11.1(ar228)*, *pab-1(ar232)* extends life span (from an average of 17 days post-L4 in the control strain to an average of 26 days in the mutant, an increase of 1.5-fold; Figure 3) and causes gigantism, with worms averaging 1.4-fold greater volume (Figure 3). Previous microarray analysis does not indicate strong germ-line enrichment for *pab-1* or *pab-2* transcripts ($\log_2$ ratio for +/*glp-4* of +0.1 for *pab-1* and −1.25 for *pab-2*; REINKE *et al.* 2004).

Identification and characterization of *glp-3(ar229); glp-3* is *eft-3*: A third allele from our screen mapped to a small interval on LGIII (see MATERIALS AND METHODS). We noted that mutations in *glp-3*, a gene that maps genetically to the region, had been analyzed previously at the phenotypic level and conferred a similar zygotic germ-line proliferation defect (KADYK *et al.* 1997). We performed complementation analysis (see MATERIALS AND METHODS) with *glp-3(q145)* and *ar229*, and they failed to complement, indicating that *ar229* is an allele of *glp-3*.

We next examined genes in the region, looking for

predicted essential genes with an X-linked paralog. *eft-3*, an EF-1-α ortholog, satisfied these criteria. One of several closely related genes, *eft-4*, resides on the X chromosome. Interestingly, one alternative splice form of *eft-4* apparently encodes a protein identical to *eft-3* [WormBase WS130]. In addition to identical protein sequences, the nucleotide sequences of the coding regions of the two genes are very similar (94% identical over 1392 bases), precluding the possibility of separate RNAi-based functional analysis of these two genes. Sequence analysis of *glp-3(q145)* in the *eft-3* region revealed a single base pair change (G to A) that would result in an the amino acid change A301T. Our mutant, *ar229*, contains a C-to-T transition in *eft-3* that encodes a S414F amino acid change. This analysis thus identified *glp-3* as *eft-3*.

Another gene, *glp-4*, confers a severe zygotic germline proliferation defect (Beanan and Strome 1992). We examined the predicted genes in the region to which *glp-4* maps (LGI, 21.4 ± 2 cM) for candidate genes for which previously reported RNAi experiments conferred a sterility or lethality phenotype (Stp, Ste, Lvl, Lva, or Emb) and for which a closely related paralog exists on the X chromosome. For genes with no available RNAi information, we checked for X-linked paralogs. No obvious candidates were identified. *rpl-31* maps very close to *glp-4*, but no change in the *rpl-31* coding sequence was detected in the DNA of *glp-4(bn2)* animals (data not shown).

X/autosome duplications of highly conserved genes in the *C. elegans* genome: We examined the *C. elegans* genome for other X/autosome gene pairs and found that 395 X-linked genes have a paralog on an autosome. Of these, 168 have a highly conserved homolog in human ($E \leq 10^{-50}$ and ≥75% identity over the length of the protein), an indication of likely "cell-essential" function. Of these 168 X-linked genes, 156 have been assayed by RNAi, and 128 have no reported phenotype in any large-scale RNAi study (*i.e.*, were scored as "wild-type" in every reported assay) (Fraser *et al.* 2000; Gonczy *et al.* 2000; Piano *et al.* 2000; Maeda *et al.* 2001; Kamath *et al.* 2003; Simmer *et al.* 2003). Of these 128 genes, 23 have an autosomal paralog that conferred an Emb, Ste, or Stp phenotype in one or more of the above studies, indicating likely germ-line function. Large-scale studies thus provide evidence suggestive of germ-line/soma subfunctionalization for ~16% of these highly conserved X/A gene pairs. No definitive conclusion can be reached regarding the remainder of these X/A pairs for various technical reasons, including likely false negatives in these studies (Piano and Gunsalus 2002; Fernandez *et al.* 2005), lack of an RNAi assay for the autosomal paralog (19 cases), or possible redundancy among members of multigene families (which may prevent the detection of phenotypes for single-gene knockouts). Thus, a more directed combined bioinformatic and functional analysis of highly conserved paralogous gene pairs will be required to assess on a genomic scale the prevalence of the X/A trend we observed. Nonetheless, our finding that three alleles from a nonbiased screen for germ-line proliferation defects led to the identification of three X/A duplicated genes is striking.

