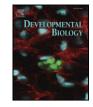
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# Involvement of HMG-12 and CAR-1 in the *cdc*-48.1 expression of *Caenorhabditis elegans*

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#### ABSTRACT

*Caenorhabditis elegans* possesses two p97/VCP/Cdc48p homologues, named CDC-48.1 (C06A1.1) and CDC-48.2 (C41C4.8), and their expression patterns and levels are differently regulated. To clarify the regulatory mechanisms of differential expression of two p97 proteins of *C. elegans*, we performed detailed deletion analysis of their promoter regions. We found that the promoter of *cdc-48.1* contains two regions necessary for embryonic and for post-embryonic expression, while the promoter of *cdc-48.2* contains the single region necessary for embryonic expression. In particular, two elements (Element A and Element B) and three conserved boxes (Box a, Box b and Box c) were essential for *cdc-48.1* expression in embryos and at post-embryonic stages, respectively. By using South-Western blotting and MALDI-TOF MS analysis, we identified HMG-12 and CAR-1 as proteins that bind to Element A and Element B, respectively, from the embryonic nuclear extract. Importantly, we found the decreased expression of p97 in embryos prepared from *hmg-12(RNAi)* or *car-1(RNAi)* worms. These results indicate that both HMG-12 and CAR-1 play important roles in embryonic expression of *cdc-48.1*.

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#### Introduction

p97 (also called VCP in higher eukaryotes and Cdc48p in yeast) is a member of the type II AAA (ATPases associated with diverse cellular activities) proteins and is evolutionarily conserved from archaebacteria to human (Woodman, 2003; Wang et al., 2004). As is characteristic of the AAA protein, p97 forms a homo-hexamer that hydrolyzes ATP to achieve its various cellular functions (Zhang et al., 2000). The common function of p97 is believed to catalyze the ATP-dependent protein conformational change; unfolding of proteins and disassembly of protein complexes (Zhang et al., 2002).

p97 is involved in a variety of cellular processes, including ERAD (endoplasmic reticulum-associated protein degradation) (Ye et al., 2001, 2003), homotypic membrane fusion (Kondo et al., 1997), transcriptional regulation (Dai et al., 1998; Rape et al., 2001; Shcherbik et al., 2003), cell-cycle regulation (Moir et al., 1982; Dai and Li, 2001), and other protein quality control activities (Wang et al., 2004; Ye, 2006). These specificities are mainly determined by differential binding of distinct cofactors/adaptor proteins, such as Ufd1/Npl4, Ubx2, p47, and p37 to the

E-mail addresses: ogura@gpo.kumamoto-u.ac.jp (T. Ogura), yamanaka@gpo.kumamoto-u.ac.jp (K. Yamanaka). N-terminal domain of p97 (Kondo et al., 1997; Schuberth and Buchberger, 2005; Uchiyama et al., 2006; Ye, 2006; Pye et al., 2007). Recent studies of gene-disrupted mutants have revealed that p97 is essential for *Saccharomyces cerevisiae* (Fröhlich et al., 1991), *Drosophila melanogaster* (León and McKearin, 1999), *Caenorhabditis elegans* (Yama-naka et al., 2004), and mouse (Müller et al., 2007), suggesting that p97 plays a fundamental role within both unicellular and multicellular organisms. In addition to many essential cellular functions, p97 is involved in the clearance of protein aggregates, such as polyglutamine-induced protein aggregation (Yamanaka et al., 2004; Kobayashi et al., 2007). It is interesting to mention that inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia has been demonstrated to be caused by mutations in p97 (Watts et al., 2004).

*C. elegans* uniquely possesses two p97 homologues, named CDC-48.1 (C06A1.1) and CDC-48.2 (C41C4.8), although p97 is encoded by a single gene in most of other organisms, such as human, mouse, and yeast (Yamanaka et al., 2004). Our recent studies showed that their functions are redundantly essential for embryogenesis. Simultaneous depletion of both CDC-48.1 and CDC-48.2 caused a complete embryonic lethal phenotype (Yamanaka et al., 2004), especially they play an important role in the progression of meiosis in embryo (Sasagawa et al., 2007a). In addition to their function in embryogenesis, it was revealed that two p97 of *C. elegans* play essential roles in ERAD pathway, as the mammalian p97 does (Ye et al., 2001; Mouysset et al., 2006; Sasagawa et al., 2007b).

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Although many reports have described the biochemical properties and the functions of p97, little is known about the expression mechanism of p97 gene. We previously described that the levels and expression patterns of two p97 proteins are differentially regulated (Yamauchi et al., 2006). It has been demonstrated that p97 expression levels correlate with the recurrence and prognosis of several human cancers (Yamamoto et al., 2003; Tsujimoto et al., 2004). Recently, it was revealed that the pre-B-cell leukemia transcription factor 1 (PBX1) is a key factor for transcription of human p97 (Qiu et al., 2007). Thus, it is very important to understand the expression regulation of p97 for not only biological but also medical fields. In the present study, to clarify the regulatory mechanisms of differential expression of two p97 proteins of C. elegans, we performed detailed analysis of their 5' upstream regions. We identified two proteins, HMG-12 and CAR-1, involved in embryonic expression of cdc-48.1, and discuss the promoter structure of two p97 genes.

#### Materials and methods

#### C. elegans culture condition

Wild-type Bristol N2 strain of C. elegans was cultured at 20  $^{\circ}$ C as described previously (Brenner, 1974).

#### Sequence analysis

Sequence alignment was performed using the GENETYX software (Genetyx). DotPlot comparison was carried out using the CLC DNA Workbench software (CLC bio). The nucleotide sequences of 5' regions of *cdc-48.1* and *cdc-48.2* of *C. elegans* and *Caenorhabditis briggsae* were obtained from WormBase (http://www.wormbase.org).

#### Construction of gfp reporter genes and transgenic worms

Various promoter regions of cdc-48.1 and cdc-48.2 were amplified from p06-1 and p41-1 plasmids by PCR with primers (p06 RV and each of p06-1 FW, p06-2 FW, p06-3 FW, p06-4 FW, p06-5 FW, p06-6 FW, and p06-12 FW for cdc-48.1, and p41 RV and each of p41-1 FW, p41-2 FW, p41-3 FW, p41-4 FW, and p41-5 FW for cdc-48.2) (Table 1). The resultant fragments were inserted into the pPD95.77 vector to create a translational fusion to green fluorescent protein (GFP). Site-directed mutagenesis was performed on p06-1 with mutant primers using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) to create p06-8, p06-9, p06-10, and p06-11. For internal deletion constructs. p06-7 was prepared from p06-1 and other constructs (p06-13 to p06-21) were prepared from p06-7 as follows. Upstream and downstream regions from a deletion region were amplified with primers (p06-1 FW and each #1 primer of p06-13 to p06-21 for upstream regions, and p06 RV and each #2 primer of p06-13 to p06-21 for downstream regions) (Table 1), and purified with Wizard SV Gel and PCR Clean-up system (Promega), Two PCR products were mixed and were annealed in the reaction mixture containing 1× PCR buffer, 0.2 mM dNTPs at 95 °C for 10 min and then 30 °C for 20 min. After addition of Ex-Tag polymerase (TaKaRa), the reaction mixtures were incubated at 72 °C for 10 min, and then the ordinal PCR reaction was carried out using p06-1 FW and p06 RV as primers. All constructs were confirmed by sequencing.

Establishment of transgenic worms was carried out as described previously (Yamauchi et al., 2006). At least three independent transgenic lines for each GFP construct were isolated and analyzed. Worms were mounted on 3% agarose pads for fluorescence microscopy on an Olympus BX51 microscope equipped with a CCD camera. Adobe Photoshop 6.0 was used for output of images.

#### Preparation of embryonic nuclear extracts and gel mobility-shift assay

For preparing large quantities of embryos, liquid culture of *C. elegans* was applied using *Escherichia coli* OP50 as a food source, and embryos were obtained by treatment of gravid hermaphrodites with NaOH and hypochlorite (Stiernagle, 2006). Preparation of embryonic nuclear extract was performed as described previously (Lichtsteiner and Tjian, 1995).

Oligonucleotides corresponding to sequences of Element A and Element B were prepared for gel mobility-shift assay and South-Western blot analysis as a probe. Sense and anti-sense oligonucleotides (Table 1) were mixed in TEN buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl), incubated at 95 °C for 10 min, and then gradually cooled down to the room temperature. Four picomols of the annealed dsDNAs were labeled using DIG Oligonucleotide 3'-End Labeling Kit (Roche Diagnostics). The embryonic nuclear extract was pre-incubated with 2  $\mu$ g of poly[dl-dC] in 40  $\mu$ l of binding buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 5% glycerol and 1 mM DTT) on ice for 10 min, and incubated with 1 ng of the DIG-labeled probe at 25 °C for 15 min. Reaction mixtures were loaded onto non-denaturing 6% polyacrylamide gel and subjected to electrophoresis in 0.25× TBE. The resultant DNA-protein complexes were transferred to a nylon membrane (Hybond N+, GE Healthcare) and detected by using DIG Gel Shift Kit (Roche Diagnostics). Unlabeled single-stranded and double-stranded oligonucleotides were used as competitors.

#### Two-dimensional gel electrophoresis

Two-dimensional (2D) gel electrophoresis of embryonic nuclear extract of C. elegans was carried out by the method described by Otani et al. (2001) with modifications. Briefly, 130  $\mu$ g of embryonic nuclear extract were prepared in 100  $\mu$ l of lysis buffer [7 M urea, 2 M thiourea, 5% N-cyclohexyl-3-aminopropanesulfonic acid (CHAPS), 2% IPG buffer (GE Healthcare), 50 mM 2-mercaptoethanol] by gentle shaking at room temperature for 1 h. The supernatant obtained after centrifugation at 13,000×g at 4 °C for 30 min was treated with 10 µl of DNase/RNase solution (2.5 mg/ml of DNase L and 2.5 mg/ml of RNase A) at room temperature for 30 min. After addition of equal volume of rehydration buffer [8 M urea, 0.5% CHAPS, 20 mM DTT, 0.5% IPG buffer and 0.04% Bromophenol Blue (BPB)] to the sample, first-dimension isoelectric focusing gel electrophoresis was carried out with Immobiline DryStrip pH 3-10 NL (7 cm) (GE Healthcare) at 200 V for 10 min, 400 V for 10 min, 1000 V for 1 h, and 1500 V overnight. After electrophoresis, the strip was soaked in equilibration buffer (50 mM Tris-HCl pH 8.7, 6 M urea, 30% glycerol, 1% SDS, 17.7 mM DTT and 0.04% BPB) and then in equilibration buffer containing 0.24 M iodoacetamide instead of DTT at room temperature for 30 min with gentle shaking.

Second-dimension SDS-PAGE was performed with a 17.5% acrylamide gel at 2.5 mA/ gel for the first 2 h and at 5 mA/gel for additional 6 h. After electrophoresis, gels were fixed in 10% trichloroacetic acid solution for 1 h and then stained with Coomassie brilliant blue R250 (CBBR).

#### South-Western blot analysis

Fractions of embryonic nuclear extract were separated by 2D electrophoresis as described above and then transferred onto nitrocellulose membranes (PROTRAN, Whatman). Membranes were stained in dyeing buffer (50% methanol and 0.1% CBBR) for 1 min and then destained in breaching buffer (50% methanol and 10% acetic acid) for 5 min. Subsequent treatments of membrane were performed as described previously (Ohri et al., 2004). Finally, the binding buffer containing 25 ng/ml of DIG-labeled probes and 0.25% BSA was added and incubated overnight at room temperature. Membranes were then washed four times in binding buffer at room temperature for 10 min each. The chemiluminescent detection of hybridized proteins was performed with a DIG Luminescent Detection Kit and Nitro-Block II (TM) (Roche Diagnostics).

