

The cDNA Sequence and Expression of the AAA-family Peroxin Genes *pex-1* and *pex-6* from the Nematode *Caenorhabditis elegans*

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ABSTRACT—We cloned cDNAs encoding two peroxins, PEX-1 and PEX-6, of the nematode *Caenorhabditis elegans*. Peroxins are proteins that play essential roles in peroxisome biogenesis and are encoded by *pex* genes. Among the peroxins, PEX-1 and PEX-6 constitute the subfamily 2 of AAA (ATPases associated with diverse cellular activities) proteins. Each cDNA agreed well with the respective mRNA in size (3.4 kb for *pex-1* and 2.3 kb for *pex-6*) and did not carry any spliced leader sequence. The *pex-1* cDNA was composed of 24 exons, which were encoded by a genomic region containing three open reading frames (ORFs), c11h1.4, c11h1.5, and c11h1.6; the predicted ORF c11h1.5 was encompassed in the 15th intron. Although many exon-intron borders in *pex-1* were inconsistent with those predicted for c11h1.4 and c11h1.6, those in *pex-6* coincided with those for the ORF f39g3.7. The *pex-1* and *pex-6* genes encoded proteins with 996 and 720 amino acid residues, respectively. Both *pex-1* mRNA and *pex-6* mRNA were detectable mainly in intestinal cells throughout the life cycle of *C. elegans*.

INTRODUCTION

Peroxisins are proteins that play essential roles in peroxisome biogenesis and are encoded by increasing numbers of *pex* genes (Distel *et al.*, 1996). Among the peroxins, Pex1p (yeast protein)/PEX1 (mammalian protein) and Pex6p/PEX6 belong to the AAA (ATPases associated with diverse cellular activities) protein family (Erdmann *et al.*, 1991; Kunau *et al.*, 1993). Although no common biological function for AAA proteins has yet been elucidated, they share one or two copies of a conserved sequence of 200–250 amino acid residues, which is termed AAA cassette and encompasses the Walker motifs A and B for ATPases (Walker *et al.*, 1982; Karata *et al.*, 1999) and the second region of homology (SRH) (Swaffield *et al.*, 1992; Karata *et al.*, 1999). The AAA family is divided into 17 subfamilies according to the number of AAA cassettes, the extent of their sequence conservation, the function of the protein, and so on (Beyer, 1997). Subfamily 2 (SF2), which consists of only two members, Pex1p/PEX1 and Pex6p/PEX6, presents two AAA cassettes of which the one located closest to the C-terminus is highly conserved, while the other one diverges from the consensus sequence considerably. The two

SF2 member proteins interact with each other in an ATP-dependent manner (Tamura *et al.*, 1998; Faber *et al.*, 1998), the overexpression of one suppresses the allele-specific defect of the other (Geisbrecht *et al.*, 1998; Faber *et al.*, 1998), and the interaction seems to be crucial for peroxisome biogenesis (Geisbrecht *et al.*, 1998); the two proteins have been speculated to be involved in the fusion of pre-peroxisomal vesicles (Titorenko and Rachubinski, 2000).

The peroxisome biogenesis disorders (PBDs) are a group of lethal autosomal-recessive diseases caused by mutations in *pex* genes. Zellweger syndrome (ZS, alternatively called cerebro-hepato-renal syndrome) is the most severe PBD, which is classified into ten complementation groups (CGs). The genes *PEX1* and *PEX6* are responsible for CG1 (alternatively called CG-E) (Reuber *et al.*, 1997; Portsteffen *et al.*, 1997) and CG4 (or CG-C) (Tsukamoto *et al.*, 1995; Yahraus *et al.*, 1996), respectively. ZS is characterized by brain malformation that has been attributed to an impediment in gliophilic neuronal migration (Evrard *et al.*, 1978). The principal effect of defective peroxisomes on neuronal cells could be studied with the *Caenorhabditis elegans* model, which has many biological advantages, especially its fully established cell lineage (Schnabel and Priess, 1997), completed genomic sequence (The *C. elegans* Sequencing Consortium, 1998), and RNA-mediated interference (RNAi) for the analysis of gene function (Fire *et al.*, 1998). Although *C. elegans* peroxisomes have

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been detected (Togo *et al.*, 2000), none of the peroxins has yet been identified. Here, we report the cDNA cloning and expression of *C. elegans* genes encoding two putative peroxins that belong to SF2, designated as *pex-1* and *pex-6*.

