Development 125, 3585-3597 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5228

Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in *Caenorhabditis elegans*

Yang Hong*, Richard Roy* and Victor Ambros

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA
*The first two authors contributed equally to this work

Accepted 10 July; published on WWW 25 August 1998

SUMMARY

C. elegans cki-1 **encodes a member of the CIP/KIP family of cyclin-dependent kinase inhibitors, and functions to link postembryonic developmental programs to cell cycle progression. The expression pattern of *cki-1::GFP* suggests that *cki-1* is developmentally regulated in blast cells coincident with G_1, and in differentiating cells. Ectopic expression of CKI-1 can prematurely arrest cells in G_1, while reducing *cki-1* activity by RNA-mediated interference (RNAi) causes extra larval cell divisions, suggesting a role for *cki-1* in the developmental control of G_1/S. *cki-1* activity is required for the suspension of cell cycling that occurs in dauer larvae and starved L1 larvae in response to environmental signals. In vulva precursor cells (VPCs), a pathway of heterochronic genes acts via *cki-1* to maintain VPCs in G_1 during the L2 stage.**

Key words: Cyclin-dependent kinase inhibitor (CKI), Cell cycle, RNA-mediated interference (RNAi), Vulva precursor cell (VPC), *Caenorhabditis elegans*, Ribonucleotide reductase (*rnr*), Dauer pathway, Starvation, GFP

INTRODUCTION

The proper development of a multicellular organism requires the precise orchestration of cell proliferation and differentiation. Despite considerable progress toward understanding the mechanisms of cell cycle progression in single cells, less is known about the coordination of the cell cycle with development in a multicellular context. Developmental control of cell cycle is likely implemented by signals acting on limiting components of the cell cycle machinery (Edgar and Lehner, 1996). For example, in *Drosophila*, the timing and number of embryonic cell cycles is controlled by the developmental regulation of Cyclin E, String (cdc25), and E2F, which are limiting cell cycle activators (Duronio and O'Farrell, 1994, 1995; Knoblich et al., 1994; Duronio et al., 1995; Richardson et al., 1995; Sauer et al., 1995; Lehner and Lane, 1997), and by the activity of Dacapo, the *Drosophila* member of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs) (de Nooij et al., 1996; Lane et al., 1996). Cells at the anterior side of the morphogenetic furrow in the fly eye imaginal disc are synchronized in G_1 of their penultimate cell cycle by *roughex* and *rca1*, which regulate cyclin A (Thomas et al., 1994; Dong et al., 1997). In *C. elegans*, the number of cell divisions in diverse cell lineages is limited by the activity of *cul-1*, which likely acts by regulating the degradation of G_1 cyclins (Kipreos et al., 1996; Mathias et al., 1996).

CKIs block cell division by inhibiting cyclin-dependent kinase activity necessary for the G_1/S transition (Harper and Elledge, 1996) and hence these proteins are potential mediators of the developmental control of G_1 progression. Two groups of CKIs have been described (Sherr and Roberts, 1995). These include the Ink4 family, members of which specifically inhibit Cyclin D-Cdk4/6 activity, and the CIP/KIP family which includes p21CIP1, p27KIP1 and p57KIP2 that exhibit promiscuous CDK-inhibitory activity. The CIP/KIP family of CKIs are highly expressed during terminal differentiation in mammalian cells (Parker et al., 1995; Macleod et al., 1995; Lee et al., 1995; Matsuoka et al., 1995), and cell culture experiments suggest a clear role for CKIs in promoting cell cycle exit for certain terminally differentiating cells (Casaccia-Bonnefil et al., 1997). In the *Drosophila* eye imaginal disc, Dacapo is expressed in terminally differentiating cells, and not in the cells anterior to the morphogenetic furrow that are temporarily held in G_1, suggesting a major role for Dacapo at the termini of cell lineages, rather than in precursor cells. Although there is evidence implicating CKIs in the developmental regulation of G_1 in precursor cells, it is relatively circumstantial. Genetic disruption of p27KIP1 or p57KIP2 in mice leads to organ hyperplasia and endocrine tumors, apparently reflecting enhanced proliferation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Zhang et al., 1997). However, it was not possible to determine directly whether the extra cells in these mouse mutants resulted from shortened G_1 phases in their progenitor cell cycles.

The nematode *C. elegans* is an excellent model for the study of developmental cell cycle control, since this animal develops according to a nearly invariant cell lineage (Sulston and Horvitz, 1977) that is conveniently accessible to observation and experimentation. Many of the postembryonic cell cycles

of *C. elegans*, particularly those of extended duration, are regulated at the level of the G_1/S transition (Hedgecock and White, 1985; Euling and Ambros, 1996). Genetic pathways have been identified that developmentally regulate postembryonic cell cycles in *C. elegans*. These pathways include the *daf* genes that control entry into and exit from the developmentally arrested dauer larva stage (Riddle et al., 1997), the *gon* genes that control cell cycle progression in somatic gonad lineages (Sun and Lambie, 1997) and the heterochronic genes, that control the length of G_1 in the vulva precursor cell (VPC) cell cycle (Ambros and Horvitz, 1985; Euling and Ambros, 1996). Although it is not known how the heterochronic pathway affects G_1 progression selectively in the VPCs, it likely regulates components of the general cell cycle machinery critical for G_1 progress.

In this study we show that *C. elegans* developmental regulators may control the timing of the G_1/S transition by temporally regulating the activity of *cki-1*. The *cki-1* promoter confers a temporal and cell type-specific pattern of reporter gene expression that coincides with developmental cell cycle progression, consistent with a role for *cki-1* in the developmental control of G_1/S in diverse cell types. Using misexpression and RNA-mediated interference (RNAi) to perturb *cki-1* activity we show that a major role for *cki-1* is in the developmental regulation of G_1/S progression throughout postembryonic development. For the vulva cell lineage, we demonstrate that *cki-1* may act as a downstream effector of heterochronic genes to control the timing of VPC cell division.