#### Identification of proteins by MALDI-TOF MS

The CBBR-stained protein spots were excised from the 2D-PAGE gels. The gel pieces were destained with 200 µl of 30% acetonitrile containing 25 mM ammonium bicarbonate for 10 min, dehydrated with 50  $\mu$ l of 100% acetonitrile for 5 min, and dried for 15 min under vacuum. The proteins were digested in 5 µl of trypsin solution [10 µg/ml of sequencing grade modified trypsin (Promega) in 50 mM ammonium bicarbonate] at 37 °C for over night. The MALDI-TOF samples were prepared by the sandwich method with a slight modification as described previously (Kussmann et al., 1997). Matrix thin layer was made with 0.5  $\mu$ l of  $\alpha$ -cyano-4-hydroxy-cinnamic acid (CHCA) solution [1 mg/ml of CHCA (Sigma) in 50% acetonitrile containing 0.1% TFA and 25 mM ammonium bicarbonate] on the sample plate (Applied Biosystems). Aliquots (1.5  $\mu l)$  from the trypsin digests were dropped onto the thin layer, dried in air, and covered with 1 µl of CHCA solution. Mass spectra were acquired by the Voyager DE-STR (Applied Biosystems) in the mass range of 700–3500 m/z. Two trypsin autolysis peaks at  $[M+H]^+$  842.5098 and  $[M+H]^+$  2211.1046 were used for internal calibration of each spectrum. The proteins were identified by the MS-Fit ProteinProspector (http:// prospector.ucsf.edu/) at ±20 ppm for mass tolerance against NCBInr.

#### Purification of His-tagged recombinant proteins

For construction of His-tagged recombinant proteins, the coding regions of *hmg-12* and *car-1* genes were amplified from the cDNA clones (yk1230g1 and yk1535g3, respectively) by PCR with primers listed in Table 1, and cloned into *Nhel* and *Bam*HI sites, and *Ndel* and *Hind*III sites of the pET28b vector (Novagen), respectively. The resultant plasmids were used to transform *E. coli* BL21(DE3) and transformants were grown at 37 °C in LB medium supplemented with 30 µg/ml kanamycin. The expression of His-tagged recombinant proteins was induced by the addition of 1 mM IPTG. Cells from 1-l culture were harvested and suspended in 22.5 ml of buffer A (50 mM Tris–HCl pH 7.5, 500 mM NaCl, and 20 mM imidazole). After sonication, cleared cell lysates were obtained by ultracentrifugation (100,000×g) at 4 °C for 1 h and incubated with Ni<sup>2+</sup>–NTA agarose (Qiagen) at 4 °C for 1 h. The Ni<sup>2+</sup>–NTA agarose resin was washed with buffer A and then with buffer B (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 20 mM imidazole, Eluted fractions were finally eluted with buffer B containing 200 mM imidazole. Eluted fractions were analyzed by SDS-PAGE and protein concentration was determined by using BCA Protein Assay Kit (Pierce).

For gel mobility-shift assay, the DIG-labeled probe corresponding to sequences of element A was incubated with 50, 75, and 100 ng of purified HMG-12 protein at 25  $^{\circ}$ C for 15 min. The detection of probe-HMG-12 complex was performed as described above.

#### RNA interference (RNAi) and Western blot analysis

A part of *hmg-12* and *car-1* genes was amplified from cDNA clones (yk1230g1 and yk1535g3, respectively) by PCR with primers listed in Table 1, and cloned into the