MATERIALS AND METHODS

Organisms and cDNA library

The Bristol N2 strain of *C. elegans* was used throughout this study according to the methods compiled by Lewis and Fleming (1995). Synchronized populations of the nematode were obtained as described (Maebuchi *et al.*, 1999). *C. elegans* λ ZAPII cDNA library, made with poly(A)-rich RNA from the whole animal, was a generous gift from Dr. Alan Coulson (The Sanger Center, Cambridge, UK). *Saccharomyces cerevisiae* strain MB328 (*MATa*, *his3*, *leu2*, *ura3*, *pex1*) was isolated by the method of Erdmann *et al.* (1989) and identified as a *pex1* mutant by genetic complementation analysis.

Cloning of the *pex-1* cDNA

The primers S1 (sense, 5'-TCCCCCGGGGAAGACGTCGGT-GGAATGTTT) and A1 (antisense, 5'-GCTCTAGAGGCTAACGTCAC-TTTTCTGACCTATC) amplified an 808-bp sequence when the cDNA library was used as a template for polymerase chain reaction (PCR); the sequence corresponded to the open reading frame (ORF) c11h1.6. The product was cloned into the plasmid pUC119 (Takara) generating pEMB835 (Fig. 1A). The sequences extending to the 5' and 3' termini of the mRNA were obtained by RACE (rapid amplification of

cDNA ends) (Frohman *et al.*, 1988). Products amplified by two successive rounds of 5' RACE were cloned into the plasmid pBluescript SK(-) (Stratagene) providing the plasmids pEMB838 and pEMB842. Primers used were as follows: S2 (5'-GACTCGAGTCGACATCGA-TTTTTTTTTTTTTTTTTT), S3 (5'-GACTCGAGTCGACATC G), A2 (5'-CGGGATCCGCCAACGAATCCAGCTCATCAAAG), A3 (5'-AACTGCAGCACTG GCTCCAATATATTTTCGAC), A4 (5'-CGGAATTCCT-CCAACGGATTTCGAAAAGTTG), A5 (5'-AACTGCAGGTCGGAATA-TTTCACAACGG), A6 (5'-CGGGATCCCTCGGCTTCTTCA GTTCT-TTTCCG), and A7 (5'-AACTGCAGCGGAACGACTTTCAAACGAA-TTGC). The 3' untranslated region of the *pex-1* cDNA was obtained by 3' RACE with primers S4 (5'-GGGGAGCTCTGGGTAATAATC-GAAGATGGACAAG), S1, and (dT)₁₇, and cloned into pBluescript SK(-) generating the plasmid pEMB855. The probe P1 was prepared by PCR with the primers S1 and A4.

Cloning of the *pex-6* cDNA

A cDNA that carried the ORF f39g3.7 was obtained by screening the λ ZAPII cDNA library as described (Bun-ya *et al.*, 1997) and cloned into pBluescript SK(-); the resulting plasmid was pEMB732 (Fig. 1B). The probe P2 used for screening was prepared by PCR with primers S5 (5'-GAAGAGTGCGAATGTGTTT) and A8 (5'-CCGATGAG-TTTCAGAAG C). The 5' region of *pex-6* cDNA was amplified by 5' RACE with primers S2, S3, A8, A9 (5'-CTCGGATCCGTGGTAATTG-TGAAGGAGG), and A10 (5'-CTCGGATCCAGAATCCCT AGAGACC-AACATG). The RACE product was identified with the probe P3 that was amplified with the primers S6 (5'-AATTGATTTATCTACTT-TGAGG) and A10, and the longest product was cloned into pBluescript SK(-) to generate the plasmid pEMB807.

Other methods

Cells of mixed-stage animals were disrupted by grinding in liquid nitrogen and the poly(A)-rich RNA was isolated using a μ MACS mRNA isolation kit (Miltényi Biotec). The *pex-1* and *pex-6* mRNA were detected by northern blot analysis with the antisense RNA probes, which were labeled with a digoxigenin-labeled nucleic acid detection kit (Boehringer Mannheim). The templates used for *in vitro* synthesis of the sense and antisense RNA probes were derivatives of pEMB842 and pEMB732; the derivatives carried the cDNA fragments of *pex-1* (nucleotides +1 to +450) and *pex-6* (nucleotides -70 to +305). The preparation of Celf (*C. elegans* homolog of initiation factor A4) mRNA and the procedure of whole-mount *in situ* hybridization were performed as described (Maebuchi *et al.*, 1999). TBLASTN searches of the *C. elegans* ACEDB database were run on the server at The Sanger Center (<http://www.sanger.ac.uk>). The CLUSTAL W program (Thompson *et al.*, 1994) was used for sequence alignments.