MATERIALS AND METHODS

Worm strains

Nematode strains were grown and maintained as described by Wood et al. (1988). All animals were grown at 20°C unless otherwise indicated. The following strains were used: VT284 *lin-14(ma135)/szT1*, VT573 *lin-4(e912);lin-14(n179ts)*, VT815 *unc-36(e251);maIs109*, VT825 *dpy-20(e1282);maIs113*, VT826 *gon-2(q388ts);maIs109*, VT827 *maEx154*, VT828 *maEx155*, VT829 *maIs103*;*maEx156*, VT774 *unc-36(e251);maIs103*, VT843 *lin-4(e912);maIs113*, VT844 *lin-14(ma135);maIs113*, VT845 *lin-29(n1440);maIs113*, EJ186 *gon-2(q388ts)*, CB1282 *dpy-20(e1282ts)*, NH2466 *dpy-20(e1282ts); ayIs4*, CB1372 *daf-7(e1372ts)*, CB1370 *daf-2(e1370ts)*.

Germline transformation

Worms were transformed by microinjection as previously described (Epstein and Shakes, 1995). *maEx154* was generated by co-injecting *hsp16-41::cki-1* (pVT365; 20 μg/ml), *hsp16-41::cki-1::GFP* (pVT365G; 20 μg/ml), and *rol-6(su1006)* (pRF4; 100 μg/ml). *maEx155* was generated by co-injecting *egl-17::CKI-1::GFP* (pVT363G; 20 μg/ml) and *rol-6(su1006)* (pRF4; 125 μg/ml). *maEx156* was generated by co-injecting *egl-17::CKI-1* (pVT363; 60 μg/ml) and *rol-6(su1006)* (pRF4; 120 μg/ml). *maIs109* was generated by co-injecting *cki-1(P2kb)::GFP* (pVT353; 20 μg/ml) and unc-*36* (Rlp16; 50 μg/ml). *maIs113* was generated by co-injecting *cki-1::GFP* (pVT371; 20 μg/ml) and *dpy-20* (pMH86; 100 μg/ml). *maIs103* was generated by co-injecting *rnr::GFP* (50 μg/ml), *unc-36* (Rlp16; 50 μg/ml) and pBluescript (100 μg/ml). Extrachromosomal arrays were integrated by gamma irradiation from a ^{137}Cs source (Epstein and Shakes, 1995).

Plasmid constructions

pVT371(*cki-1::GFP*) is a transcriptional fusion between 8 kb of *cki-1* upstream sequence from cosmid T05A6 and the NLS::GFP sequence from pPD95.02, and includes 1.5 kb of 3′ sequence from *cki-1*. pVT353 (*cki-1(P2kb)::GFP*) is identical to pVT371 except that it has only 2 kb of *cki-1* upstream sequence. pVT352 and pVT352G are identical to pVT353 except that the NLS::GFP sequence has been replaced by the coding sequence for CKI-1 or CKI-1::GFP, respectively. pVT363 (*egl-17*::*CKI-1*) and pVT363G (*egl-17*::*CKI-1::GFP*) were made by replacing the 2kb promoter sequence in pVT352 and pVT352G with 4.5 kb of the *egl-17* promoter from NH354 (Burdine et al. 1998). This promoter fragment also contains the EGL-17 signal peptide coding sequence (MLKVLLTLMLS) which was fused in frame with CKI-1. pVT365 (*hsp16-41::CKI-1*) and pVT365G (*hsp16-41::CKI-1::GFP*) were made by inserting the CKI-1 (from pVT352) or CKI-1::GFP (from pVT352G) coding sequence with 1.5kb 3′ *cki-1* sequence into pPD79.83.

The large subunit of the ribonucleotide reductase gene (*rnr-1*) was identified by the *C. elegans* sequencing consortium on chromosome I. 1.0 kb of promoter sequence upstream of the predicted translational start was amplified by PCR. This fragment, which contains putative E2F binding sites, was inserted into pPD95.67 to yield pVT501 (*rnr::GFP*).

RNAi

The *cki-1* cDNA was amplified from an oligo dT-primed *C. elegans* cDNA library (kindly provided by Robert Barstead). The *Xma*I fragment was inserted into pBluescript and the resulting construct was linearized with *Bam*HI and *Hin*dIII to synthesize antisense and sense *cki-1* RNA, respectively. 1 μg of the gel-purified template was used for in vitro transcription reactions and the RNA was then phenol/chloroform extracted, ethanol precipitated and annealed (Fire et al., 1998). Double stranded RNA was injected at concentrations of 1-2 mg/ml and injected animals were recovered, transferred daily to new plates, and the resulting F_1 progeny were monitored for phenotypic abnormalities.

Microscopy and photography

Images of live animals anesthetized with 1 mM levamisole were captured with an Optronics DEI750 integrating color CCD video camera and a Scion CG-7 RGB video capture board on a Power Macintosh 8500AV. GFP fluorescence images were taken with exposures ranging from 1/8 to 2 seconds. The digital images were processed using Adobe Photoshop. Images used to display relative levels of GFP expression were all processed identically.

RESULTS

cki-1 encodes a G_1 cyclin-dependent kinase inhibitor

The *C. elegans* genome project identified two loci on chromosome II (*cki-1* and *cki-2*) that are predicted to encode proteins of the p21/p27 class of CKI (de Nooij et al., 1996; Lane et al., 1996). *cki-1* was chosen for this investigation due to its greater sequence similarity to p21/p27. The genomic structure of *cki-1* predicted using GeneFinder (Epstein and Shakes, 1995) was confirmed by cDNA sequencing (Kipreos, personal communication; data not shown).