#### Table 1

Oligonucleotides used in this study

GP conserve for de 43 pho 44 pho 45			
pin-1     PM     CATEGOACHTITECCATEGATICATE       pin-2     PM     CATEGOACHTITECCATEGATICATE       pin-3     PM     CATEGOACHTITECCATEGOTT       pin-3     PM     CATEGOACHTITECCATEGOTT       pin-3     PM     CATEGOACHTITECATEGOTT       pin-3     PM     CATEGOACHTITECAACACHTITEGAACHTITEGAACHTICATE       pin-3     PM     CATEGOACHTITEGAACHACHTITEGAACHTITEGAACHTICATEGOTT       pin-3     PM     CATEGOACHTITEGAACHACHTITEGAACHTICATEGOTT       pin-3     PM     CATEGOACHTITEGAACHACHTITEGAACHTITEGAACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTITEGOACHTICAACHACHTITEGAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTITEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTICATEGOACHTICAACHACHTITEGOACHTICAACHACHTICAA			
pbp:2     PW     CATGOLARATTICLCTICCTC       pb:3     PW     CATGOLARATTICLCA       pb:4     PW     CATGOLARATTICCCA       pb:4     PW     CATGOLARATTICCCA       pb:4     PW     CATGOLARATTICCCA       pb:5     PW     CATGOLARATTICCCA       pb:5     PW     CATGOLARATTICCCA       pb:5     PW     CATGOLARATTICCA       pb:6     PW     CATGOLARATTICCA       pb:7     PW     CATGOLARATTICCA    <	p06	RV	TGCGGGATCCGTAACTGAGATTTGGAGCTGG
pbs3     PW     CCATCCATCATACATACCCACA       pbs4     PW     CCATCCATCATACATACATACATACATACATACATACAT	p06-1	FW	GCATGCAACATTTGCCATTGGATGACC
pbs3     PW     CCATCCATCATACATACCCACA       pbs4     PW     CCATCCATCATACATACATACATACATACATACATACAT	•	FW	GCATGCAGACTATTCTCCTCTCGTCTC
pbs-5     PW     CCNTCCIT/CAMATACCITYCGAMAAC       pbs-5     PW     CCNTCCITYCGAMACC       pbs-7     1     TTAMAACCARACACATTICAAACCAAACCATTICAAACCAACC	•		
jpb:5     IV     CCATCCTTICCGTERATTCGCATAACC       p06-6     IV     CCATCCTTICGGTERATTGCATAACC       p06-7     1     TEDAAAACCATTIGCAACCATTICAACAACC       p06-8     IV     CCATCCTTICACATTICAACCATACTTICAACCAACCATTICACAACCAAC	•		
p66.6     PW     CCATCCITCUTTACAATATT       p67.7     1     CCATCCITCUTTCAAATATT       p66.8     PW     CCATCCITCUTCAATACTTACAATAA       p66.9     PW     CCATCCITCUTCAAATATAACTATAAATAA       p66.9     PW     CCATCCITCUTCAAATATAACTATAAATAAATAAATAAATAAATAAA	•		
pbi-7     1     TETAMAMCGARCAETTEGATAETTACT       pbi-8     NV     CERTAETTACAETTAGAATTACT       pbi-9     NV     CERTAETTACATTACTTACATTACATTACAATTACAACCAACC		FW	CGATGCTTTGCGTACACITGTTGAAAAC
2     AMATECTITICAMACITAGANIA       06-8     PV     ICAMATECTITICAMACITAGACITAGANIA       06-9     PV     CATACICAMACITAGACITAGANIAACCANTICA       06-10     PV     CONTINUAACCANTICACACICAMACICACCANTICAACCANTICA       06-10     PV     CONTINUAACCANTICACACICAMACICACCANTICAACCANTICACACICAMACICACCANTICACACICACACICACAACCACCACCACACACACACACA	p06-6	FW	GCATGCTGTGTCGTTTTTAGAAATAATTT
2     AMATECTITICAMACITAGANIA       06-8     PV     ICAMATECTITICAMACITAGACITAGANIA       06-9     PV     CATACICAMACITAGACITAGANIAACCANTICA       06-10     PV     CONTINUAACCANTICACACICAMACICACCANTICAACCANTICA       06-10     PV     CONTINUAACCANTICACACICAMACICACCANTICAACCANTICACACICAMACICACCANTICACACICACACICACAACCACCACCACACACACACACA	p06-7	1	TCTAAAAACGACACAGTTTGAAAAGGATTTTCACT
p06-8     PV     CGAMARCETTICAAACTAAAACAACC       p16-9     PV     CGATCATTAAAACTAAACCACATTICTAAAACAACCACACCACACACA	F		
No. 1000     NV     CGTCCTTTATATTTACTTACAAACCGTTTCA       p05-9     NV     CGTCTTTAAAACTTCGTAAAACCGATTCAAAACGATTCGTAAAACGATTCGTAAAACGATTCGTAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACAAAACGATTCGTACACAAAACGATTCGTACACAAAACGATTCGTACACAAAACGATTCAAAAACGATTCGCACCACAAAATGAAAACGATTCACACACGATTCACAAAACGATTCACACACGATTCACAAAACGATTCACACACGATTCACAAAATTCAAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAATTCAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAATTCAAAAATCGTACACCACCAAAAAACGATTCACCACCACAAAATTCAAAAATCGTACACCACCACAAAAATCAAAAATCGTACACCACCACAAAAATCAAAAATCGTACACCACCACAAAAACGAAAAACGAAAATTCACAAAAACGAAAATTCACAAAAACGAAAATTCACAAAAATCAAAAAACGAAAATTCACAAAAACGAAAAACGAAAAACGAAAAACGAAAAAACGAAAAAA	-06.9		
p66-9     PV     CANTACCAMACTITUTAAAAASCCC       p06-10     PV     CCCTENTICAAAAASCCTITUGTCAAAACCCCT       p06-10     PV     CCCTENTICAAAAACCCTACTACACCACCT       p06-11     PV     CCCCACCTTUTAAACCTCCTACAACCACCACACACACACA	p06-8		
NV     CCCTCTTTCAAAACTCTCCCTCAAAACTCTTCTCAAAACTCTCCTC		RV	
p66-10     PW     TCGTAPACTTCTGTAMACCAGCATTATATACCAGCAGA       p66-11     PW     CACCACCAGCAGACAGCAGACAGCAGACAGCAGACAGCAG	p06-9	FW	GAATCACCAAACACTTTTTGAACCCACTTGTTGAAAACACGC
p66-10     PW     TCGTAPACTTCTGTAMACCAGCATTATATACCAGCAGA       p66-11     PW     CACCACCAGCAGACAGCAGACAGCAGACAGCAGACAGCAG		RV	GCGTGTTTTCAACAAGTGGGTTCAAAAAGTGTTTGGTGATTC
NV     TICTACATCACCACCATACATCACTURCACTCACCACCA       D6-11     NV     CACCACTCATACATCACCURCACTCACTCACCCACCACATATACAACACCACCACCA       D6-12     NV     CCACACCATTCATACCCCCACCCATATACAACACCACCCACCA	n06-10	FW	TCCCTACACTTCTTCAAAACACAACTCTATATCCCTCCATCAT
p06-11     PW     CCCCCCCCTTCACATCCCCCCCCCCCCCCCCCCCCCCC	00010		
FV     CCACACACATTICANATITICAACCCCATAGA       p06-12     PW       p06-13     1       p06-14     1       p06-15     1       p06-16     2       p06-17     1       p06-18     1       p06-19     2       p06-10     1       p06-11     2       p06-12     1       p06-13     1       p06-14     2       p06-15     1       p06-16     1       p06-17     1       p06-18     1       p06-16     1       p06-17     2       p06-18     1       p06-19     1       p06-18     1       p06-19     1       p06-17     2       p06-18     1       p06-19     1       p06-18     1       p06-19     1       p06-10     1       p06-11     1       p			
p66-12     PW     CCATECECCATECTCAATECAT       p66-13     1     ATACCACATECACTACCTACTACT       p66-14     1     CCACACCATECTCAAATECATT       p66-15     1     CCACACATECTTICAAATECATECACCATECACCATECACATECACCATECAC	p06-11	FW	CACGCTTCTATATGCGTGGCCACTGACAATTTTCAAATCTGTGTCG
p66-13     1     ATTICGACCATACCTIFACATITICATAGATT       p66-14     2     CACATACCATACCATACCATACATTICATAGATT       p66-14     CCTCCAAAATCATTACGATCATACCA       p66-15     1     CCTCCAAAAATCATACCATCCAAACCATTACAC       p66-16     1     CCTCCAAAAATCATTACCAAACCATACCT       p66-17     1     CCTCCAAAAAATCATACCACCAACCACACCCAAACCATTACCAC       p66-17     1     CCTCCAAAAAATCATACCTCCACCATCCACCCCACCCC		RV	CGACACAGATTTGAAAATTGTCAGTGGCCACGCATATAGAAGCGTG
p66-13     1     ATTICGACCATACCTIFACATITICATAGATT       p66-14     2     CACATACCATACCATACCATACATTICATAGATT       p66-14     CCTCCAAAATCATTACGATCATACCA       p66-15     1     CCTCCAAAAATCATACCATCCAAACCATTACAC       p66-16     1     CCTCCAAAAATCATTACCAAACCATACCT       p66-17     1     CCTCCAAAAAATCATACCACCAACCACACCCAAACCATTACCAC       p66-17     1     CCTCCAAAAAATCATACCTCCACCATCCACCCCACCCC	p06-12	FW	GCATGCGGGTGATGCTGCAAATCAT
2     ACAATCAAAATCTTACCAAAATCATTT       96-14     1       96-15     2       96-15     1       96-16     2       96-16     2       96-17     1       96-18     1       96-19     1       96-20     2       96-19     1       96-20     2       96-30     1       96-31     2       96-42     ACCTCATTCACTGACATCACCTATAATCCCC       96-43     1       96-44     2       96-54     1       96-57     2       96-58     1       96-59     1       96-670     1       96-50     1       96-51     2       96-520     1       96-520     1       96-521     2       96-521     1       96-521     2       96-521     2       96-521     2       96-520     1       96-520     1       96-521     2       96-521     2       96-521     2       97     2       97     2       97     2       97     2       97	•		
p06-14     1     CCACACIGNTITICAMANICATTICCACCATCACC       p06-15     1     CCTCACATICATITICACANICATITICACACCATCACC       p06-16     1     CCTCACANICATITITACANIACCITCITITICA       p06-16     1     CCTCACANICATITITACANIACCITCITITICC       p06-17     1     CCTCCANICATITITACANIACCICATICACICACICACICACCACICACCACICACCACCACICACCAC	p00-15		
2     CCTCCAANTATTICAANTAGCTCTCGTATTIAG       p6-15     1     ACTCTATTICAANTAGCTCTCGTATTICAAC       p6-16     1     ATTCTATTICAATAATACGTCTCACC       p6-16     1     ATTCTATCTACTCGCACATCACT       p6-17     1     CCTCCAATATACTCCC       p6-18     1     ACCCTCATTICACCATTACACTCCACTACATACACT       p6-17     1     CAACCCACTTACACTCACATACATACCCC       p6-18     1     TCTCAAAACCGACTACATACACTCCATTACCCC       p6-19     1     ATTCTCACACTCACATCACTACATCCATACACCC       p6-19     1     ATTCTACACTCACACTCAATTCCGTTTTACAAACCGACACTACACTCCACCC       p6-19     2     ATTCTACACTCACACTCACACCCCCATTCTCCCACTCCCCCC			
p66-15 1 CCTCATTGCCAAATCATTTGCCACCATCACC 2 CCTCATTGCCAATCATTTATGCCAATCACTTTATTGCCAACCATCACCACCACCACCACCACCACCACCACCACCA	p06-14		
p66-15 1 CCTCATTGCCAAATCATTTGCCACCATCACC 2 CCTCATTGCCAATCATTTATGCCAATCACTTTATTGCCAACCATCACCACCACCACCACCACCACCACCACCACCA		2	GCTGCAAATCATTTTTACAAATACGTCTCGTTATTTAG
2     GCTGCAATATCACAGTATTATTCC       p66-16     1     ARTGATCTACGCACCACCACCCCCACTTCC       p66-17     2     CAACCTATTCCGTTCCACCTAAACTGCCTTCCCG       p66-18     1     CAACCCACTTACATTCCACCCCACCTAACTTCCACTCCACCGATACATAC	p06-15		
p66-16 ALT	P00 10		
2ACCCTAGICCCACCTACATACATTACCCCp66-171CANACCGACTATACATCCCCCGACTACATACATACTACCACCp66-182ATTTCATCCCCACGACTACAACTCGCACTATACATTCCCACGACTACAACTCGCATTTCAACTCGCATTTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCAACTCGCATTCACCACCACTTCCCACCCA			
p06-171CAAACGCAGTTACACTCCAGTGAAAATATGCAGAAAAATATGAAAAG 2p06-181TTCTAMAAGCCAGAATTTTTCGGTATACTCGATTCAAATGAGT 2p06-191AAATTGCAGTATGCGGTGTGTTGAACTGCGTTTC 2p06-202CATCAGCGTTAGTAAAATGATTTTGCAAATTATTp06-212CATCAGCGTTGGGTGTTTGAAATGATTp06-222CATCAGCGTTGGCAGTTAGTAAATGATTTTGCAAATGATTp06-231CATCAGCGTTGGCAGTTGGCGGTTTGGAAATGAATGATTp06-242TACAAATGCCGTGGCAGCAGTTGGCGGTTp06-212TACAAATGCCGGCAGCAGTGGCGGTp06-211CATCAGCTGTGGCGCTGGCGCAGTGGGCGTp06-212TACAAATGCGGCAGAATGGCAAGTGGGGGTp06-212TACAAATGCGGCAGCAGTGGGCGTp06-212CATCAGCGTGGCGCGCGCGGTGGTGGTGGTGGTGGGGGGTp145TACAAATGCGGCAGTGGTGGTGGGGGGGGGGGGGGGGGG	pu6-16		
2ATTICATCICGACTICGAATCACCGTAACTCCGTTTTCAp06-181TTCTAAAAACCACACACACGTTCGTAACTCCGTTTCAAAAAGp06-191ANATTICAAAAACCACACACACACACACACACACACACACAC		2	ACGCTGATGTGCGTTCCAGCTACATACATTACCGC