RESULTS

Composite cDNA encoding PEX-1 and PEX-6

We searched the *C. elegans* genome database for orthologs of SF2 proteins and found two open reading frames, c11h1.6 and f39g3.7, which encode polypeptides with the highest similarity to Pex1p/PEX1 and Pex6p/PEX6, respectively. Gene-specific probes were prepared by PCR amplification of a *C. elegans* cDNA library with appropriate primers: S1 and A1 for *pex-1* (Fig. 1A) and S5 and A8 for *pex-6* (Fig. 1B). Since c11h1.6 encodes only the region of the highly conserved AAA cassette, we applied the RACE procedure directly to isolate the 5' and 3' portions of the *pex-1* cDNA. Three cDNA fragments cloned in pEMB842, pEMB838, and pEMB855 corresponded to the 5' distal, central, and 3' distal regions of the mRNA, respectively, and constituted a cDNA of

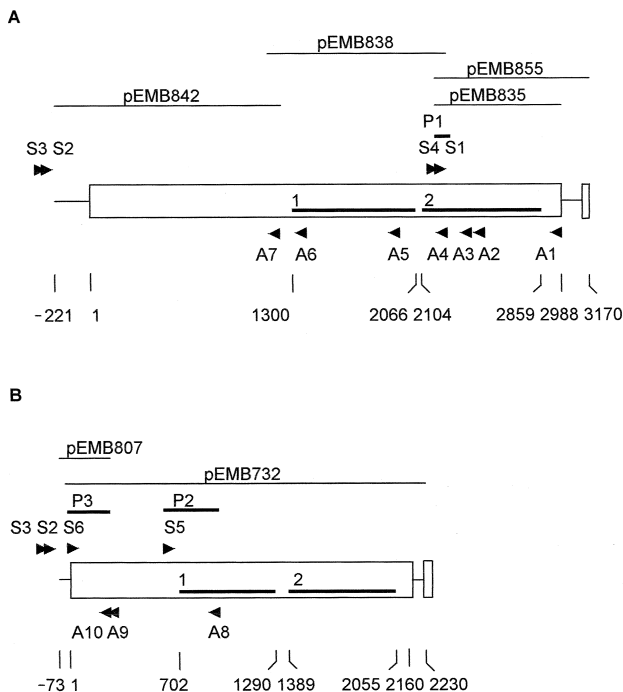


Fig. 1. Schematic structures of the composite cDNAs encoding PEX-1 (A) and PEX-6 (B). Thin lines indicate the cDNA clones (A: pEMB835, pEMB838, pEMB842, and pEMB855; B: pEMB732 and pEMB807) and thick lines indicate probes (A: P1; B: P2 and P3). The open box represents the ORF, in which bars show the first and second AAA cassettes (1 and 2), and the open rectangles denote the poly(A) sequence. Arrowheads mark positions of sequences corresponding to PCR primers. The nucleotides on the mRNA-identical strand are numbered; position +1 is A of the start codon of the ORF. The nucleotide sequences of *pex-1* and *pex-6* are available at DDBJ/EMBL/GenBank under the accession numbers AB054992 and AB010968, respectively.

3391 nucleotides (nt) (Fig. 1A). The main body of *pex-6* cDNA was obtained by plaque hybridization because the region encoded by f39g3.7 extended beyond the AAA cassette sequences. This clone, pEMB732, together with clone pEMB807 obtained by 5' RACE constituted the cDNA of 2303 nt (Fig. 1B). The lengths of the *pex-1* and *pex-6* cDNA coincided well with those of mRNA detected by northern blot analysis of the poly(A)-rich RNA from a mixed-phase culture; 3.4 kb for *pex-1* and 2.3 kb for *pex-6* (Fig. 2). Therefore, we concluded that the composite cDNA corresponded to the respective full-length mRNA. No spliced leader (SL) sequence was detected at the 5' end of either cDNA.