To test whether *cki-1* encodes a cell cycle inhibitor, we examined the effects of ectopic expression of *cki-1* on cell cycle progression. First, we used a heat shock promoter to express CKI-1 and CKI-1::GFP fusion proteins, resulting in embryonic and postembryonic cell cycle arrest (Fig. 1A; Table 1). Second, we employed the *egl-17* promoter (Burdine et al., 1998) to express CKI-1 or CKI-1::GFP specifically in a single vulva precursor cell, P6.p. The *egl-17* promoter is activated

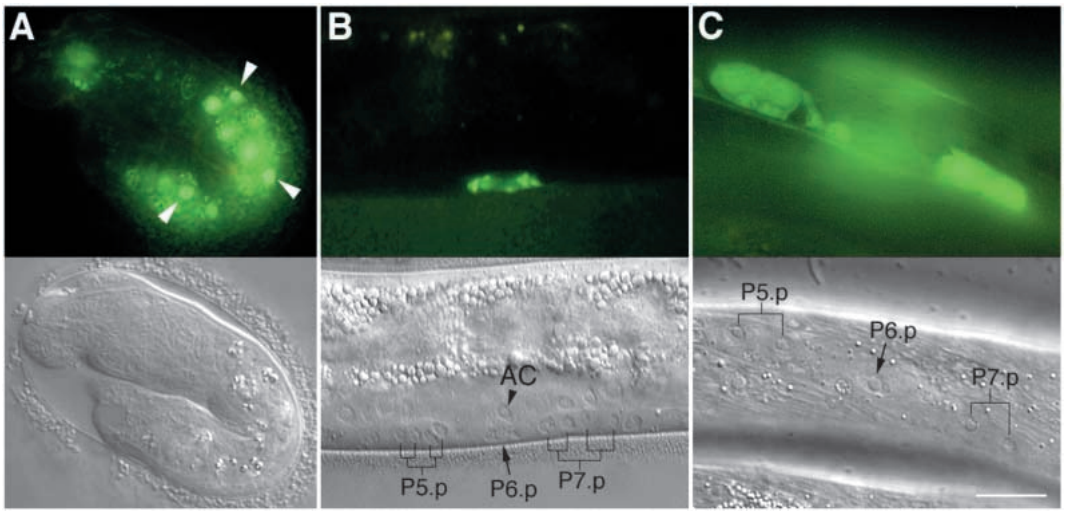

Fig. 1. G_1 arrest by ectopic expression of CKI-1 and CKI-1::GFP. In A both CKI-1 and CKI-1::GFP were expressed together (each from the heat shock promoter *hsp16-41*) (Stringham et al. 1992; Fire et al. 1990). In B a CKI-1::GFP fusion protein was expressed from the *egl-17* promoter. In C the CKI-1 protein was expressed from the *egl-17* promoter, and the GFP fluorescence corresponds to *rnr ::GFP*, which acts as an S phase reporter. In this and subsequent figures where GFP fluorescence and DIC images of the same field are paired vertically, the upper and lower images are of the same focal plane. (A) Embryo arrested at the two-fold stage (see also Table 1). Arrowheads point to nuclear-localized CKI-1::GFP fluorescence in intestine cells. (B) At the late L3 stage, when P5.p and P7.p have each divided twice, P6.p is undivided (lower panel) and is the single cell expressing *egl-17::CKI-1::GFP* (upper panel). Fluorescence is detected in the nucleus of P6.p, and also outside the nucleus, likely owing to the presence of part of the *egl-17* signal sequence in the fusion gene (see Materials and Methods). (C) In this late L3 stage animal, the daughter cells of P5.p and P7.p express *rnr::GFP* strongly, yet the undivided P6.p (which is presumably expressing *egl-17::CKI-1*) shows no detectable *rnr::GFP* expression. The background GFP fluorescence is from the dividing somatic gonad cells in another focal plane. AC: anchor cell. Scale bar, 5 μm. All animals in this and subsequent figures are oriented anterior left and dorsal up (in C the animal is twisted because it is co-transformed with *rol-6(su1006)*).

specifically in P6.p during the late G_1 of the cell cycle (V. Ambros, unpublished). Driven by the *egl-17* promoter, the CKI-1::GFP fusion protein was expressed specifically in P6.p and blocked P6.p from dividing (Fig. 1B; Table 1). The *egl-17::CKI-1* transgene was transformed into animals carrying an S phase-specific reporter (Duronio and O'Farrell, 1994) consisting of the worm ribonucleotide reductase (*rnr)* promoter fused to GFP. In these doubly transgenic animals, no *rnr::GFP* expression was detectable in the non-dividing P6.p, whereas P5.p and P7.p divided in the L3 and their daughter cells showed strong *rnr::GFP* expression (Fig. 1C; Table 1). We conclude that CKI-1 expression cell-autonomously blocks G_1/S.

Developmental expression of *cki-1::GFP*

There are no predicted coding sequences within 20 kb upstream of *cki-1*. To begin an analysis of *cki-1* promoter activity, the CKI-1 protein coding sequence was excised from a clone containing 8 kb of *cki-1* upstream and 1.5 kb of *cki-1* 3′ downstream sequence and replaced with GFP coding sequence. The resulting transcriptional fusion, pVT371, was transformed into worms and attached to a chromosome to generate *maIs113* (see Materials and Methods). Unless otherwise noted, the term *cki-1::GFP* will be used in reference to *maIs113*.