Unlike many autosome/X-linked gene pairs in mammals and Drosophila, retrotransposition is not the mechanism for the *rpl*, *pab*, and *eft* duplications: The implications of gene duplication, genome organization, and large-scale gene silencing mechanisms in the germ line are relevant to other organisms. Indeed, in an analogous process to *C. elegans* X chromosome germ-line silencing (Fong *et al.* 2002; Kelly *et al.* 2002), the X chromosome is inactivated in mouse during meiosis, a general phenomenon referred to as meiotic sex chromosome inactivation (MSCI) (McKee and Handel 1993). The possibility of an autosome/X-linked distribution of gene duplicates and correlated germ-line/soma functional separation has been noted and proposed as one possible "coping" mechanism to allow important genes to function despite MSCI in mammals (McKee and Handel 1993 and references therein; Handel 2004). There are six documented cases of mammalian genes with autosome/X duplications in which only the autosomal copy is expressed during spermatogenesis. For five of these genes, the X-linked copies contain introns while the autosomal genes are intronless, a hallmark of duplicative retrotransposition to the autosome from an ancestral X-linked gene (see Handel 2004 and references therein). Moreover, in humans there are five active duplicates of the cytoplasmic PABP, at least two of which, including the X-linked PABPC5, are intronless, again suggesting retrotransposase-mediated gene duplication (see Mangus *et al.* 2003 and references therein). Studies in Drosophila also suggest a high rate of X-to-autosome gene duplication by way of retrotransposons with a significant enrichment of testes-specific expression in the intronless autosomal members of the gene pairs (Betran *et al.* 2002). We therefore examined the predicted intron structure (WormBase WS130) of the duplicated *C. elegans rpl*, *pab*, and *eft* genes identified in this study and found no evidence for retrotransposition: all autosomal and X-linked copies contain introns and, in the cases of the *rpl-11*, *rpl-25*, and *eft* duplicates, some predicted intron/exon boundaries are conserved to the base. These results suggest that mechanisms of gene duplication other than retrotransposition may be important for duplications that evolve separate germ-line and somatic functions in *C. elegans*.

Duplicated genes in *C. briggsae*: To determine if the duplication of the three *rpl* genes, the *pab* genes, and the *eft* genes arose prior to the evolutionary separation of lineages that gave rise to *C. elegans* and *C. briggsae* or if these duplications are derived within the *C. elegans* branch, we searched for closely related homologs of these genes in *C. briggsae*. The presence of closely related paralogs within the *C. elegans* genome required some