Analysis of the cDNA sequences, which have been deposited in DDBJ/EMBL/GenBank under the accession numbers AB054992 (*pex-1*) and AB010968 (*pex-6*), revealed

that the ORF of *pex-6* was identical to f39g3.7 but that of *pex-1* was not identical to any ORF(s) predicted by the GENEFINDER program. The *pex-1* cDNA (and its ORF) was composed of 24 exons, encoded by a genomic region containing three ORFs, c11h1.4, c11h1.5, and c11h1.6 (Fig. 3). The region predicting c11h1.4, which consisted of 14 exons, encoded the first 15 exons of the *pex-1* ORF, ten of which were shared with c11h1.4. The spacer region between c11h1.5 and c11h1.6 encoded the 16th and 17th exons of *pex-1*. Exons 18 through 24 were shared with c11h1.6, although the 18th exon was extended to the 5' side by 10 nucleotides from the start codon of c11h1.6. There was no significant deviation from the splice-site consensus sequences with two exceptions of 5' splice sites having AG/GC (instead of AG/GU) in the 6th and 15th introns, which were not recognized by the program. The ORF c11h1.5 that has a different orientation from the others was encompassed in the 15th intron. The *pex-1* and *pex-6* genes encoded proteins of 996 and 720 amino acid residues, respectively.

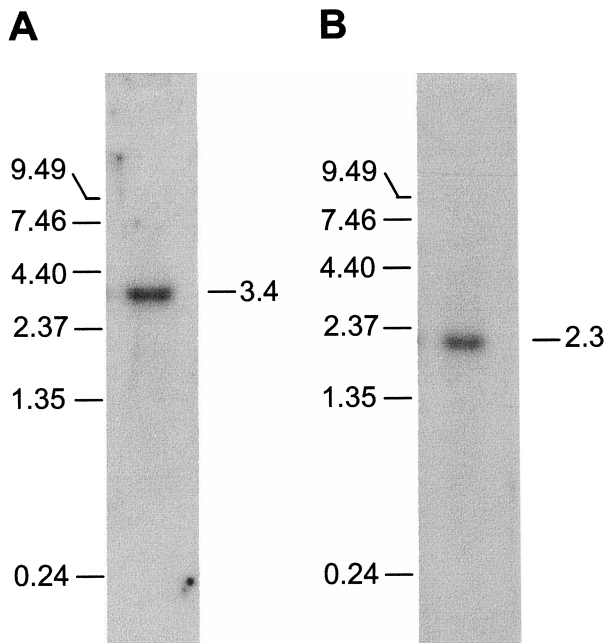


Fig. 2. Northern blot analysis. The poly(A)-rich RNA (about 5 μ g) prepared from mixed-phase cultures was separated by electrophoresis in the presence of 2.2 M formaldehyde, transferred onto nylon membranes and probed with *pex-1* (A) or *pex-6* (B) digoxigenin-labeled antisense RNA. The sizes of markers are shown on the left and those of the transcripts detected are shown on the right.

Identification of PEX-1 and PEX-6

To identify the tentatively termed *pex-1* and *pex-6* genes as genuine peroxin genes, we compared their amino acid sequences with those of biologically established SF2 members of the AAA protein family because no *pex* mutants were available in *C. elegans* and the expression of PEX-1 in a *pex1* mutant (strain MB328) of the yeast *S. cerevisiae* did not complement the mutation (data not shown). There were five pairs of SF2 sequences, which were confirmed to be peroxins by their mutant phenotype. Their sources and the number of amino acid residues in parentheses of Pex1p/PEX1 and Pex6p/PEX6 were: *S. cerevisiae* (1043, 1030) (Erdmann *et al.*, 1991; Voorn-Brouwer *et al.*, 1993), *Pichia pastoris* (1157, 1165) (Heyman *et al.*, 1994; Spong and Subramani, 1993), *Yarrowia lipolytica* (1023, 1025) (Titorenko *et al.*, 2000; Nuttley *et al.*, 1994), *Hansenula polymorpha* (1074, 1135) (Kiel *et al.*, 1999), and *Homo sapiens* (1283, 980) (Reuber *et al.*, 1997; Portsteffen *et al.*, 1997; Yahraus *et al.*, 1996). The sequence similarities, especially in the region outside the AAA cassettes, were moderate and *C. elegans* PEX-6 was smaller than the putative orthologs. We therefore compared the sequences of three motifs in the two AAA cassettes; Walker motifs A and B