2ATTICATACCIGACIGTAACTCCGTTUTCAp06-181TUTAAAAACCACAACATTUTGAAATAATp06-191AAATTACCACAACATTUTGAAATAATTp06-201ATATGCCGTTAGTAAAAAGGATACCACATTUGAAATAATp06-201CCTCCAATCATTUTTACATTACACCGATACCCp06-201CATAGCCGTTAGGATACCATTUTGACATCGp06-201CATAGCCGTTGGATACACCATCGCATCGCTp06-201CATAGCTGTGCATCCAACCCATTUTGACATCGp06-201CATAGCTGTTGCATCCCAACCCGTTGGATCGCGTTp06-211CATAGCTGTTGCATCCCAACCCCATCGTCCATTUTGACTCGp06-211CATAGCTGTTGCATCCCAACCCGATCGTGCCATTUTGCCGTGp06-211CATAGCTGTGCATCCCAACCGCATCGTCCATTUTGCCGTGp06-211CATAGCTGCCACTTGCCAACCGATCGCAACCGATGCGAATCCCAACCGCGTTAp06-211CATAGCTGCAACCGACTGCTCCATTUTGCCATTGCp06-211RVCTCCAAGCAACCGCTGCTCCTTTTGCCGTGp11-1RVCTCCAGCACTGCCACTTGCCAACCACCGTACCAACCAAp11-2RVCTCCACGCACTGCACACTGCGCAACCGCTACCAACCGCTACCAACCGCTACCAACCGCTACCAACCGCTACCAACCGCTACCAACCGCTACCAACCGCTACCAACACCGTACCAACCGCTACCAACACCGTACCAACCGCTACCAACACCGTACCAACCGCACCACCAACCA	p06-17	1	GAAACGCAGTTTACAGTCCAGGATGAAAATATGAAAAG
p66-18     1     TTCTAAAACCGACACTITITICGAAATAACCGACACACTITITICGAAATAACCGACACCGACACCCCCCGACACCCCCCCCCC			
2AMANTEGREGATICAMANAGE TERCENT $p06-19$ 1ATATEGCATAGE TERCENT CEGACE TERCENT CEGACE ACTIVE CARACTER CEGATE CEGATE CARACTER CEGATE CEGATE CARACTER CEGATE CEGATE CARACTER CARACTER CEGATE CEGATE CECATE CEGATE CEG	p06 19		
p66-19     1     ATATECCECTAGIAAAAATCATTECCACCATCACC       p66-20     1     CATCACCCTTTEGAATCATTECCAACCETACC       p66-20     1     CATCACCCTTTEGAATCCCAACCETACCCT       p66-20     2     TACAAAACTECTCCAACCECTATECCT       p66-21     1     CATCACCCTTTEGAATCCCAACCETATECCT       p66-21     2     CATCACCCTTTEGAATCCCAACCECTATECCCATTEGCCT       p66-21     2     CATCATTECCAACCECATTECCCATTEGCCT       p66-21     2     CATCATTECCAACCECATTECCCATCCCCATTEGCCT       p66-21     2     CATCATTECCAACCECATTECCCATTEGCCT       p67     FW     CCAAGCCACTTACCTCACCCCCTCTCCATTEGCCTG       p64     FW     CCAAGCACCACTTECTACACCACCACCACCACCACCACCACCACCACCACCACC	poo-19		
2CCTCCAAATCATTTTTACTAACGCCATATCGTTTCp06-201CATACACCTTTGGCATCTCACACCACACACGATTGGCATCTGCACACCACACCTTGGCATCTGCACACCACACCTTGGCATCTGCACATCGCATTGGCATCTGCACATCGCATTGGCATCTGCACATCGCATTGGCATCTGCACATCGCATTGGCATCTGCACATCGCATTGGCATCTGCACATGGCATTGGCATCTGCACATGGCATTGGCATCTGCACATGGCATTGGCATTGGCACTTGGCACTTGGCATTGGCACTTGGCATTGGCAGTTGGCATTGGCAGTTGGCATTGGCAGTTGGCATTGGCAGTTGGCATTGGCAGTTGGCATTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGCAGTTGGGGGGGG			
p06-20 1 1 CACACCTTCGCCACCCACTTCTACATCG 2 1 CACACTTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	p06-19	1	ATATGCCGTTAGTAAAAAATGATTTGCAGCATCACC
p06-20 1 1 CACACCTTCGCCACCCACTTCTACATCG 2 1 CACACTTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC		2	GCTGCAAATCATTTTTTACTAACGGCATATCGTTTC
2     TACAAAGTCCTCCATCTCCAAACCCTTATCTCCGT       p06-21     1     ACTCTTATTTCCAAATCTCCCAATCTCCCATCTCCCGTTA       p1     RV     CAAGCATTCCAAACCTTATTTCCC       p41     RV     CCCAGCATCCCAACCTCCTCATTTCGCCTG       p41-2     FW     CTCCAGCACTTCCAACACTTCCAATTTCCCAACACA       p41-3     FW     CTCCAACCGCACGTTACACCGCAGAACAA       p41-4     FW     CTCCAACGCACGTTACATCACACAA       p41-5     FW     CTCCAACGCATTCCAACCGTTACACCCC       p41-5     FW     CTCCAACGCATTCCAACGTTCCAACACCC       p41-5     FW     CTCCACACGCTTCCTCAACCCTCACCC       p41-5     FW     CTCCACACCGTTCCTCAACCCTCACCCC       p41-6     FW     CCTACCTCCACGCTTCCTCAACCCTCACACCC       p41-7     FW     CCTACCTCCACCTCCAACCCTCCAACCCCCC       p41-8     FW     CCTACCTCCACCTCCAACCCTCCAACCCCCCCCCCC       p41-9     FW     CCTACCTCCAACCCTCCAACCCTCCAACCCCCCCCCCC	n06-20		CATCAGCGTTTGGGATGCAGCACTTTGTAGATCG
p06-21     1     ACTCITITTCCAATTCCCAATTCCCCAATCCCCTTA       CFP onstructs for cdc-48.2     FW       p41     RV     CCAAGCATTCCCACGTCCTCCATTTTCGCCTG       p41-1     FW     CTGCAAGCATTCCCAGCTCGTCATTTCGCCTG       p41-2     FW     CTGCAGCACGTTAACCAGGATGCAAACAA       p41-3     FW     CTGCAGCACGTTAACCAGGAGGATGCTATACCAGCACA       p41-4     FW     CTGCAGCACGTTAACCAGCGTTGCTAA       p41-5     FW     CTGCAGCAGCGTGCAGACGCTTGCTGAA       p41-4     FW     CTGCAGCAGCGTGCTGCAAGCGTGCAAACGCC       p41-5     FW     CTGCAGCAGCGTGCTGCAAGCGAGA       p41-6     FW     CTGCAGCACGTTGCTGCAGCAGGAGA       p41-7     FW     CTGCAGCACGTTGCTGCAGCGAGA       p41-8     FW     CTGCAGCACGTTGCTGCAGCGAGA       p41-9     FW     CTGCAGCCACGTGCGAGGAGA       p41-9     FW     CTGCAGCCCTTGCTGCAGCGAGA       p41-9     FW     CTGCAGCCCATCGAGCGAGA       p41-9     FW     CTGCAGCCCCCGCGAGAGA       r     FW     CTGCAGCCCCCGCGCGAGAGA       r     FW     CTGCAGCCACTCGCGCGCGCGCGCGAGAGAGAGA       r     FW     CTGCAGCCACTCAGCGCGCGCGCGCGCGCGCGCGCGAGAGAGA	p00 20		
2     CATATCGTTTCGAGAATTGCAAATAACAGTTATTTTTCC       GFP constructs for cdc-48.2     FV       p41     RV     CCAAGGATCCCAGCTCGTCCATTTGGCGTG       p41-1     FW     CTGCAGCAGTTTCATGAGGAAAACAA       p41-2     FW     CTGCAGACGGTGAACGAGCGTTGATGG       p41-3     FW     CTGCAGACGGTGAACGGTTGTAATGGAGAACAA       p41-4     FW     CTGCAGACGGTGAAGCGTTGAATGGAGAGCG       p41-5     FW     CTGCAGGAGGTGTGAATGGAGAGCG       p41-6     FW     CTGCAGGCATCCTCTTCTTTGATTCGATT       HMG-12 overexpression     FW     CCTAGCTCAGACGTGCGAAGGAGGAGAGAGAGA       rRV     CATATGTCGAATCAAACACCCGTAC     FW       rCAR-1 overexpression     FW     CCTGCAGCTCGCGAAGAG       rRV     RV     CATATGTCGAATCAAACACCCGTAC       rGar-1 sexion     FW     CCTGAGTCGAGCGTGCGCGAGA       rrCTAGACTTCAGCCGCGCGG     FW     RV       rCTAGCTTACTCGACGCGGCGCGG     FW     CTCGAGTCAGACGTGCGCGCGGG       rc1 sexion     FW     CTCGAGTCAGACCGTGCGCGGG       rc1 sexion     FW     CTCGAGTCGAGCGGCGCGGGGGGGGGGGGGGGGGGGGGG	00.04		
GPC constructs for cdc-48.2 p41 RV CCAAGGATCCCAGCTCGTCCATTTGCGCCTG p41-1 PW CTGCAGCAGTTTACATGAGAAACAA p41-3 PW CTGCAGCAGTTTACATGAGAGGGGTGTTATCG p41-3 PW CTGCAGACGGTAGAACGGTGTGTATCG p41-4 PW CTGCAGAGAGAGGGTGTATACGAGGC p41-5 PW CTGCAGAGCAGTGTGAATGCAGAGGC p41-5 PW CTGCAGGCAGTCTCTTGTAATGAGGCC p41-5 PW CTGCAGGCAGTCTCTTGTTGAATGAGGCC p41-5 PW CTGCAGGCAGTCTCTGTGAAGAGAG p41-6 PW CTGCAGGCAGTCTCTGTGAAGAGAG p41-7 PW CTGCAGGCAGCTGTGCTGAAGAGAGA p41-7 PW CTGCAGGCAGTCTCTGGTGAAGAGAA p41-7 PW CTGCAGGCAGCTGCGGAAGAGAGA p41-7 PW CTGCAGGCAGCTGCGGAAGAGAA p41-7 PW CTGCAGGCAGCTGCGGAAGAGAA p41-7 PW CTGCAGGCAGCTGCGGAAGAGAA p41-7 PW CTGCAGGCGCGCGGCGGAAGAGAGAA p41-7 PW CTGCAGGCGGCGGGGAAGAGAGAGAGAGAGAGAGAGAGAG	p06-21		
p41RVCCAAGGATCCAAGTTCACATTTTGCCTGp41-1FWCTGCAGCAGTTCACATCAGCAGTGTATACGAAACAAp41-2FWCTGCAGAACGTACAACGCTTTATCGp41-3FWCTGCAGAACGTCTAAACACAAp41-4FWCTGCAGAACGTGTAAACGAAGCCGTTGTGAAp41-5FWCTGCAGACGTGTGATACGAGCGTGAAACGAArMG-12 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACrAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAraft-10 overexpressionFWCTCGAGTCCAACCGTGCGAAGACGAAGAGAraft-10 overexpressionFWCTCGAGTCGAACCGTGCGAAGAGAraft-11 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAAraft-12 RNAiFWCTCGAGTCAGACGTTGCTGAAGAGAGAGAGAGAGAGAGAG		2	CATATCGTTTCGAGAATTGCAAATAACAGTTATTTTTCC
p41RVCCAAGGATCCAAGTTCACATTTTGCCTGp41-1FWCTGCAGCAGTTCACATCAGCAGTGTATACGAAACAAp41-2FWCTGCAGAACGTACAACGCTTTATCGp41-3FWCTGCAGAACGTCTAAACACAAp41-4FWCTGCAGAACGTGTAAACGAAGCCGTTGTGAAp41-5FWCTGCAGACGTGTGATACGAGCGTGAAACGAArMG-12 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACrAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAraft-10 overexpressionFWCTCGAGTCCAACCGTGCGAAGACGAAGAGAraft-10 overexpressionFWCTCGAGTCGAACCGTGCGAAGAGAraft-11 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAAraft-12 RNAiFWCTCGAGTCAGACGTTGCTGAAGAGAGAGAGAGAGAGAGAG			
p41RVCCAAGGATCCAAGTTCACATTTTGCCTGp41-1FWCTGCAGCAGTTCACATCAGCAGTGTATACGAAACAAp41-2FWCTGCAGAACGTACAACGCTTTATCGp41-3FWCTGCAGAACGTCTAAACACAAp41-4FWCTGCAGAACGTGTAAACGAAGCCGTTGTGAAp41-5FWCTGCAGACGTGTGATACGAGCGTGAAACGAArMG-12 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWGCTAGCTCAGACGTTGCTGAAGAGAArAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACrAft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCATATGTCGAATCAAACACCGTACraft-10 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAraft-10 overexpressionFWCTCGAGTCCAACCGTGCGAAGACGAAGAGAraft-10 overexpressionFWCTCGAGTCGAACCGTGCGAAGAGAraft-11 overexpressionFWCTCGAGTCAGACGTTGCTGAAGAGAAraft-12 RNAiFWCTCGAGTCAGACGTTGCTGAAGAGAGAGAGAGAGAGAGAG	GFP constructs for cdc-48.2		
p41-1 p41-2 p41-3FWCTGCAGCCAGTTACCTGAGAGAAACAA PWp41-3 p41-3 p41-4FWCTGCAGAATTACCAGAGCTGTTATCG PWp41-4 p41-5FWCTGCAGAGAGGTGTGAATCGAGAGCC CGAGCACTCTTCTTTGATCGATTHMG-12 overexpression RV c7000000000000000000000000000000000000		DV	CCAACCATCCCACCTCCATTTTCCCCCTC
p41-2 p41-3 p41-3 p41-4FW FW CTGCAGAAGGTTATGGAAGGC CTGCAGAAGGTTATGGAAGGC CTGCAGAAGGTTGTATGGAAGGC p41-5HMG-12 overexpression CAR-1 overexpressionFW RV RVGCTAGCTCAGACGTTGCTGAAGAGAA GGATCCTTAGTCTGAGCGAA GGATCCTTAGTCTGAGCGAAGAA GGATCCTTAGTCTGAGCAATCGAAGAA GGATCCTTAGTCTGGAATCAAACACCGTAC AAGCTTATTGTCGGATCAAACACCGTAC AAGCTTACTTCGAGCGAGCGAGAGAA CGATCTAGTCTGAGCGAGCGAGAGAA CGATCTATTGTCGGAAGAGAA CGATCTAGTCTGAGCGAGCGTTGCTGAAGAGAA CGATCTATTGTCGGAAGAGAAACACCGTAC CGAGTCAAGACGAGGAGAGAA CGATCTAGTCGGAAGAGAAACACCGTGG RVcar-1 RNAi 			
p41-3 p41-4 p41-5FWCTGCAGAGCGTTGGAACGCTTTGTGAA CTGCAGGAGCACTGTGAATGAGAGCC CTGCAGGAGCACTGTGAATGAGAGCC CTGCAGGAGCACTGTGATGAGAGCC CTGCAGGAGCACTGTGATGAGAGCC CTGCAGGAGCACTGTGAATGAGAGCC CGAGCCATCGATGCAAGAGAA GGATCCTTAGTCTGAGCCATCGAAGAGAA CGA71 overexpressionFW RVCCTAGCTCAGCGTTGCTGAAGAGAA GGATCCTTAGTCTGAGCAGTGC AAGCTTATTGTTCGGCAGCTGCGCA-1 overexpression RV RVFW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGGCAGCTGCGhmg-12 RNAi RV RVFW RVCTCGAGTCAGACGTTGCTGAAGAGA AGCTTATTGTTCGGCAGCTGCGcar-1 RNAi element AFW RVCTCGAGTCATCCAACCTAACCGTGG TCTAGACTTCCCGCGTTTCCCTCGTTCCCGTCTCCGTTCCCGTCTACGACGAGAGAGA	•		
p41-4 p41-5FWCTGCAGAGAGAGTGTGAATGAGAGGC CTGCAGCCATCTTCTTTTGATTCGATTHMG-12 overexpressionFW RVGCTAGCTCAGACGTTGCTGAAGAGAA GCATCCTTAGTCTGAGCCATCGAAGAGACAR-1 overexpressionFW RVCATATGTCGAATCAAACACGGTAC AAGCTTATTGTTCGGCAGCGCGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAA AGCTTATTGTTCGGCAGCTTGCTGAAGAGAA TCTAGACTTCAGCGGCAGCTTTCTTCcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAA TCTAGACTTCAGCGGCAGCTTTCCcel mobility-shift assay element AFws SenseAGCTTATTCTCCGCTCTCGTTTCACATCTCTGTTCTCCGTCTATGATCGA CGATCGATAGACGAGAGAGATGTGAAAACGAGAGGAGGAGAGAATAGCTA CGATCGATAGACGAGAGAACGAAGGAAGGAGGAGGAATAGCTA CGATCGATAGACCGAGAGAACGAAGGAGGATGTGCAAACGAAGGAGGAGGAGAATAGCTA GCATCGATCACAAACGCGAGAAGGAACGAAGGAGGAGGAGAATAGCTA Element BSense Sense AGACTATCTCACAAAGTCCTGCAT	p41-2	FW	CTGCAGAATTACCAGAGTGTGTTATCG