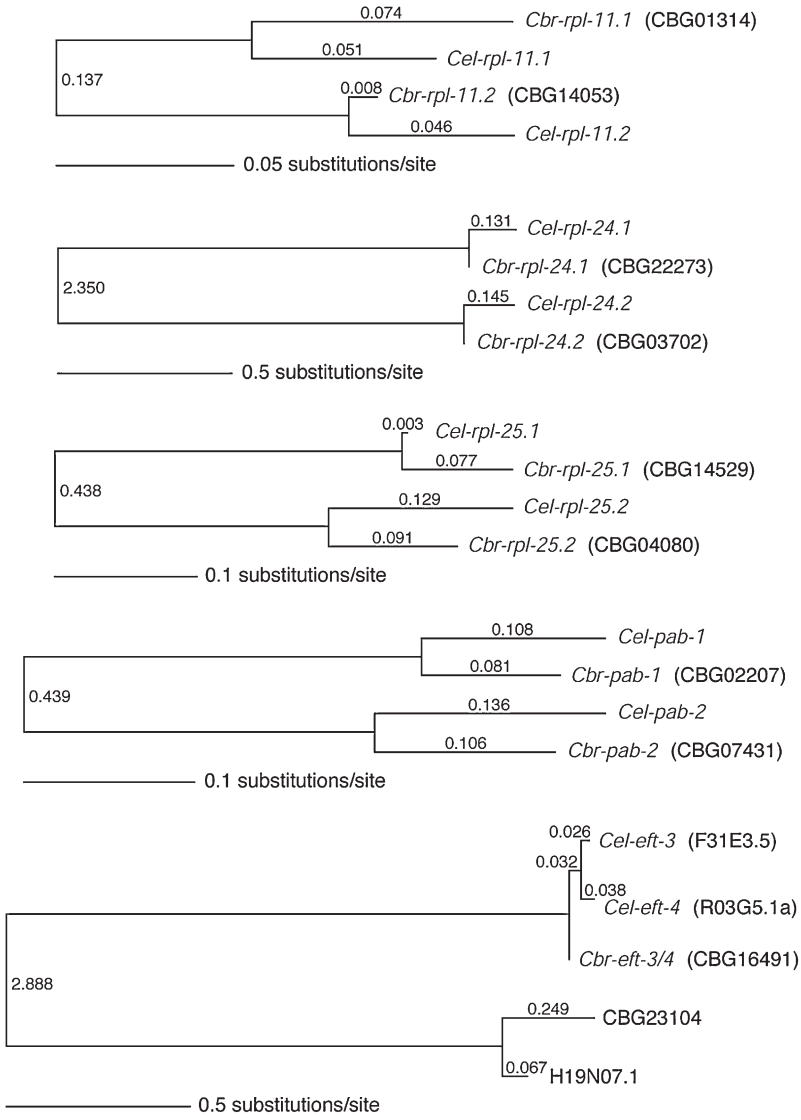

FIGURE 4.—Phylogenetic relationships of *pab*, *rpl*, and *eft* gene pairs in *C. elegans* (*Cel*) and *C. briggsae* (*Cbr*). Branch lengths (substitutions per site, estimated by likelihood) are indicated on the branches and with a scale for each set of genes. For each set of homologs, all possible unrooted phylogenies were compared using a log-likelihood ratio test; in all cases, the maximum likelihood (ML) tree was significantly supported ($P < 0.05$) over all other trees. Jackknife analyses demonstrated 100% support for the internal branch in 2000 Monte-Carlo replicates for each set of four or five homologous genes. Note that H19N07.1 is the *C. elegans* ortholog of the CBG23104 gene in *C. briggsae*; these genes are paralogs of the *eft-3/4* genes in these two species. After diverging from *C. briggsae*, the *eft-3/4* ortholog in a *C. elegans* ancestor duplicated to produce *eft-3* and *eft-4*, which are therefore both orthologs of the single *C. briggsae eft-3/4* gene.

care in assignment of the corresponding orthologs in *C. briggsae*, in part because similarity alone may not reflect the degree of phylogenetic relationship since rates of evolution can be very different in different gene lineages. Therefore, rather than relying solely on the best BLASTP match, we examined syntenic regions between the two species and performed a maximum likelihood phylogenetic analysis (see MATERIALS AND METHODS; Figure 4). The results indicate that each of the *rpl-11*, *rpl-24*, *rpl-25*, and *pab* genes has a single corresponding ortholog in *C. briggsae*. Our BLASTP, synteny, and phylogenetic analyses agree for the three *rpl* genes and the *pab* genes, identifying the same pairs of genes as putative orthologs. Our analysis is consistent with the hypothesis that four duplications (three *rpl* genes and the *pab* genes) preceded the divergence between *C. elegans* and *C. briggsae*. Unlike the *rpl* and *pab* genes, the duplication event that produced *eft-3* and *eft-4* appears to be more recent, having occurred within the *C. elegans* lineage after diverging from the lineage leading to *C. briggsae* (Figure 4). It is likely that the *C. briggsae* chromosome that shares synteny with the *C. elegans* X chromosome is the *C. briggsae* sex chromosome, but genetic analyses in *C. briggsae* will be required to verify this prediction.

To determine if the related duplicated *C. briggsae* genes (Figure 4) also show functional separation in the germ line and soma, we performed RNAi for each gene of the four gene pairs (Table 2). Because *C. briggsae* is not amenable to RNAi analysis by feeding, double-stranded RNA was delivered by injection into the germ line. We found that injection of dsRNA corresponding to the *C. briggsae* orthologs of the autosome-linked member of each A/X pair caused complete sterility (no embryos) in the injected animal within 12 hr of injection (Table 2). In contrast, all or most of the worms injected with the orthologs of the X-linked *C. elegans* genes produced embryos and/or live progeny. The latter injected animals, however, displayed various degrees of embry-

onic lethality and/or were subfertile, suggesting the possibility of germ-line function for these genes. Larval arrest and growth defects were also observed among the progeny of these animals (Table 2), however, suggesting that these genes act in the soma as well as the germ line. As was observed from RNAi in *C. elegans*, injection of dsRNA corresponding to each gene of the *C. briggsae* *rpl-24* pair (an A/A pair in *C. elegans*) produced almost identical results. In addition, for the *C. briggsae* orthologs of the *C. elegans* autosome members of X/A pairs, many progeny produced within the first 12 hr after injection became sterile adults, whereas adult sterility was not a prevalent phenotype among early progeny from the other experiments (Table 2).