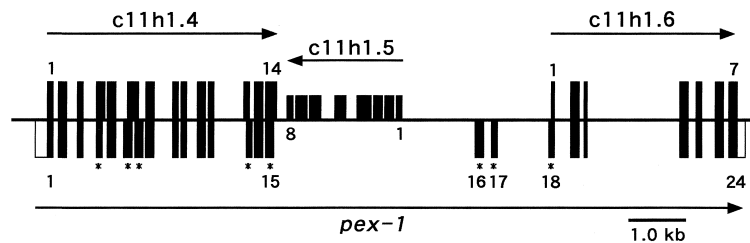


Fig. 3. Genomic organization of the *pex-1* gene. The closed rectangles above the line, which indicates part of the genomic sequence cloned in the cosmid c11h1, are exons for three ORFs predicted by the GENEFINDER program. The names and orientation of the ORFs are as indicated. The rectangles below the line denote exons determined by the *pex-1* cDNA sequence; closed rectangles indicate the translated region and open rectangles indicate the untranslated regions. The asterisks mark exons not correctly predicted. The numbers indicate the first and last exons as well as the 15th through 18th exons for *pex-1*.

Motif			Walker A	Walker B	SRH	
Consensus sequence			GPPGCGKT - - - - -	hhhhDDhD - - - - -	TSxxxxxDxAhRPGRhD	
			T	EE	N E	
1 st AAA cassette	PEX-1	<i>Cel</i>	508	GGNGSGKT - (52) -	CLFLDDFD - (56) -	IVSRKIELEQLTKEDRCE
		<i>Pex1p</i>	523	GTSGSGKS - (50) -	LLILEDDL - (55) -	LIEHDFQLRAPDKEARKQ
		or <i>Yli</i>	435	GSRGSGKS - (50) -	VLFLDDID - (55) -	VFEELFHLKSPDKEARLA
		PEX1	461	GKQGIIGKT - (52) -	LIVLDNVE - (64) -	FVSETWSLRAPDKHARAK
		<i>Sce</i>	504	GASGSGKT - (51) -	VLVLENLD - (55) -	LIQQEFSLKAPDKELRKE
	<i>Hpo</i>	599	GGKGSKGS - (51) -	VVLLDDLD - (64) -	IFQCVQHIQPPNQEQRCE	
	<i>Hsa</i>			*	*	
	PEX-6	<i>Cel</i>	265	GASGSGKR - (47) -	VLFRNSN - (48) -	LALYTFSAEFMDENDRKT
		<i>Pex6p</i>	570	LSRAIGKS - (49) -	IVFIKHIE - (49) -	KFKFEIVLGVPSERLTL
		or <i>Yli</i>	477	AKRGVGS - (47) -	VVVLQHLE - (49) -	RFQFEIEIGVPSERQRQ
PEX6		483	TTNNVGKA - (53) -	VIFLAHLD - (57) -	HMRFEILVVPSEARLR	
<i>Sce</i>		571	MARCVGKA - (49) -	ILYIRHIE - (50) -	KFKFDISINVPTEPERKL	
<i>Hpo</i>	470	GPPGCGKT - (47) -	VLLLTAVD - (53) -	AFPHLELVPALSEQRRLS		
<i>Hsa</i>			*	*		
2 nd AAA cassette	PEX-1	<i>Cel</i>	769	GPSGCGKT - (47) -	ILFFDELD - (40) -	TSRIDLIDDALLRPGRFD
		<i>Pex1p</i>	840	GYPGCGKT - (47) -	ILFFDEFD - (39) -	TSRPDLIDSALLRPGRLD
		or <i>Yli</i>	704	GYPGCGKT - (46) -	ILFFDEFD - (39) -	TSRPDLIDPALLRPGRLD
		PEX1	738	GYPGCGKT - (47) -	ILFFDEFD - (39) -	TSRPDLIDSALLRPGRLD
		<i>Sce</i>	771	GYPGCGKT - (47) -	VLFFDEFD - (39) -	TSRPDLIDSALLRPGRLD
	<i>Hpo</i>	881	GPPGTGKT - (47) -	ILFFDEFE - (39) -	TSRPDLIDPALLRPGRLD	
	<i>Hsa</i>			*	*	
	PEX-6	<i>Cel</i>	500	GSPGCGKT - (47) -	VIFFDEID - (43) -	TNRPDLLDNSLMTTPGRFD
		<i>Pex6p</i>	859	GPPGTGKT - (47) -	VVFFDELD - (44) -	TNRPDLLDEALLRPGRFD
		or <i>Yli</i>	760	GPPGTGKT - (47) -	VVFFDELD - (43) -	TNRPDLLDEALLRPGRFD
PEX6		772	GPPGTGKT - (47) -	VIFFDEID - (42) -	TNRPDLLDEALLRPGRFD	
<i>Sce</i>		853	GPPGTGKT - (47) -	VIFFDELD - (44) -	TNRPDLLDEALLRPGRFD	
<i>Hpo</i>	744	GPPGTGKT - (47) -	IIFFDELD - (41) -	TNRPDLLDPALLRPGRFD		
<i>Hsa</i>			*	*		