Embryonic expression of *cki-1::GFP* begins at the comma stage in the pharyngeal primordium as pharyngeal muscles begin terminal differentiation (Wood et al., 1988). *cki-1::GFP* is highly expressed in most cells during late embryogenesis, when cells are either differentiating or undergoing cell cycle arrest prior to hatching. At hatching, and in L1 animals maintained in the absence of food, *cki-1::GFP* is detected in Q, M, Z1, Z4 and the V cells. The expression in these cells

Table 1. Ectopic expression of CKI-1 and CKI-1::GFP inhibits cell cycle

A. Heat shock driven expression of CKI-1 and CKI-1::GFP*

	% embryos
Arrested embryos with GFP expression	94.8 (*n*=115)
Arrested at comma or earlier stage	19.1
Arrested at 1.5 fold stage	19.1
Arrested at 2 fold stage	33.0
Arrested at 3 fold stage	23.5
	Number of intestinal nuclei per animal†
GFP plus animals	21.4±2.5 (*n*=12)
GFP minus animals	30.6±1.7 (*n*=7)

B. *egl-17::CKI-1::GFP* transgenic animals

% P6.p cells undivided‡	62.5 (*n*=54)

C. *egl-17::CKI-1; rnr::GFP* transgenic animals

% undivided P6.p cells that were GFP negative§	100.0 (*n*=10)

*Eggs were heat shocked at 33°C for 2 hours, and after recovering at 20°C for 24-48 hours unhatched embryos were scored using DIC microscopy.

†L2 and L3 larvae were scored for the number of intestinal nuclei. In wild-type animals, approximately 20 intestinal nuclei are present at hatching, and some of these divide in the late L1, increasing the number of intestinal nuclei to about 30-35. Eggs were heat shocked at 33°C for 2 hours, and after recovering at 20°C for 24 hours, larvae were heat shocked again at 33°C for 2 hours followed by another 24 hour recovery at 20°C (Kuwabara and Kimble, 1995).

‡P6.p cells were scored only if: (1) P5.p and P7.p each had at least two daughters, and (2) CKI-1::GFP expression in P6.p cell was detectable.

§Undivided P6.p cells were scored for *rnr::GFP* expression only if: (1) P5.p and P7.p each had at least two daughters, and (2) *rnr::GFP* expression was uniformly strong in all P5.p and P7.p daughter cells.

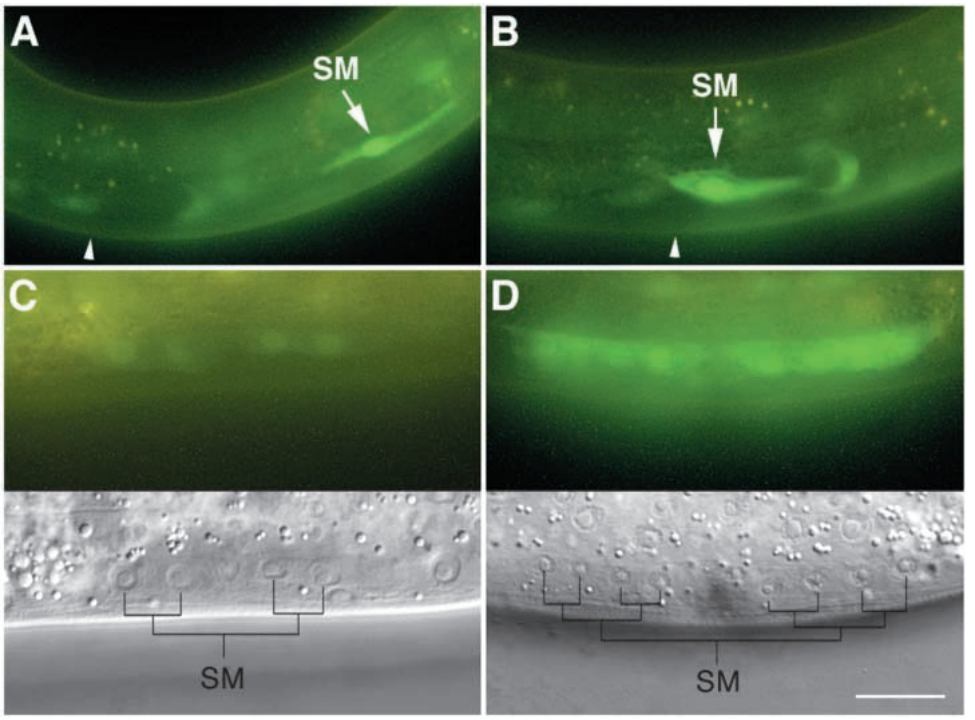

Fig. 2. Developmental expression pattern of *cki-1::GFP* in sex myoblasts (SM) and sex muscle cells. (A) Early L2 and (B) late L2; the SM cell (arrow) shows strong *cki-1::GFP* expression during migration towards the middle of the gonad (arrowhead). (C) Four dividing SM daughter cells at late L3 stage show much weaker *cki-1::GFP* expression than SM (see A and B). (D) The eight SM daughter cells that differentiate into sex muscles at the L3 molt show high *cki-1::GFP* expression. Scale bar, 5 μm.

fades after feeding, when cell division resumes. Throughout postembryonic development, *cki-1::GFP* is expressed with a temporal and cell type-specific pattern that corresponds to developmental patterns of cell cycle progression (see below and Table 2). Expression is also strong in many postmitotic neurons and muscle cells. *cki-1::GFP* expression tends to be stronger in newly differentiated cells and then gradually decreases. *cki-1::GFP* is also expressed in dauer larvae (see below).