Therefore, the paralogs in *C. briggsae* that correspond to *C. elegans* autosome/X paralogs display consistent functional differences between paralogs, including a potential somatic role for the orthologs of *C. elegans* X-linked members of X/A pairs. Because both genes in each pair caused phenotypes that are associated with germ-line function, however, there may be a lesser degree of germ-line/soma subfunctionalization between these genes in *C. briggsae*. The extent to which this difference between *C. elegans* and *C. briggsae* is a consequence of differences in experimental methods, species-specific response to RNAi, or other developmental differences is unclear. In summary, our analysis in *C. briggsae* suggests that RNAi directed against the *C. briggsae* orthologs of *C. elegans* autosomal members of the three autosome/X gene pairs (*rpl-11, rpl-25, pab*) caused a more profound germ-line defect than did RNAi directed against the *C. briggsae* orthologs of *C. elegans* X-linked members of these pairs or against the autosome/autosome pair (*rpl-24*).

DISCUSSION

RPL, PAB, and EFT proteins and early germ-line proliferation: Using forward genetics, we identified *rpl-11.1, pab-1,* and *glp-3/eft-3* as genes required zygotically for early germ-line proliferation. These genes encode the L11 protein of the large ribosome subunit, a cytoplasmic PABP, and an elongation factor (EF-1-α), respectively. All are important for translation and are likely essential for cell proliferation. Their critical roles in early germ-line proliferation suggest that the processes of reinitiation and maintenance of early proliferation from the quiescent state of the germ-line precursor cells Z2 and Z3 is dependent on proper ribosome biogenesis and/or active translation. That the primordial germ cells are born and locate properly to the somatic gonad in the mutants suggests that the maternal copy is sufficient for these events and/or that the X-linked paralogs of each gene are functionally redundant in the germ line until Z2 and Z3 resume cell division. We also identified a critical germ-line proliferation function for *rpl-25.2* that is not shared by its X-linked paralog, *rpl-25.1.*

Gene duplication, genome organization, and the germ line: Our phenotypic analysis of the three duplicated *rpl* genes and *pab* genes and previous analysis of *glp-3* (Kadyk *et al.* 1997) in *C. elegans* suggest that the functional role of autosomal members of each autosome/X-linked pair is critical in the germ line and that the X-linked copy is largely if not exclusively active in the soma. We also identified orthologous genes in the *C. briggsae* genome, and in four of the five cases (all but *eft-3/eft-4*), the gene duplication preceded the evolutionary divergence of these two species. Among the paralogous pairs within *C. briggsae* that correspond to X/A pairs in *C. elegans*, some degree of subfunctionalization of these gene duplicates occurs, whereas the pair corresponding to the autosome/autosome duplication does not display the same degree of subfunctionalization. Given that the germ-line phenotypes were much more severe in the *C. briggsae* orthologs of the autosome-linked members of X/A pairs in *C. elegans*, if the chromosome that carries the paralogs is, indeed, the *C. briggsae* X chromosome, we speculate that germ-line-expressed genes may be similarly underrepresented on the X chromosome of *C. briggsae*, as are germ-line-expressed genes in *C. elegans* (Reinke *et al.* 2000, 2004). Interestingly, the same mode of X chromosome silencing that operates in the *C. elegans* germ line appears to operate in *C. briggsae* (Kelly *et al.* 2002).

That a similar pattern of germ-line function of the autosomal copy of autosome/X-linked paralogs is abundant among many cell proliferation-essential genes is evidenced by our genetic identification of three independent loci (from three alleles) that are required for robust germ-line proliferation. Given that these three mutants (of seven obtained from a forward genetic screen covering 11,586 genomes that were screened in a way that could identify putative severe proliferation defective mutants; Pepper *et al.* 2003a; E. J. A. Hubbard, unpublished data) uncovered autosomal copies of duplicated genes with the same kind of autosome/X chromosome distribution, we speculate that other genes essential for early germ-line proliferation may be encoded by the autosome duplicate of autosome/X-linked pairs of genes encoding proteins essential for cell proliferation.

Recent studies of X-linked genes in mammals suggest that only genes expressed after the onset of male germ-line X chromosome silencing (MSCI) are underrepresented on the X chromosome (Khil *et al.* 2004). In *C. elegans* the X chromosome lacks histone modifications that correlate with transcriptional activation throughout the adult male germ line and in the zone of mitosis and early meiosis in adult hermaphrodites (Fong *et al.* 2002; Kelly *et al.* 2002). Therefore, it is conceivable that in addition to genes required in spermatogenesis, genes required in earlier stages of germ-line development in *C. elegans* may be particularly sensitive to selective pressure keeping them off the X chromosome.

Identification of correlations between the genomic

distribution of gene duplicates and germ-line function may be mutually informative in other species with X chromosome silencing. That is, the presence of X-linked "cell-essential" genes with autosomal duplicates could identify genes required for germ-line development during X chromosome silencing, while those that are not duplicated could point to genes that are required at a time in development when the X is not silenced. This approach may be useful in identifying autosomal genes required for fertility.