Fig. 4. Motifs in the first and second AAA cassettes. PEX-1 and PEX-6 (the DDBJ/EMBL/GenBank accession numbers are AB054992 and AB010968, respectively) of *C. elegans* (*Cel*) were aligned with the Pex1p/PEX1 and Pex6p/PEX6 orthologs, respectively and thereafter the AAA cassette regions were taken from the final alignments. The abbreviations of their sources and the accession numbers are: *Ppa*, *P. pastoris* (Z36987 and Z22556); *Yli*, *Y. lipolytica* (AF208231 and L23858); *Sce*, *S. cerevisiae* (M58676 and L20789); *Hpo*, *H. polymorpha* (AF129873 and AF129874); *Hsa*, *H. sapiens* (AF030356 and U56602). The numbers on the left represent the position of the first amino acid residue and those in parentheses indicate the residue number present between the motifs. Similar residues present in four or more sequences are shaded gray, and then four or more identical residues among them are blackened. The asterisks denote residues that distinguish the Pex1p/PEX1 and Pex6p/PEX6 sequences. The consensus sequences for Walker motifs A and B as well as SRH are shown above the alignments; h and x represent hydrophobic residues and any amino acid residue, respectively.

for nucleotide binding and SRH with unknown function (Fig. 4). The first AAA cassette was unique for SF2 proteins and the second located closest to the C-terminus was characteristic for all AAA proteins. PEX-1 and PEX-6 of *C. elegans* showed a high degree of similarity to respective five cognate proteins not only in the motif sequences but also in distances between the motifs. In addition to a considerable sequence similarity in the respective N-terminal region (data not shown), three crucial residues (Fig. 4, marked by asterisks) identified the *C. elegans* PEX-1 and PEX-6 as genuine orthologs of Pex1p/PEX1 and Pex6p/PEX6, respectively.

Spatio-temporal pattern of gene expression

The mRNAs of both *pex-1* and *pex-6* were detectable throughout the life cycle of *C. elegans* (Fig. 5). The extent of expression relative to that of *CeIF*, which is a homolog of eucaryotic initiation factor 4A and was used as the control for equal sample loading (Krause, 1995), was higher at the larval stage L3 and lower at the young adult stage (YA) than the other stages: embryo (E), larvae (L1, L2 and L4), or egg-laying adult (EA). The developmental patterns of gene expres-

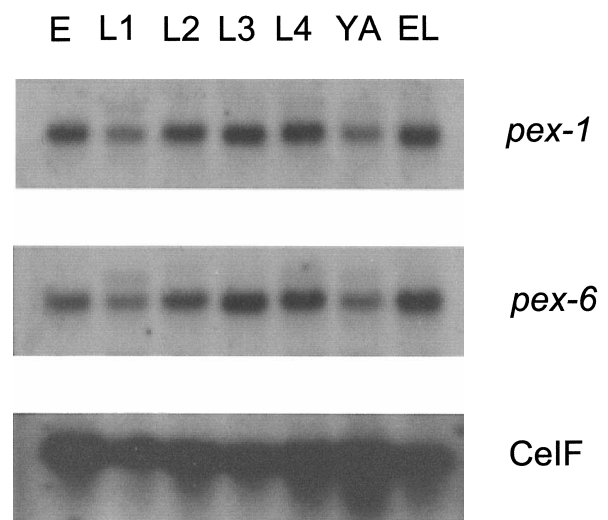


Fig. 5. Amounts of *pex-1* and *pex-6* mRNA at each developmental stage. Poly(A)-rich RNA (5 µg of each) from embryos (E), larvae (L1, L2, L3, and L4), young adults (YA) and egg-laying adults (EA) were assessed by northern blot analysis as in Fig. 2. The *pex-1*, *pex-6*, and *CeIF* mRNAs were detected with the appropriate probes.