Table 2. Upstream elements of *cki-1* gene confer differential cell type and temporal expression pattern

Developmental stage	Cell/tissue type	GFP expression*	
		maIs113	*maIs109*
Mid-embryogenesis	Pharynx primordium	++	+++
Late embryogenesis	Numerous cells†	++	+++
Early L1	Z1/Z4 gonad cells	++	+++
	Hypodermal V cells	++	–
	Hypodermal P cells	–	–
	Q cell	++	nd
	M cell	++	+++
L2	Sex myoblasts	++	–
	Distal tip cells	++	+++
Early L2	Pn.p cells	+/–	–
L2 molt	Pn.p cells	++	–
L3 molt	Vulva cells	++	+++
	Sex muscles	++	+++
L4 molt	Seam cells	++	–
All larval stages	Neurons	++	+++
	Intestine cells	++	–
dauer	Lateral hypodermis, SM, VPC, intestinal cells	+++/++	–

*GFP expression from *maIs113* is driven by the 8kb *cki-1* promoter while *maIs109* is driven by the proximal 2kb *cki-1* promoter (see Materials and Methods). In general, GFP expression from *maIs109* is higher than from *maIs113*. –, no detectable expression; +++, strongest expression; +/–, expression is visible, but very weak.

†Expressing cells were not identified specifically, but include neurons and muscle cells.

Lateral hypodermal V lineage: V cells express *cki-1::GFP* strongly (Fig. 4D) until they divide in the mid-L1, when fluorescence decreases significantly. Seam cells express at a moderately high level during resting phases between molts, and at relatively reduced levels during division. Expression increases substantially at the L4 stage coincident with seam cell terminal differentiation (Fig. 4H).

SM lineage: The two sex myoblasts (SMs) are born in the late L1, migrate to the lateral midline (Sulston and Horvitz, 1977) and, in the mid-L3, divide to produce sex muscles. *cki-1::GFP* expression is high during SM migration, reduced during SM division and high again as the sex muscles differentiate (Fig. 2A-D).

P lineage: *cki-1::GFP* expression is undetected in P cells, but at the L1 molt, the progeny of the Pn.a neuroblasts express high levels of *cki-1::GFP*. *cki-1::GFP* expression in P3.p-P8.p (the VPCs) is described below.

Somatic gonad: *cki-1::GFP* expression in Z1 and Z4 (Fig. 4B) diminishes prior to cell division in the mid-L1. *cki-1::GFP* is strongly expressed in Z1.aa and Z4.pp, the distal tip cells (DTCs), beginning in the L2 (Fig. 4F), but is undetectable in the rest of the Z1/Z4 lineage until the late L3 and early L4, when cki-*1::GFP* expression in the somatic gonad increases dramatically at the onset of terminal differentiation (Fig. 3E).

Intestine: After the L1 molt, intestine cells undergo cycles of endoreplication (Hedgecock and White, 1985). *cki-1::GFP* is expressed in the intestine after the L1 molt and throughout the larval stages.

cki-1::GFP expression correlates with developmental arrest

To determine if *cki-1::GFP* expression correlates with developmental G_1 arrest, we compared the timing of expression of *cki-1::GFP* to that of the S phase reporter, *rnr::GFP*. The expression pattern of *rnr::GFP* is consistent with the previously reported timing of S phase in the lateral and ventral hypodermal lineages (Hedgecock and White, 1985; Euling and Ambros, 1996; R. Roy, unpublished data). For example, VPC G_1 extends from the mid-L1 until shortly after the L2 molt when the DNA content of VPCs doubles (Euling and Ambros, 1996) and *rnr::GFP* expression appears in VPCs (Fig. 3). *cki-1::GFP* expression is first detected in late L1 or early L2 VPCs, and peaks at the L2 molt. *cki-1::GFP* expression is almost undetectable in dividing vulva cells, while *rnr::GFP* expression is strong. After the final vulva cell division, *cki-1::GFP* fluorescence increases markedly and *rnr::GFP* fades (Fig. 3A-F).

A reciprocal pattern of *cki-1::GFP* and *rnr::GFP* expression also occurs in animals that are subject to conditional cell cycle arrest; in the absence of food, newly hatched L1 larvae do not begin larval development, and cell division is arrested in G_1 as evidenced by a lack of *rnr::GFP* expression. *cki-1::GFP* expression is very high in these arrested larvae, and then fades by about 8-12 hours postfeeding, as cells re-enter the cell cycle and activate *rnr::GFP* expression (Fig. 3G-J). Similarly, under crowded or starved conditions, L2 larvae differentiate into developmentally arrested dauer larvae (Riddle et al., 1997) in which *rnr::GFP* expression is reduced while *cki-1::GFP* expression is elevated (Table 2).

cki-1 upstream elements control spatial and temporal expression patterns

To test whether 5′ upstream sequences of *cki-1* affect the developmental expression pattern of a *cki-1::GFP* reporter, we removed the distal 6 kb of 5′ upstream sequence from the *cki-1::GFP* construct to generate *cki-1(P2kb)::GFP*. *maIS109*, the integrated transgene containing *cki-1(P2kb)::GFP*, is expressed in some of the cells that express the 8 kb construct, and not in others (Table 2; Fig. 4). Specifically, *maIS109* is not expressed in resting V cells, the VPCs, SM or in terminally differentiating seam cells. This result suggests that the distal 6 kb of *cki-1* 5′ upstream sequences contains enhancer elements required for *cki-1* expression in these cells. Replacing the *cki-1* 3′ untranslated