p41-5FWCTGCAGGCATCTCTTTTTGATTCGATTHMG-12 overexpressionFW RVGCTAGCTCAGACGTTGCTGAGAGAGA GCATCCTTAGTCTGAGCCATCGAAGAGACAR-1 overexpressionFW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGCAGCGCGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA AGCTTATTGTTCGCGCAGCTGCGcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA CGCAGCTCCATATCCGCGCGcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA TCTAGACTTCAGCGCGCGTTTCCcar-1 RNAiFW RVSensecar-1 RNAiFW RVAGCTTATTCTCCAGCTGCGGAGAGAGAGAGAGAGAGAGAG	p41-3	FW	CTGCAGAACGGTAGAACGCTTTGTGAA
p41-5FWCTGCAGGCATCTCTTTTTGATTCGATTHMG-12 overexpressionFW RVGCTAGCTCAGACGTTGCTGAGAGAGA GCATCCTTAGTCTGAGCCATCGAAGAGACAR-1 overexpressionFW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGCAGCGCGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA AGCTTATTGTTCGCGCAGCTGCGcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA CGCAGCTCCATATCCGCGCGcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAGA TCTAGACTTCAGCGCGCGTTTCCcar-1 RNAiFW RVSensecar-1 RNAiFW RVAGCTTATTCTCCAGCTGCGGAGAGAGAGAGAGAGAGAGAG	n41-4	FW	CTGCAGAGAGAGTGTGAATGAGAGGG
HMG-12 overexpression       FW       GCTAGCTCAGACGTTGCTGAAGAGAA         GAR-1 overexpression       FW       GCTAGCTCAGACCATCGAAGAGAA         CAR-1 overexpression       FW       CATATGTCGAATCAAACACCCGTAC         hmg-12 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGAA         car-1 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGAA         car-1 RNAi       FW       CTCGAGTCACACCGTGCG         car-1 RNAi       FW       CTCGAGTCACACCGTGCGCG         car-1 RNAi       FW       CTCGAGTCACACCGTGCGCGCG         car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGG         car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGG         car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGG         car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGGG         car-1 RNAi       FW       CTCGAGTCACAAGCTAACCGTGGG         car-1 RNAi       FW       CTCTAGTCTCCATCTCCATCCCGTGCTGCGTTCC         car-1 RNAi       FW       CTCTAGTCTCCATCCAAGCTAACCGTGGG         car-1 RNAi       FW       CTCTAGTCTCCATCTCCATCTCCGTGCTGCGTGCG         car-1 RNAi       FW       CTCTAGTCCACGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	*		
FW RVGCTAGCTCAGACGTTGCTGAAGAGAA GGATCCTTAGTCTGAGCCATCGAAGAACAR-1 overexpressionFW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGGCAGCTGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA AGCTTATTGTTCGGCGGCAGTTTCTTCcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAA TCTAGACTCCAGCGCGGGcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATGCGGGGcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTCCCCTCGTTTCCCGTCTCGTTTCCCGTCTATCGATCG	p41-2	1. AA	CIGCAGGCATCICITCITIGATICGATI
FW RVGCTAGCTCAGACGTTGCTGAAGAGAA GGATCCTTAGTCTGAGCCATCGAAGAACAR-1 overexpressionFW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGGCAGCTGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA AGCTTATTGTTCGGCGGCAGTTTCTTCcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGAA TCTAGACTCCAGCGCGGGcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATGCGGGGcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTCCCCTCGTTTCCCGTCTCGTTTCCCGTCTATCGATCG			
RV       GGATCCTTAGTCTGAGCCATCGAAGA         CAR-1 overexpression       FW         FW       CATATGTCGAATCAAACACCGTAC         hmg-12 RNAi       FW         FW       CTCGAGTCAGACGATGCTGCAGAGAGA         rcr1 RNAi       FW         RV       CTCGAGTCAGACGTTGCTGAAGAGAGA         car-1 RNAi       FW         RV       AAGCTTACTTCCAGCGCAGCTTGCTG         Carse Sense       AAGCTTACTTCCCAGCTGCGTTTCCCATCTTGGTTCTCCGTCTATCGATCG	HMG-12 overexpression		
CAR-1 overexpression       FW       CATATGTCGAATCAAACACCGTAC         FW       RV       CATATGTCGGAATCAAACACCGTAC         hmg-12 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGA         car-1 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGAGA         car-1 RNAi       FW       AGCTTACTTCCAAGCTAACCGTGG         car-1 RNAi       FW       AGCTTACTTCCAAGCTAAGCGGAGAGAGAGAGAGAGAGAG		FW	GCTAGCTCAGACGTTGCTGAAGAGAA
CAR-1 overexpression       FW       CATATGTCGAATCAAACACCGTAC         FW       RV       CATATGTCGGAATCAAACACCGTAC         hmg-12 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGA         car-1 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGAGA         car-1 RNAi       FW       AGCTTACTTCCAAGCTAACCGTGG         car-1 RNAi       FW       AGCTTACTTCCAAGCTAAGCGGAGAGAGAGAGAGAGAGAG		RV	GGATCCTTAGTCTGAGCCATCGAAGA
FW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGGCAGCTGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA TCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA TCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTTCAGCGCGCAGTTTCCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTTCCATGCGGCGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCCGTCTACGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCGGGGTTTCCcar-1 RNAiFW RVGTCGATCGATAGACGGGGGGAACCGAAGAGGGGGGGGGG			
FW RVCATATGTCGAATCAAACACCGTAC AAGCTTATTGTTCGGCAGCTGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA TCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA TCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTTCAGCGCGCAGTTTCCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTTCCATGCGGCGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCCGTCTACGTGG TCTAGACTCCATATCCGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTGCGGTTTCCcar-1 RNAiFW RVAAGCTTACTTCCAAGCGGGGTTTCCcar-1 RNAiFW RVGTCGATCGATAGACGGGGGGAACCGAAGAGGGGGGGGGG	CAP 1 outproversion		
RVAAGCTTATTGTCGGCAGCTGCGhmg-12 RNAiFW RVCTCGAGTCAGACGTTGCTGAAGAGA TCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGGTTTCCGel mobility-shift assay element AFW RVAAGCTTACTTCCCAGCGTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCFelment BSense Anti-senseAGACTATTCTCCTCTGCGTCTGCATTGAAACGAGAGAGAG	CAR-1 Overexpression		
hmg-12 RNAi       FW       CTCGAGTCAGACGTTGCTGAAGAGA         FW       RV       CTCGAGTCAGACGTTGCTGAAGAGA         car-1 RNAi       FW       AAGCTTACTTCCAGCGGCAGTTTTCTTC         car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGG         Gel mobility-shift assay       FW       CTCGAGTCAGACCTTACTCCAGCTTACCGTGGG         Gel mobility-shift assay       Sense       AGACTATTCTCCTCTCGTCTCGTTTCCACATCTCTTCGGTCTCACGATCGGAGAGAGA		FW	
FW     CTCGAGTCAGACGTTGCTGAAGAGA       RV     TCTAGACTTCAGCGGCAGTTTTCTTC       car-1 RNAi     FW       FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     AAGCTTACTTCCAAGCTAACCGTGG       Gel mobility-shift assay     rcTAGACTCCATATTCCGCGGTTTCC       element A     Sense     AGACTATTCTCCTCGTCTCGTTTCACATCTCTTCGGTCTATCGATCG		RV	AAGCITATTGTTCGGCAGCTGCG
FW     CTCGAGTCAGACGTTGCTGAAGAGA       RV     TCTAGACTTCAGCGGCAGTTTTCTTC       car-1 RNAi     FW       FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     AAGCTTACTTCCAAGCTAACCGTGG       Gel mobility-shift assay     rcTAGACTCCATATTCCGCGGTTTCC       element A     Sense     AGACTATTCTCCTCGTCTCGTTTCACATCTCTTCGGTCTATCGATCG			
FW     CTCGAGTCAGACGTTGCTGAAGAGA       RV     TCTAGACTTCAGCGGCAGTTTTCTTC       car-1 RNAi     FW       FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     AAGCTTACTTCCAAGCTAACCGTGG       Gel mobility-shift assay     rcTAGACTCCATATTCCGCGGTTTCC       element A     Sense     AGACTATTCTCCTCGTCTCGTTTCACATCTCTTCGGTCTATCGATCG	hmo-12 RNAi		
RVTCTAGACTTCAGCGGCAGTTTTCTTCcar-1 RNAiFW RVAAGCTTACTTCCAAGCTAACCGTGG TCTAGACTCCATATCCGCGGTTTCCGel mobility-shift assay element ASense AGACTATTCTCCTCTGTCTCGTTTCACATCTCTTCGTTCTCCGTCTATCGATCG	1116 12 1011		
car-1 RNAi       FW       AAGCTTACTTCCAAGCTAACCGTGG         FW       RV       TCTAGACTCCATATCCGCGGTTTCC         Gel mobility-shift assay       Element A       Sense         Anti-sense       AGACTATTCTCCTCTCGTCTCCGTTTCACATCTCTTCGTTCTCCGTCTATCGATCG			
FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     TCTAGACTCCATATCCGCGGTTTCC       Gel mobility-shift assay     F       element A     Sense       Anti-sense     AGACTATTCCCTCGTCGCGTTTCACATCTCTTCGGTCTCGCGTCTATCGATCG		RV	TCIAGACTTCAGCGGCAGTTTTCTTC
FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     TCTAGACTCCATATCCGCGGTTTCC       Gel mobility-shift assay     F       element A     Sense       Anti-sense     AGACTATTCCCTCGTCGCGTTTCACATCTCTTCGGTCTCGCGTCTATCGATCG			
FW     AAGCTTACTTCCAAGCTAACCGTGG       RV     TCTAGACTCCATATCCGCGGTTTCC       Gel mobility-shift assay     F       element A     Sense       Anti-sense     AGACTATTCCCTCGTCGCGTTTCACATCTCTTCGGTCTCGCGTCTATCGATCG	car-1 RNAi		
RV     TCTAGACTCCATATCCGCGGTTTCC       Gel mobility-shift assay element A     Sense       Anti-sense     AGACTATTCTCCTCTGGTCTCGCTTTCACATCTCTTCGGTCTCGGCAGAGAGAG		FW	AAGCTTACTTCCAAGCTAACCGTGG
Gel mobility-shift assay       element A       Sense     AGACTATTCTCCTCTGGTCTCGCTTTCACATCTCTCGGTCTCGCTTATCGATCG			
element A Sense AGACTATTCTCCTCTGTCTCGTTTCACATCTCTCGTCTCGTCTCGTCTCGTCTCGACAGAGAGAG		ICV	
element A Sense AGACTATTCTCCTCTGTCTCGTTTCACATCTCTCGTCTCGTCTCGTCTCGTCTCGACAGAGAGAG			
Sense     AGACTATTCTCCTCTGCTCTCGTTTCACATCTCTCGTCTCGTCTCGATCGA	Gel mobility-shift assay		
Sense     AGACTATTCTCCTCTGCTCTCGTTTCACATCTCTCGTCTCGTCTCGATCGA	element A		
Anti-sense     CGATCGATAGACGAGAAGAAGAGAGAGAGAGAGAGAGAGA		Sense	AGACTATTCTCCTCTCGTCTCGTTTCACATCTCTTCGTTCTCCGTCTATCGATCG
Element B Sense TATCGATCTACAAAGTGCTGCAT			
Sense TATCGATCTACAAAGTGCTGCAT		Allu-sellse	CONTCONTROACCORACOAACAACAACATOTOAAACCACACACACACACACAACAACAACAACAACAACAA
	Element B		
Anti-sense ATGCAGCACTTTGTAGATCGATA		Sense	TATCGATCTACAAAGTGCTGCAT
		Anti-sense	ATGCAGCACTTTGTAGATCGATA