Evolutionary hypotheses: We propose three possible evolutionary hypotheses regarding functional gene duplicates of cell-essential genes, X chromosome germline silencing, and genome organization. From our study and from other published reports, we found data supporting each of the hypotheses. Therefore, we suggest that each of these hypothetical mechanisms is likely at work.

One hypothesis is that gene duplication (regardless of whether it occurred before or after the evolution of germ-line X chromosome silencing) generally results in "subfunctionalization" (Lynch and Conery 2000) into germ-line/soma roles. That is, when there is positive selection to subfunctionalize into different expression compartments, it is generally the case that these compartments correspond to germ-line and soma compartments. Such a general trend might result, for example, if many regulatory regions in the genome had elements controlling germ-line *vs.* soma expression and if, by chance alone, transposed duplicates "land" near such regulatory elements or if germ-line- or soma-specific elements are lost during duplication/transposition. This hypothesis predicts that autosome/autosome and autosome/X duplicates should show similar degrees of germ-line/soma subfunctionalization. An example of an autosome/autosome duplicate pair that exhibits germ-line/soma subfunctionalization is the *iff-1* and *iff-2* gene pair that encodes eIF5A homologs. These duplicated genes fulfill separate germ-line/soma functions (Hanazawa *et al.* 2004). An alternative hypothesis to account for the behavior of this pair is that the member of this pair that is functioning in the soma, although it is not located on the X, may be silenced by the same mechanism that silences genes on the X chromosome.

A second hypothesis is that gene duplication (again, regardless of whether it occurred before or after the evolution of X chromosome silencing) results in subfunctionalization *due to* X chromosome silencing. That is, X chromosome silencing provides the conditions for positive selection for subfunctionalization. In this case, we predict that relative to autosome/autosome duplicates, the autosome/X duplicates should show a bias toward germ-line/soma subfunctionalization. The *rpl-11* duplicate genes are examples of this kind of subfunctionalization: RNAi directed against *rpl-11.1* disrupts germ-line development, while *rpl-11.2(RNAi)* has a more profound effect on somatic development. While autosome/autosome duplicates may show subfunctionalization, under this mechanism they would not necessarily be biased toward germ-line/soma compartmentalization. This hypothesis would also predict that strict germline/soma subfunctionalization would not occur unless one duplicate is on the X chromosome. In this regard, the *rpl-24* genes provide an example of autosomal duplicates that do not show germ-line/soma subfunctionalization and remain active in both the germ line and the soma.

A third hypothesis is that subfunctionalization results from genetic drift, not from positive selection. Here, the only role of selection would be to maintain the new function that resulted from random drift (*i.e.*, purifying selection). In this case, gene copies that end up on the X chromosome lose their germ-line expression capability by drift, since another autosomal gene is redundant for this function. This hypothesis predicts that X-linked copies are expressed only somatically, but autosomal copies are expressed in both the germ line and the soma where there might be strong purifying selection to maintain somatic expression and identical protein sequences. Examples of this phenomenon are the *rpl-25* and *pab* genes. For both of these pairs, removal of the X-linked pair had little phenotypic consequence (suggesting that the autosomal gene acts redundantly with the X copy in the soma) while removal of the autosome-linked duplicate resulted in sterility due to germ-line proliferation defects.

To understand the relationships between gene duplication, X chromosome silencing, and germ-line function and the relative roles of the proposed evolutionary mechanisms, it will be important to correlate specific gene function with silencing and duplication over the entire genome. Germ-line-acting genes required specifically for early germ-line proliferation will likely appear among the sterile and embryonic-lethal phenotypic classes that have been identified by large-scale RNAi screens (as are the autosomal *rpl* and *pab* duplicates) (Fraser *et al.* 2000; Gonczy *et al.* 2000; Piano *et al.* 2000; Hanazawa *et al.* 2001; Maeda *et al.* 2001; Colaiacovo *et al.* 2002; Kamath *et al.* 2003; Simmer *et al.* 2003). Although available data from large-scale RNAi screens identify genes required for fertility, these data do not delineate the precise germ-line defect that underlies the sterility phenotype. A large-scale screen to pinpoint more precisely the defects that cause sterility in *C. elegans* (*e.g.*, proliferation defects *vs.* gametogenesis defects) is underway (E. J. A. Hubbard, unpublished data). Together with genome-wide informatic analysis of gene duplicates, these data should help elucidate further the correlation between gene duplication, X chromosome silencing, genome organization, and germ-line development.

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