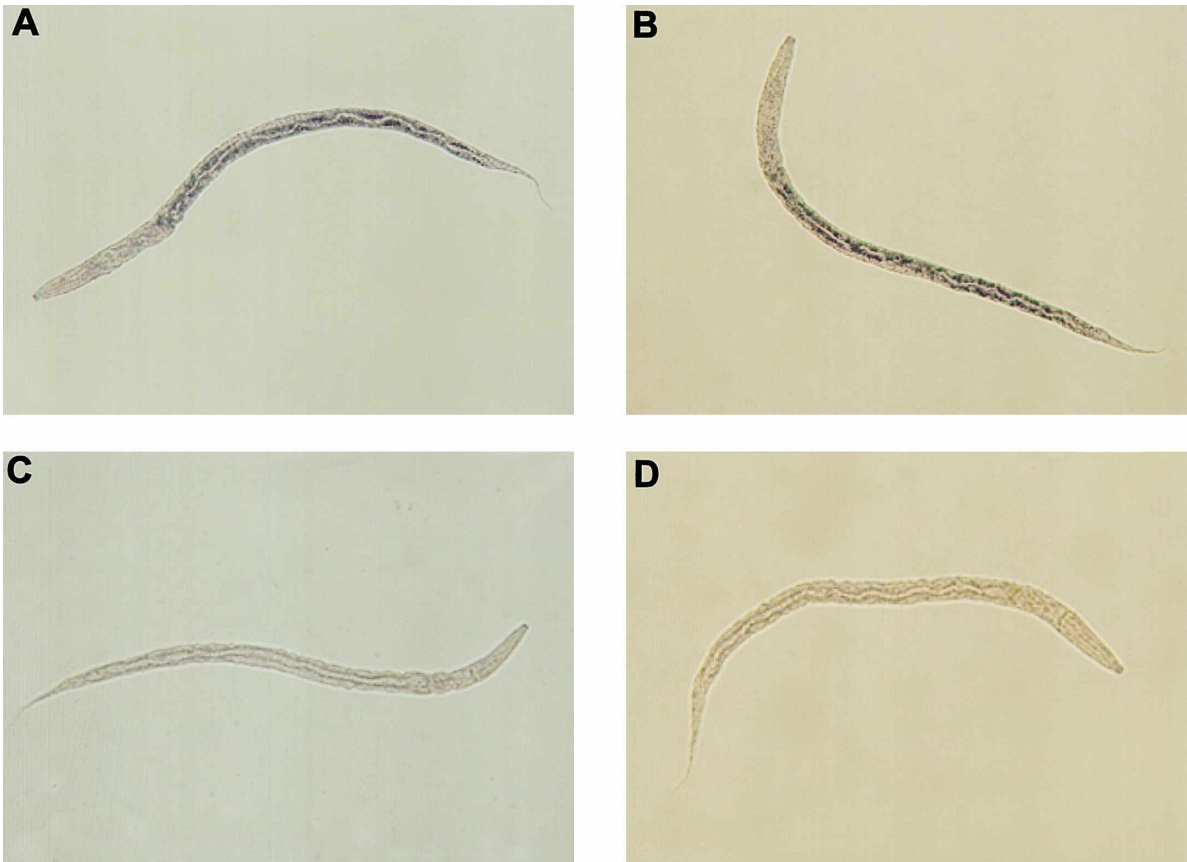


Fig. 6. Expression of the *pex-1* and *pex-6* mRNA *in situ*. Fixed L4 larvae were probed with the digoxigenin-labeled antisense RNA of *pex-1* (A) or *pex-6* (B) or with the sense RNA of *pex-1* (C) or *pex-6* (D) and incubated with alkaline phosphatase-labeled sheep anti-(digoxigenin) Fab fragment. Alkaline phosphatase catalyzed the formation of a dark blue precipitate in cells containing the *pex-1* or *pex-6* mRNA.

sion for *pex-1* and *pex-6* were apparently parallel to each other, though the results were semiquantitative because of their extremely low level of expression. The tissue distributions of the *pex-1* and *pex-6* mRNA were examined by whole-mount *in situ* hybridization using antisense RNA probes (Fig. 6, A and B). Specimen used was L4 larva because in younger larval stages the somatic cell division is not completed and mature cuticle at adult stages tends to reduce the accessibility of RNA probes. Both of these mRNAs were detected mainly in intestinal cells.