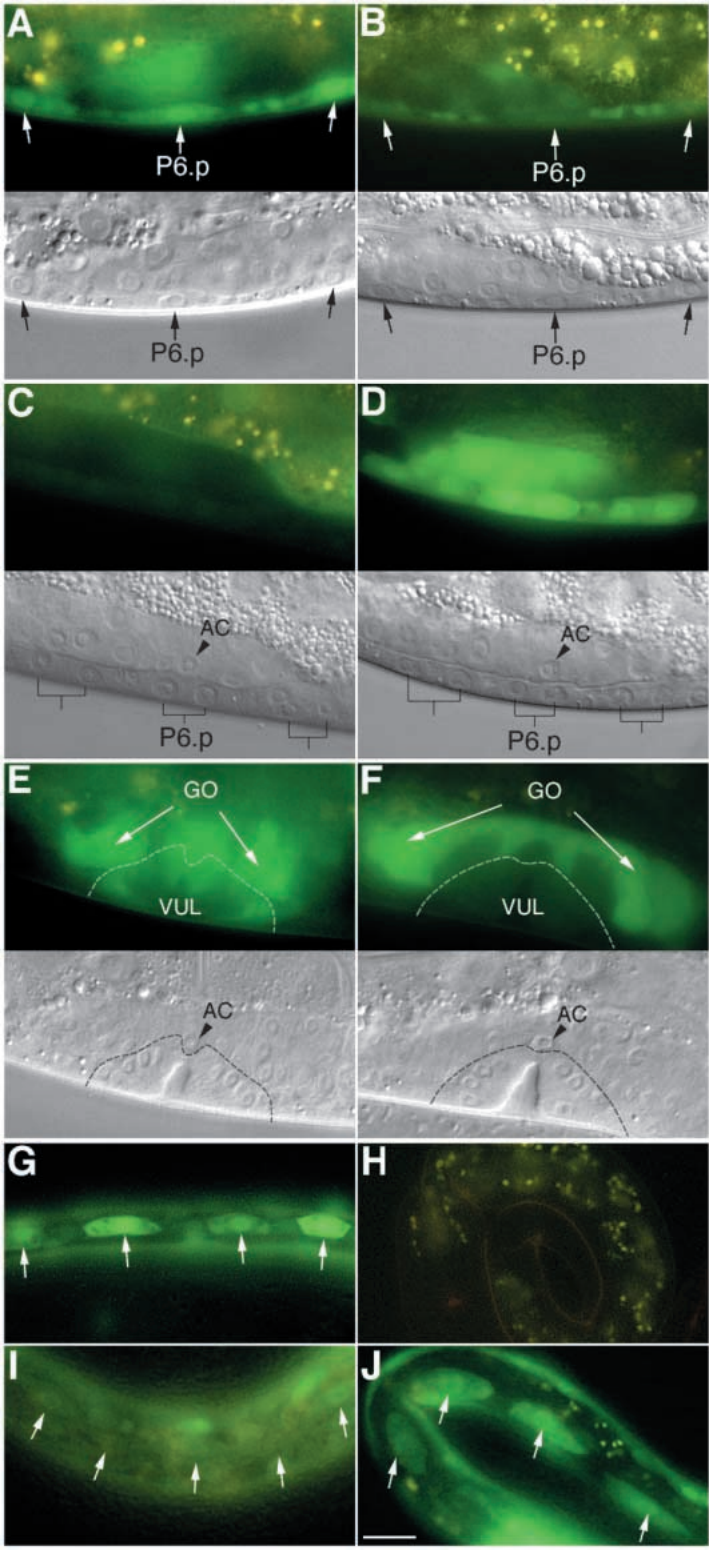

Fig. 3. *cki-1::GFP* expression correlates with developmental G_1 arrest. (A-F) Reciprocal expression pattern of *cki-1::GFP* (A, C, and E) and *rnr::GFP* (B,D,F) during VPC development. (A,B) L2 molt: *cki-1::GFP* expression is high in G_1-arrested VPCs (arrows) at L2 molt (A). In contrast, in *rnr::GFP* animals of the same stage, GFP is low or undetected in VPCs (B). (C,D) Late L3: VPCs have each produced two daughter cells and *cki-1::GFP* is not detectable (C), while in *rnr::GFP* animals, GFP expression is strong (D). (E,F) Early L4: *cki-1::GFP* is strong in differentiating vulva cells (VUL; circumscribed) and some differentiating somatic gonad cells (GO) (E). In *rnr::GFP* animals, GFP is not detected in differentiating vulva cells but is strongly expressed in dividing ventral uterine cells (F). (G-J) *cki-1::GFP* (G,I) and *rnr::GFP* (H,J) expression in starved and fed L1s. Newly hatched L1 animals in the absence of food show strong *cki-1::GFP* expression in lateral hypodermal V cells (G), but undetectable *rnr::GFP* expression (H). L1 8-10 hours postfeeding shows significantly reduced *cki-1::GFP* expression in V cells (I), while *rnr::GFP* is detectable in V cells (J). Arrows in G, I and J indicate V cells. AC, anchor cell. Scale bar, 5 μm.

region (UTR) in the 8 kb *cki-1::GFP* construct with the *unc-54* 3′UTR did not alter the pattern of GFP expression (data not shown).

cki-1::GFP expression in the L1 somatic gonad depends on *gon-2* activity

During normal gonadogenesis, Z1 and Z4 divide in the mid-L1, while in *gon-2* animals Z1 and Z4 are severely delayed or totally blocked from dividing (Sun and Lambie, 1997). This early requirement for *gon-2* activity by Z1/Z4, together with the temporal pattern of expression of *cki-1::GFP* in these cells, suggests that *gon-2* may be required for down-regulating *cki-1* in Z1 and Z4 in the L1. To test whether this decrease in *cki-1::GFP* expression depends on *gon-2* activity, we examined the expression of *cki-1::GFP* in Z1/Z4 of a *gon-2(q388ts)* mutant. Approximately 50% (n=11) of undivided Z1/Z4 cells strongly express *cki-1::GFP* in *gon-2(q388ts);cki-1::GFP* animals as late as the L3 or L4 (Fig. 5A) suggesting that *gon-2* activity is directly or indirectly involved in the down-regulation of *cki-1* in Z1 and Z4.