LITMUS 28 vector (New England Biolabs). Using the resultant plasmids as template, we amplified DNA fragments by PCR with the T7 primer. The PCR products were used as templates for *in vitro* transcription by T7 RNA polymerase (TaKaRa). To knock-down *hmg-12* and *car-1* expression, we took the soaking RNAi method (Maeda et al., 2001). Briefly, 100 to 200 L1 larvae were soaked in 12  $\mu$ l of 1.25 × M9 buffer containing 15 mM

spermidine, 0.25% gelatin and  $\sim\!8\,\mu g$  of dsRNA, for 24–48 h. RNAi-treated L1 larvae were then grown into adult on seeded plates.

Lysates of embryos from *hmg-12(RNAi)* or *car-1(RNAi)* worms were prepared. Concentration of lysates was determined by DC Protein Assay kit (Bio-Rad). Western blot analysis was carried out as described previously (Yamauchi et al., 2006).

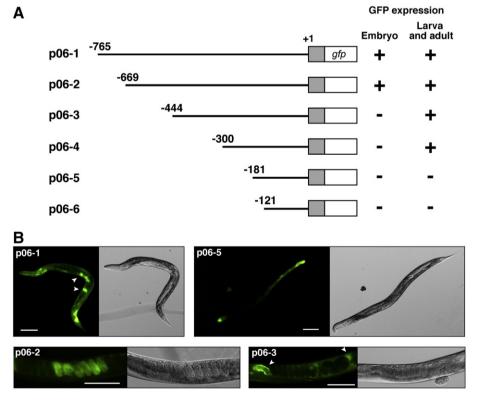
#### Promoter structure of two p97 genes of C. elegans

C. elegans possesses two genes for p97, named cdc-48.1 and cdc-48.2, whose expression patterns and protein levels were differentially regulated (Yamanaka et al., 2004; Yamauchi et al., 2006). To investigate molecular mechanisms of their expression regulation, we first injected the initial construct, p06-1, containing 765 bp upstream of the translational start site of cdc-48.1 in translational fusion to the reporter GFP (Fig. 1A), and analyzed transgenic worms. As shown in Fig. 1B, CDC-48.1::GFP derived from p06-1 was ubiquitously expressed, predominantly in spermathecae at adult stage and in embryos. We then prepared serial deletions of the cdc-48.1 promoter and analyzed expression pattern of GFP (Fig. 1). Pattern of GFP signal from p06-2 was essentially the same as that from p06-1, indicating that the region upstream of -669 bp is not necessary for a normal cdc-48.1 expression. When deleted up to the position -444 bp (p06-3), GFP expression in embryos, but not in larvae and adult, was abolished. This result suggests that a cis-regulatory element for the expression in embryos is located between -669 bp and -444 bp in the *cdc*-48.1 promoter region. When further deleted up to the position -181 bp (p06-5), GFP expression in larvae and adult was also abolished, suggesting that another cis-regulatory element is located between -300 bp and -181 bp. It should be noted that the GFP expression in posterior gut cells from p06-5 (Fig. 1B) and p06-6 (data not shown) is due to unc-54 3'-UTR sequence contained in pPD95.77 vector (Boulin et al., 2006). When deleted up to the position -54 bp, GFP expression in posterior gut cells was no more observed (data not shown). In addition, we have previously shown that cdc-48.1 transcripts were trans-spliced to SL1 at - 19 bp position (Yamauchi et al., 2006). Thus, these results suggest that the region from -121 bp to -54 bp of cdc-48.1 contains a minimal promoter sequence. It is interesting to mention that this region is highly A+T rich (75%), and that one TATA sequence is found at -67 bp to -64 bp.

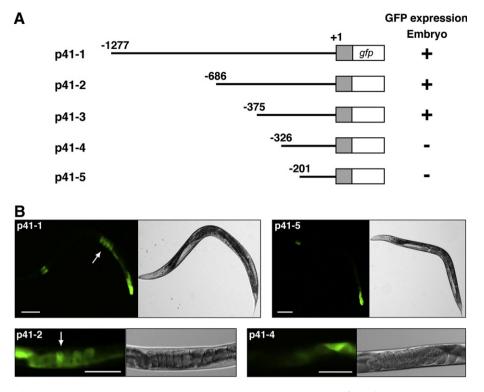
We next analyzed the expression pattern of *cdc*-48.2 in the same way as for *cdc*-48.1. In the initial construct, p41-1, containing 1277 bp upstream of the translational start site of cdc-48.2, CDC-48.2: GFP was expressed mainly in embryos (Fig. 2). Although the deletion of -1277 bp to -375 bp region did not affect GFP expression, deletion of -375 bp to -326 bp region led to clearly abolish GFP expression in embryos (p41-4 in Fig. 2). These results suggest that a *cis*-regulatory element for the expression in embryos is located between - 375 bp and -326 bp in the cdc-48.2 promoter. Again, when deleted up to the position -75 bp, GFP expression in posterior gut cells was no more observed (data not shown). We have previously shown that cdc-48.2 transcripts were trans-spliced to SL1 at -17 bp position (Yamauchi et al., 2006). These results thus suggest that a minimal promoter of cdc-48.2 exists between -200 bp and -75 bp, in which one TATA sequence is found at -155 bp to -152 bp. Taken together, promoter structure of two p97 genes of *C. elegans* seems to be quite different.

### Three conserved sequences are essential for cdc-48.1 expression at post-embryonic stages

One strategy to identify the elements regulating expression of *C. elegans* genes is to compare the promoter sequence of *C. elegans* with the homologous sequence in the related-nematode *C. briggsae*. It is known that intergenic and intronic sequences between these nematodes have diverged considerably, whereas both sequences and functions of the regulatory elements are common to these two species (Gilleard et al., 1997). In order to identify regulatory elements, we performed a dot matrix comparison (CLC DNA Workbench 3) on the promoter sequences of two p97 genes in both nematode species. We clearly found a conserved block of sequences from -256 bp to -122 bp in the *cdc-48.1* promoter (Fig. 3A), while no conserved block of sequences of sequences of sequences of the sequences of sequences from -256 bp to -122 bp in the *cdc-48.1* promoter (Fig. 3A), while no conserved block of sequences of sequences of sequences of sequences from -256 bp to -122 bp in the *cdc-48.1* promoter (Fig. 3A).



**Fig. 1.** Deletion analysis of the *cdc-48.1* promoter region. (A) GFP reporter constructs in which decreasing regions of the *cdc-48.1* promoter were translationally fused to GFP are shown schematically. (B) Each construct was microinjected into *C. elegans* and transgenic worm lines were observed under the microscope. Photos are in pairs; the right panel shows the Nomarski optics view and left panel shows the fluorescence view. The spermathecae expressing GFP are indicated by white arrowheads. Scale bar, 100 µm. Expression patterns are also summarized in panel A. When GFP expression was positive or negative, represented by + or –, respectively.



**Fig. 2.** Deletion analysis of the *cdc-48.2* promoter region. (A) GFP reporter constructs in which decreasing regions of the *cdc-48.2* promoter were translationally fused to GFP are shown schematically. (B) Each construct was microinjected into *C. elegans* and transgenic worm lines were observed under the microscope. Photos are in pairs; the right panel shows the Nomarski optics view and left panel shows the fluorescence view. GFP expressing embryos in uterus are indicated by white arrows. Scale bar, 100 μm. Expression patterns are also summarized in panel A. When GFP expression was positive or negative, represented by + or –, respectively.

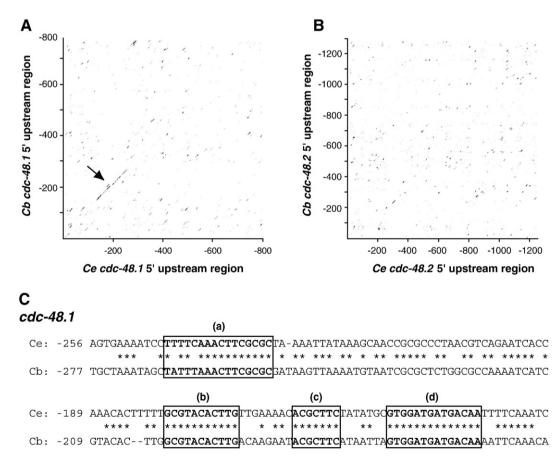


Fig. 3. cdc-48.1 promoter contains conserved blocks of sequences. (A) Dot matrix comparison of 765 bp of the 5' upstream regions of cdc-48.1 in C. elegans and C. briggsae. A conserved region is indicated by an arrow. (B) Dot matrix comparison of 1277 bp of the 5' upstream regions of cdc-48.2 in C. elegans and C. briggsae. (C) Sequence alignment of the conserved region of cdc-48.1 in C. elegans (Ce) and C. briggsae (Cb). Asterisks indicate nucleotide identity in C. elegans and C. briggsae. Four well-conserved boxes (a–d) are in bold face and boxed.

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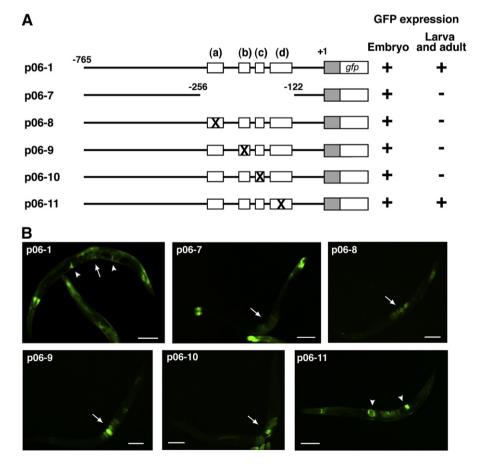
quences was found in the cdc-48.2 promoter (Fig. 3B). Sequence alignment of the conserved block is shown in Fig. 3C. In this region, four distinct, well-conserved sequences (Box a to d) were identified. Then, we analyzed whether this conserved block plays an active role in cdc-48.1 expression at post-embryonic stages. When the entire region of this conserved block was deleted (p06-7), GFP expression at larval and adult stages, but not in embryos, was abolished (Fig. 4). When deleted up to -181 bp (p06-5), no specialized GFP expression was observed as shown in Fig. 1. Furthermore, introduction of point mutations at Box a (p06-8) caused a loss of GFP expression at larval and adult stages, indicating that Box a in the conserved block plays an important role in expression at post-embryonic stages. Besides Box a, there can be identified three additional, highly conserved boxes (Box b, Box c and Box d) (Fig. 3C). When Box b or Box c was mutated (p06-9 or p06-10), GFP was no longer expressed at larval and adult stages as same as observed with p06-8 (Fig. 4). These results indicate that conserved boxes, Box a, Box b, and Box c, are essential for cdc-48.1 expression at post-embryonic stages. In contrast, the p06-11 construct in which the conserved box, Box d, was mutated showed the GFP expression normally, implying that Box d is unnecessary for cdc-48.1 expression at post-embryonic stages (Fig. 4).