DISCUSSION

The sequences of the *pex-1* and *pex-6* cDNAs did not contain any of the 22-nucleotide SL sequences, which are *trans*-spliced to the 5' terminus of about 70% of mRNA species of *C. elegans* (Krause and Hirsh, 1987; Zorio *et al.*, 1994). This raised misgivings about the completeness of the cDNA, especially for the short *pex-6* cDNA. However, 5' RACE procedures under a wide variety of conditions revealed no further 5' sequence beyond those cloned in pEMB842 or in pEMB807. This result together with the coincidence of cDNA lengths (3391 nt for *pex-1* and 2303 nt for *pex-6*) with apparent mRNA lengths (3.4 kb for *pex-1* and 2.3 kb for *pex-6*) indicated that the composite cDNAs were full-length sequences.

Eight *pex-1* exons out of 24 were not correctly predicted by the GENEFINDER program (Fig. 3, marked by asterisk). This results from two unique features of the gene: a long intron and a biased codon usage. The unusually long 15th intron (3698 nt) misled into the split of the single *pex-1* ORF into two ORFs c11h1.4 and c11h1.6 and missed the 16th and 17th exons and a part of the 18th exon. The incorrect prediction in the 5' half of the *pex-1* ORF may be due to the extremely low level of expression and an irregular codon usage. The prediction of ORF depends not only on splice site sequences but also on codon usage. Since codon usage in *C. elegans* varies among genes in a manner correlated with their expression level (Sharp and Bradnam, 1997), an extreme expression level and deviation from regular codon usage affect the prediction. The *pex-1* ORF, indeed, shows more deviation (e.g., high frequency of GUG, UCG, ACA, and CAC) than the *pex-6* ORF, which is correctly predicted.

The intestinal expression of *pex-1* and *pex-6* is similar to that of P-44, which is *C. elegans* type-II 3-oxoacyl-CoA thiolase involving in β -oxidation of acyl-compounds with an α -methyl branched-chain (Bun-ya *et al.*, 1997, 1998). Since P-44 is present exclusively in peroxisomes (Maebuchi *et al.*, 1999), the expression of *pex-1* and *pex-6* is consistent with their function as peroxins and suggests that the multifunctional intestinal cells of this organism also serve hepatic functions, for which

peroxisomes are essential.

Many lines of evidence indicate that the interaction between Pex1p/PEX1 and Pex6p/PEX6 is required for peroxisome biogenesis (Tamura *et al.*, 1998; Geisbrecht *et al.*, 1998; Faber *et al.*, 1998; Kiel *et al.*, 1999). The homo-hexamers of each protein could constitute a heterogeneous double ring structure similar to that of *N*-ethylmaleimide-sensitive factor (NSF) because the AAA module tends to form a hexameric structure (Lenzen *et al.*, 1998; Vale, 2000). In this context, it is notable that the expression patterns of *pex-1* and *pex-6* mRNAs were apparently parallel to each other (Fig. 5). Their synthesis may be co-regulated to keep the amounts of component proteins equivalent. It is plausible that the final multiprotein structure has influenced the molecular evolution of component proteins. Four pairs of yeast Pex1p and Pex6p are similar to each other in molecular size (*S. cerevisiae*, 1043 residues and 1030 residues; *P. pastoris*, 1157 and 1165; *Y. lipolytica*, 1023 and 1025; *H. polymorpha*, 1074 and 1135), while PEX6 from the animal kingdom are smaller than PEX-1 by about 300 residues (*H. sapiens*, 1283 and 980; *C. elegans*, 996 and 720). The moderate sequence similarities in the N-terminal region outside the AAA cassettes among each protein group suggest that this region evolved to fit the surface of the partner peroxin.

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