cki-1::GFP expression in the L4 seam cells depends on the activity of the heterochronic gene *lin-29*

lin-29 encodes a zinc-finger transcription factor which is required for the terminal differentiation of hypodermal seam cells at the L4 molt (Rougvie and Ambros, 1995). In *lin-29(lf)* animals, seam cells fail to withdraw from the cell cycle or to differentiate at the L4 molt, and instead they reiterate larval fates including cell divisions. In the wild type, *cki-1::GFP* is expressed strongly in terminally differentiated seam cells (Fig. 4H). In contrast, *lin-29(n1440);cki-1::GFP* animals display an intermediate level of *cki-1::GFP* expression similar to that of resting hypodermal cells between each molt (Fig. 5B). This result suggests that *lin-29* is required to activate the high level of *cki-1::GFP* expression associated with seam cell terminal differentiation.

cki-1::GFP expression in VPCs is *lin-14* dependent

The heterochronic gene *lin-14* controls the length of VPC G_1 (Euling and Ambros, 1996). To test whether *cki-1::GFP* expression in VPCs (Fig. 3A and Table 2) depends on *lin-14* activity, *lin-14(ma135); cki-1::GFP* larvae (n=11) were examined for *cki-1::GFP* expression throughout the L1 and L2. With the exception of one VPC (P3.p) with weak *cki-1::GFP* expression in one animal, VPCs displayed no detectable *cki-1::GFP* expression (Fig. 5C). Expression in other cell types appeared essentially normal. Thus, removal of *lin-14* activity reduces *cki-1* expression to below detectable levels specifically in VPCs.

The normal timing of VPC cell division requires the down-regulation of *lin-14* activity after the L1 by *lin-4* (Lee et al., 1993). In a *lin-4(lf)* mutant, where *lin-14* activity remains high throughout development, VPCs display complex defects, including delayed or blocked cell divisions, or irregular cell division patterns (Euling and Ambros, 1996). Nevertheless vulval morphogenesis never occurs in *lin-4(lf)*, indicating that high *lin-14* activity prevents vulval differentiation (Euling and Ambros, 1996). Consistent with blocks in both VPC cell cycle progression and vulval differentiation in *lin-4(e912);cki-1::GFP* animals, GFP was detected at high levels in VPCs exhibiting cell cycle delay, and was low or undetectable in the progeny of divided VPCs (Fig. 5D).

RNA-mediated interference of *cki-1* activity

If *cki-1* is critical for maintaining cells in G_1 during

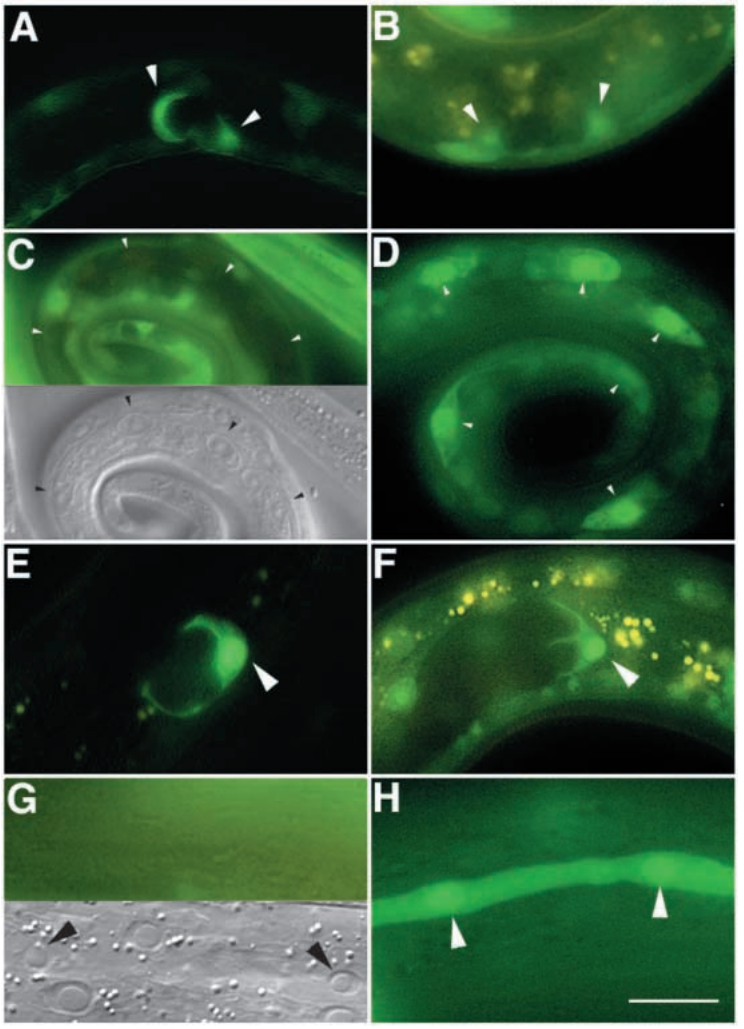

Fig. 4. *cki-1* 5′ upstream sequences control spatial and temporal expression patterns of *cki-1::GFP*. (A,C,E,G) *maIs109* animals (transformed with pVT353, containing 2 kb of *cki-1* upstream sequences driving GFP; see Materials and Methods). (B,D,F,H) *maIs113* animals (transformed with pVT371, containing 8 kb of *cki-1* upstream sequences). 2 kb (A) and 8 kb (B) constructs express GFP in resting Z1 and Z4 somatic gonad cells (arrowheads) in the early L1. The 2 kb (C) construct is not expressed, while the 8 kb construct (D) is expressed, in resting lateral hypodermal V cells (arrowheads) in the early L1. Both constructs (E,F) are expressed in distal tip cells (DTC, arrowheads), shown here at the L2 stage. The 2 kb construct (G) is not expressed, while the 8 kb construct (H) is expressed, in differentiating lateral hypodermal seam cells (arrowheads) at the L4 molt. Scale bar, 5 μm.