## Two regulatory elements are necessary for cdc-48.1 expression in embryos

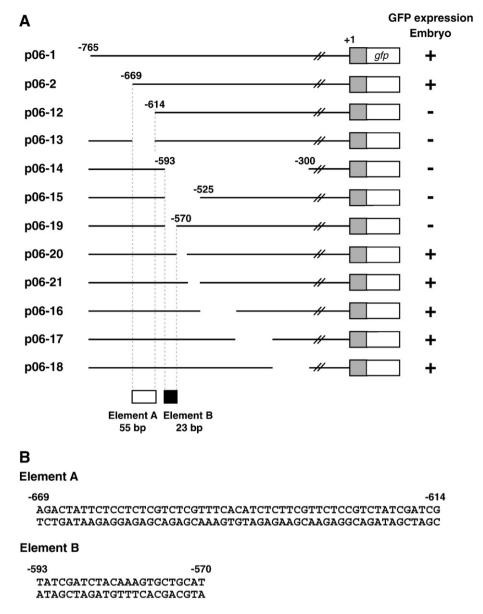
We demonstrated that regulatory elements for *cdc*-48.1 expression in embryos are located between –669 bp and –444 bp of the *cdc*-48.1 promoter (p06-2 and -3 in Fig. 1A). However, no conserved block of sequences was found in this region between *C. elegans* and *C. briggsae*  by means of a dot matrix comparison (Fig. 3A). To identify functional elements, we analyzed reporter constructs sequentially deleted from -669 bp to -444 bp more precisely. When the -669 bp to -614 bp region was deleted, GFP expression in embryos was completely abolished (p06-12 and p06-13 in Fig. 5A). Interestingly, GFP expression in embryos was also lost in worms injected with p06-14 that contains the -765 bp to -593 bp region, indicating the 55-bp region (-669 bp to -614 bp) is necessary but not sufficient for cdc-48.1 expression in embryos. To search for an additional element, we next analyzed reporter constructs sequentially deleted downstream region from -614 bp. We found that deletion of the region from -593 bp to -525 bp caused a loss of GFP expression in embryos (p06-15 in Fig. 5). Analysis of smaller internal deletions in the -593 bp to -525 bp region showed that the 23-bp region (from -593 bp to -570 bp) is additionally required for cdc-48.1 expression in embryos (p06-19, p06-20 and p06-21 in Fig. 5A). Other deletions (p06-16, p06-17 and p06-18) did not affect the GFP expression in embryos. Taken together, two regulatory regions were identified to control embryonic expression of cdc-48.1, namely one is the 55-bp region (-669 bp to -614 bp) and the other is the 23-bp region (-593 bp to -570 bp). We hereafter refer to the 55-bp region and the 23-bp region as Element A and Element B, respectively (Fig. 5B).

#### Identification of proteins that bind to Element A and Element B

In an initial attempt to identify transcription factors that contribute to *cdc-48.1* expression in embryos, we examined whether *C. elegans* embryonic nuclear extracts contain proteins that bind to Element A and Element B. Gel mobility-shift assays were conducted using DIG-



**Fig. 4.** Mutational analysis of the regions necessary for post-embryonic expression in the *cdc-48.1* promoter. (A) GFP reporter constructs in which mutated *cdc-48.1* promoter was translationally fused to GFP are shown schematically. Four boxes represent well-conserved boxes of the *cdc-48.1* promoter between *C. elegans* and *C. briggsae*. X indicates mutated site. + and – indicate that GEP expression was and was not seen. (B) Each construct was microinjected into *C. elegans* and transgenic worm lines were observed under the fluorescent microscope. GFP-expressing spermathecae and embryos are indicated by white arrowheads and arrows, respectively. Scale bar, 100 µm.

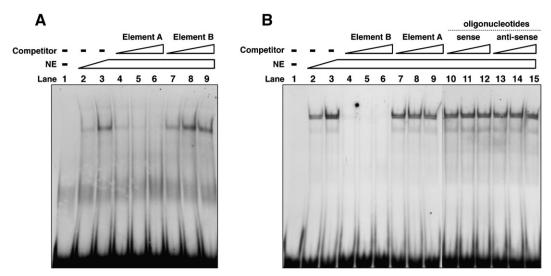


**Fig. 5.** Identification of two elements involved in embryonic expression of *cdc-48.1*. (A) GFP reporter constructs in which decreasing regions of the *cdc-48.1* promoter were translationally fused to GFP are shown schematically. Each construct was microinjected into *C. elegans* and transgenic worm lines were observed under the fluorescent microscope. + and – indicate that GEP expression was and was not seen. Two elements essential for the *cdc-48.1* expression in embryo are shown as a white box (Element A) and a black box (Element B) and their sequences are also shown (B).

labeled, double-stranded oligonucleotides containing Element A or Element B sequence as probes. Single shifted band was detected with each regulatory element (lanes 2–3 in Figs. 6A, B). To verify their binding specificities to each element, we performed the competition assays. Unlabeled oligonucleotides with the identical sequences were used as specific competitors. Both regulatory elements, binding activities were significantly reduced by co-incubation with the specific competitors (lanes 4–6 in Figs. 6A, B). Co-incubation with unlabeled oligonucleotides with non-related sequences did not affect band shift (lanes 7–9 in Figs. 6A, B). These results suggest the existence of two distinct proteins that specifically bind to Element A and Element B of the *cdc-48.1* promoter.

To identify the proteins that bind to Element A and Element B from *C. elegans* embryonic nuclear extracts, we resolved embryonic nuclear extracts by 2D gel electrophoresis, and performed South-Western blot analyses with DIG-labeled probes. Both probes hybridized to the spots resolved in the basic region: the hybridized spot with Element A, apparent molecular size and pl were 46 kDa and 9.5; the hybridized

spots with Element B, apparent molecular size and pI were 42 kDa and 9.0, 42 kDa and 8.5, and 42 kDa and 8.3, respectively (Fig. 7). Hybridized spots were excised from the gels and trypsinized, and the resulting peptides were analyzed by mass spectrometry using a MALDI-TOF on the basis of peptide mass matching. As a result, we found that proteins bound to Element A and Element B were HMG-12 (Y17G7A.1) and CAR-1 (Y18D10A.17), respectively. Note that three spots with same molecular size but with different pI detected with the Element B probe were all identified as CAR-1 (Fig. 7B), suggesting that CAR-1 has some modification, although we have not defined the type of modification. In WormBase (http://www.wormbase.org), HMG-12 is defined as a high mobility group protein, contains DNA binding domain called AT-hook motif, and has calculated molecular size 33 kDa and pI 9.64, while CAR-1 is defined as a cytokinesis, apoptosis, RNA-associated protein, and has calculated molecular size 38 kDa and pI 9.46. It should be mentioned that, although apparent pIs of both proteins are consistent with calculated ones, their apparent molecular sizes seem to be larger than calculated ones (see below).

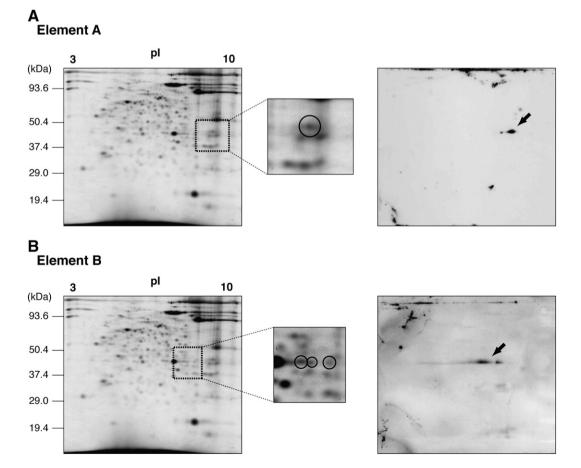


**Fig. 6.** Detection of factors that specifically bind to Element A and Element B. Gel mobility-shift assays were performed using embryonic nuclear extract and DIG-labeled probes containing Element A (A) and Element B (B) sequences. Lane 1: probe only (1 ng), 6.6 µg (lanes 2) and 11 µg (lanes 3–9) of embryonic nuclear extract were added. Competitors were co-incubated in lanes 4–15. Co-incubated competitors are: lanes 4–6, unlabeled dsDNA with sequences of Element A (A) and Element B (B) as a sequence-specific competitor, lanes 7–9, unlabeled dsDNA with sequences of Element A (A) and Element B (B) and Element B (B) as a nonspecific competitor, lanes 10–12 unlabeled sense-oligonucleotide with sequences of Element B (B), lanes 13–15 unlabeled anti-sense-oligonucleotide with sequences of Element B (B). Twenty-five-times (lanes 4, 7, 10 and 13), fifty-times (lanes 5, 8, 11 and 14) and hundred-times (lanes 6, 9, 12 and 15) of competitors were used.

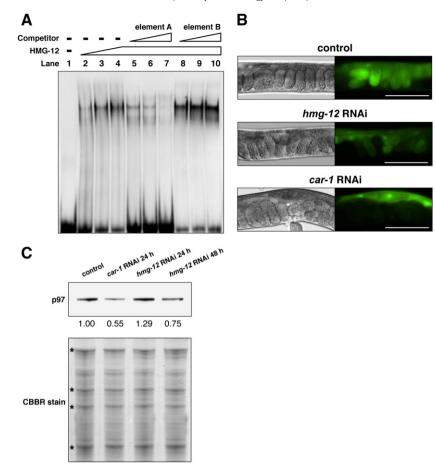
#### HMG-12 and CAR-1 are involved in embryonic expression of cdc-48.1

We then investigated whether both HMG-12 and CAR-1 regulate *cdc-48.1* expression in embryos. First, to examine whether these

proteins are indeed able to bind to each element *in vitro*, we performed gel mobility-shift assays using DIG-labeled probes and bacterially expressed recombinant proteins. Bacterially expressed recombinant HMG-12 and CAR-1 migrated on the SDS-PAGE gel at 48 kDa and 45 kDa,



**Fig. 7.** Identification of proteins that bind to Element A and Element B. South-Western blot analyses were carried out using embryonic nuclear extract and DIG-labeled probes containing Element A (A) and Element B (B) sequences. Left panels show the results of 2D gel electrophoresis of embryonic nuclear extract of *C. elegans*, and right panels show the results of South-Western blot analyses are shown at middle panels.



**Fig. 8.** Both HMG-12 and CAR-1 are involved in embryonic expression of *cdc-48.*1. (A) HMG-12 specifically binds to Element A. A gel mobility-shift assay was performed using the recombinant HMG-12 protein and the DIG-labeled probe containing Element A sequence. Lane 1: probe only (1 ng), 50 ng (lanes 2), 75 ng (lane 3) and 100 ng (lanes 4–10) of purified HMG-12 were added. Competitors were co-incubated in lanes 5–10. Co-incubated competitors are unlabeled dsDNA with sequences of Element A as a sequence-specific competitor (lanes 5–7), and unlabeled dsDNA with sequences of Element B as a nonspecific competitor (lanes 8–10). Twenty-five-times (lanes 5 and 8), fifty-times (lanes 6 and 9) and hundred-times (lanes 7 and 10) of competitors were used. (B and C) Effects of RNAi of *hmg-12* and *car-1* on embryonic expression of *cdc-48.*1 were analyzed. Transgenic worms microinjected with p06-7 (Fig. 4A) were used. The soaking RNAi method was applied. L1 larvae were soaked in M9 buffer containing dsRNA for 24–48 h. RNAi-treated L1 larvae were then grown into adult on seeded plates and observed under the microscope. (B) Photos are in pairs; the left panel and the right panel show the Nomarski optics view and the fluorescence view, respectively. Upper panel shows a control worm immersed in water, middle panel a worm of *hmg-12(RNAi)*, and lower panel a worm of *car-1(RNAi)*. Scale bar, 100  $\mu$ m. (C) Embryos were prepared from RNAi-treated worms and the amount of p97 in embryos were analyzed by Western blotting. The same amount of total proteins was resolved in each lane (1.5  $\mu$ g for CBBR staining). Four protein bands with asterisks on the CBBR stained gel were quantified and used as a loading control. Relative amounts of p97 between control and RNAi samples are also shown.

respectively (data not shown), which are consistent with apparent molecular size on the 2D gels (Fig. 7), supporting that identified spots by South-Western blot are HMG-12 and CAR-1. We found specific binding of purified HMG-12 to Element A but not to Element B *in vitro* (Fig. 8A), and the band shift showed the same pattern as the embryonic nuclear extracts were used (Figs. 6A and 8A). Unfortunately, we were not able to test the CAR-1 binding to Element B in gel mobility-shift assay because CAR-1 has not been recovered in a soluble form so far.

Second, to analyze the function of HMG-12 and CAR-1 on the expression of *cdc-48.1 in vivo*, we knocked down *hmg-12* and *car-1* expression on the worms harboring p06-7, which contains the *cdc-48.1* promoter region only involved in embryonic expression (Fig. 4A), by means of soaking RNAi method. When the worms were treated with *hmg-12* RNAi, GFP expression in embryos was only slightly decreased (Fig. 8B). However, the level of p97 proteins in embryos prepared from *hmg-12(RNAi)* worms was significantly reduced (Fig. 8C). In the case of *car-1(RNAi)*, RNAi effects were more prominent. GFP expression in embryos was also significantly reduced in embryos prepared from *car-1(RNAi)* worms (Fig. 8C). Taken together, these results indicate that both HMG-12 and CAR-1 play important roles in embryonic expression of *cdc-48.1*.

#### Discussion

C. elegans uniquely possesses two p97 homologues, and their expression patterns and levels were differently regulated: CDC-48.1 is ubiquitously expressed through all developmental stages, while CDC-48.2 is mainly expressed at embryonic stage. The amount of CDC-48.1 is almost double to that of CDC-48.2 (Yamanaka et al., 2004; Yamauchi et al., 2006). In this study, we analyzed promoter regions of C. elegans cdc-48.1 and cdc-48.2 genes to understand molecular mechanisms of differential expression of two p97 homologues. Mutation analysis of their promoter regions revealed that the promoter of cdc-48.1 contains two regions necessary for embryonic and post-embryonic expression, while the promoter of cdc-48.2 contains the single region necessary for embryonic expression (Figs. 1-5). Sequence comparison between the region necessary for embryonic expression of cdc-48.1 and cdc-48.2 did not show any significant homology (data not shown). These results suggest that differential expression patterns of two p97 proteins in C. elegans are dependent on each promoter. Mutations in the region necessary for embryonic expression of cdc-48.1 caused a loss of embryonic expression but not of post-embryonic expression (Figs. 1 and 5). Conversely, mutations in the region necessary for post-embryonic expression caused a loss of post-embryonic expression but not

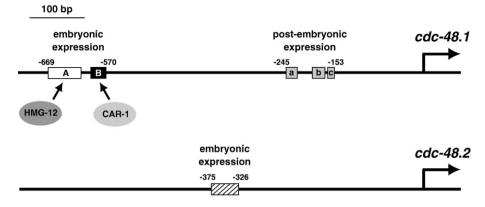


Fig. 9. Promoter structures of two p97 genes of *C. elegans*. The *cdc*-48.1 promoter contains two modules: one is essential for embryonic expression [Element A (white box) and Element B (black box)] and the other for post-embryonic expression [Box a, Box b and Box c (gray boxes)]. HMG-12 and CAR-1 would bind to Element A and Element B, respectively, and play a crucial role for embryonic expression of *cdc*-48.1. The *cdc*-48.2 promoter contains the region only involved in embryonic expression (hatched box).

of embryonic expression (Figs. 1 and 4). These results suggest that two *cis*-regulatory elements in the *cdc-48.1* promoter region function independently of each other.

Comparison of the promoter sequences between C. elegans and C. briggsae has been shown to be a powerful tool to identify elements regulating gene expression (Hwang and Lee, 2003; Hong et al., 2004; Zhao et al., 2005; Wagmaister et al., 2006). Dot matrix comparison of the promoter sequences of C. elegans and C. briggsae revealed a conserved block of sequences in the cdc-48.1 promoter, which corresponds to the region necessary for post-embryonic expression in the present study (Fig. 3). This suggests that the regulatory mechanism for postembryonic expression of cdc-48.1 would be well conserved in C. elegans and C. briggsae. A series of mutation analysis of this conserved block further defined that three well-conserved boxes, Box a, Box b, and Box c, play an important role in post-embryonic expression (Figs. 3 and 4). We have previously demonstrated that CDC-48.1: GFP was expressed ubiquitously, including several head neurons, ventral cord, vulval muscles, and spermathecae, at larval and adult stages (p06-1 in Fig. 1B; Yamanaka et al., 2004; Yamauchi et al., 2006). When even one of the three well-conserved boxes was mutated, GFP expression was abolished in all tissues, except for embryos in uterus (p06-8, p06-9 and p06-10 in Fig. 4B). These results implicated that these boxes control cdc-48.1 expression in a developmental stage specific, but not tissue specific manner. No band shift was observed in gel mobility-shift assays using embryonic nuclear extracts and probes with identical sequences with these well-conserved boxes (data not shown), suggesting that regulatory factors involved in post-embryonic expression of cdc-48.1 are mainly expressed at larval and adult stages. On the other hand, no conserved block of sequences was detected in the regions for embryonic expression of cdc-48.1 and cdc-48.2 genes by means of dot matrix comparison between C. elegans and C. briggsae (Fig. 3).

Detailed deletion analysis of the region necessary for embryonic expression in the *cdc-48.1* promoter revealed that two *cis*-regulatory elements, Element A and Element B are essential for *cdc-48.1* expression in embryos (Fig. 5). By using South-Western blotting and MALDI-TOF MS analysis, we identified HMG-12 and CAR-1 as proteins that bind to Element A and Element B, respectively (Fig. 7). HMG-12 of *C. elegans* is defined as a high mobility group (HMG) protein and belongs to HMGI/Y families (referred as HMGA) (Reeves and Beckerbauer, 2001; Okkema and Krause, 2005). In mammals, HMGA proteins bind to the minor groove of stretches of AT-rich DNA via the AT hook motif, and facilitate recruitment of other transcription factors to the enhancer (Reeves and Beckerbauer, 2001). *C. elegans* HMG-12 is also predicted to contain seven AT hook motifs in WormBase (http://www.wormbase.org). *hmg-12(RNAi)* worms show embryonic lethal phenotype (http://www.wormbase.org), suggesting that HMG-12

might be expressed in embryo similarly to CDC-48.1. Purified HMG-12 bound to double-stranded Element A, but not to non-related sequence, such as Element B (Fig. 8). Thus, HMG-12 protein specifically binds to Element A and may recruit other transcription factor (s) or may induce structural changes in chromatin to activate the *cdc*-48.1 expression in embryos. A slight but significant reduction of the levels of exogenous CDC-48.1: GFP expression and endogenous p97 proteins was indeed observed in embryos prepared from *hmg-12* (*RNAi*) worms (Figs. 8B, C), implying an important role of HMG-12 in the *cdc-48.1* expression in embryos. It should be noted that the genome of *C. elegans* has nine genes that encode putative HMG proteins including two HMGA family proteins, HMG-11 and HMG-12 (Okkema and Krause, 2005). Therefore, it is likely that other HMG proteins of *C. elegans* may compensate the *cdc-48.1* expression in *hmg-12(RNAi)* embryos to some extent.

Another protein CAR-1 was identified as a protein that binds to Element B (Fig. 7B). CAR-1 has been named for cytokinesis, apoptosis and RNA (Audhya et al., 2005). CAR-1 contains several clustered RGG motifs suggestive of an RGG box, which is found in numerous RNAbinding proteins (Burd and Dreyfuss, 1994), and belongs to a family of novel Sm-like proteins, which associate with RNA-binding proteins (Albrecht and Lengauer, 2004; Audhya et al., 2005). Therefore, CAR-1 has been implicated in RNA metabolism. In this study, by means of gel mobility-shift assays, binding activities of the protein in embryonic nuclear extracts to Element B were not changed in co-incubation with sense and anti-sense, single-stranded oligonucleotides of sequences of Element B as a competitor (Fig. 6B). This suggests that the protein binding to Element B specifically recognizes double-stranded DNA, but not single-stranded DNA. Although we were not able to confirm the binding of CAR-1 to Element B directly in vitro because of CAR-1's insolubility during purification processes, RNAi analyses demonstrated that CAR-1 undoubtedly plays a crucial role in the cdc-48.1 expression in embryos (Figs. 8B, C). It has been reported that depletion of CAR-1 significantly disrupts the spindle formation and ER organization in embryos. Therefore, CAR-1 was suggested to be required for cytokinesis and membrane trafficking in C. elegans embryos (Audhya et al., 2005; Squirrell et al., 2006). It is very interesting to mention that p97 has been demonstrated to play an important role in ER maintenance during interphase as well as in ER assembly at the end of mitosis (Uchiyama and Kondo, 2005; Uchiyama et al., 2006). Furthermore, it has been reported that RNAi of p97 causes spindle defects and induces apoptosis (Kobayashi et al., 2002; Wójcik et al., 2004). Taken together, it is thus reasonable to assume that the observed defects by depletion of CAR-1 resulted from the reduction of the cdc-48.1 expression. Then, how is cdc-48.1 expression regulated developmentally? CAR-1 is mainly expressed in germline and in early embryonic stage. However, CAR-1 level was gradually reduced after 4-cell stage (Boag et al., 2005).

Therefore, we consider that the reduction of CAR-1 level in late embryonic stage is at least one factor to regulate *cdc-48.1* expression developmentally. Further studies should be directed towards how CAR-1 regulates the *cdc-48.1* expression in embryos.

In summary, we were able to reveal the promoter structure of two p97 genes of *C. elegans*, and showed that HMG-12 and CAR-1 are involved in the *cdc-48.1* expression in embryos (Fig. 9). We are now trying to identify proteins that are required for post-embryonic expression of *cdc-48.1* and for embryonic expression of *cdc-48.2*. Since p97 is a multi-functional protein as described above, clarifying the whole picture of the expression regulation of two p97 genes in *C. elegans* will give us some clues to understand not only their common functions but also specified functions of each p97.

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