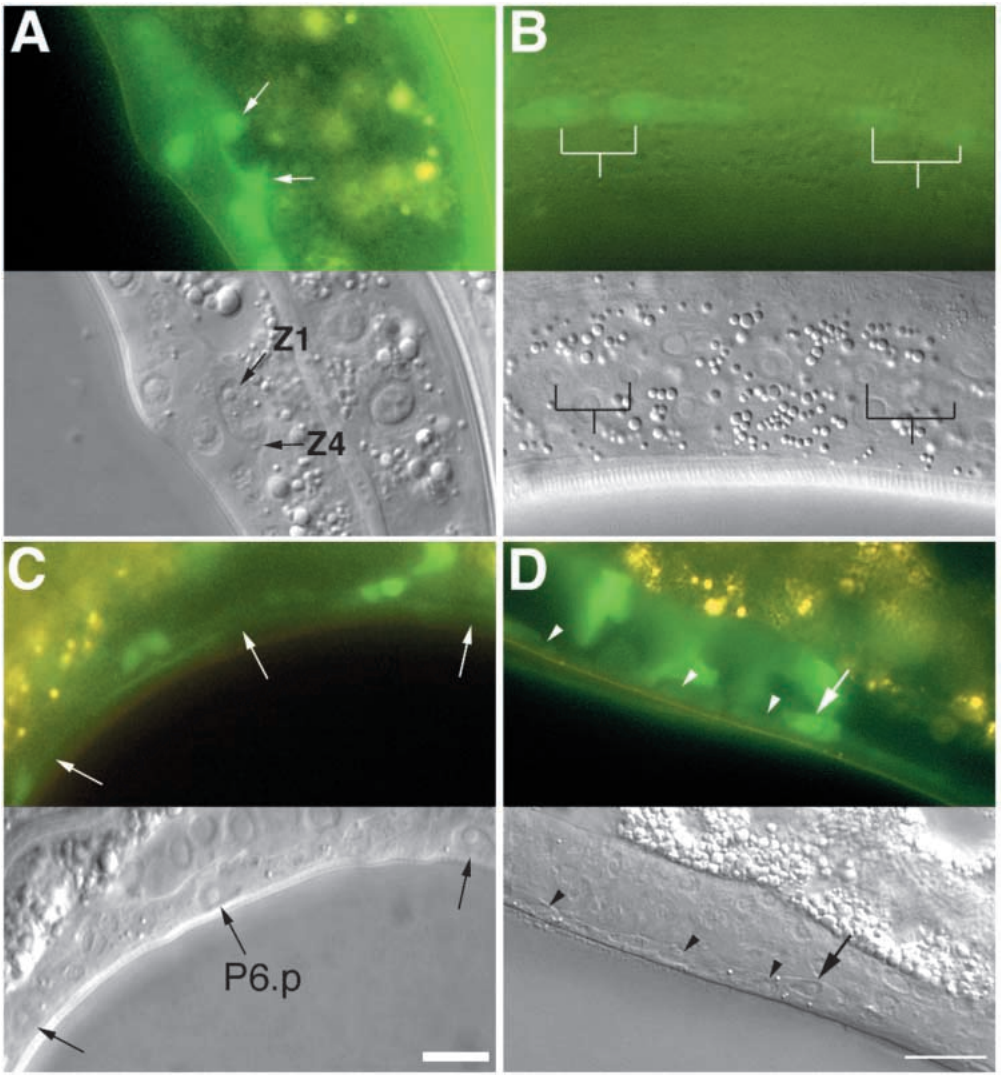

Fig. 5. *cki-1::GFP* expression in developmental mutants. (A) *cki-1::GFP* expression (upper) in undivided Z1 and Z4 cells of a *gon-2(q388ts)* L3 larva. Note the undeveloped gonad outlined by undivided Z1 and Z4 (lower). (B) *cki-1::GFP* expression (upper) in undifferentiated *lin-29* L4 seam cells is greatly reduced compared with the wild type (see Fig. 4H). Daughters of recently divided seam cells are indicated by branched lines (lower). (C) In a *lin-14(0)* animal at late L2 stage, *cki-1::GFP* expression (upper) is essentially undetectable in VPCs (compare with Fig. 3A) just prior to cell division. (D) Strong *cki-1::GFP* expression in an undivided VPC (arrow) in a *lin-4* L4 animal. In undifferentiated cells derived from the irregular division of another VPC, *cki-1::GFP* expression is virtually undetectable (arrowheads). In C the animal twisted slightly between exposures. Scale bar, 2.5 μm (A,C); 5 μm (B,D).

development, then reduced *cki-1* activity should disrupt the normal pattern of cell division, particularly in those cell types where *cki-1* temporal expression is coupled to development. To test this we used RNA-mediated interference (RNAi) (Rocheleau et al., 1997; Fire et al., 1998) to compromise *cki-1* gene activity. The RNAi method involves injecting adult hermaphrodites with double-stranded RNA corresponding to exon sequences of a target gene. The progeny of injected animals display a phenotype corresponding to loss of function of the target gene (Fire et al., 1998). *cki-1(RNAi)* animals were visibly uncoordinated and showed morphological abnormalities accompanied by misregulated cell division in numerous postembryonic cell lineages. *cki-1(RNAi)* animals show extra lateral hypodermal cell divisions in the early larval stages, accompanied by alterations in the timing of the L1 molt (data not shown). The apparent absence of strong embryonic effects of *cki-1(RNAi)* was somewhat unexpected since *cki-1::GFP* is expressed in mid- to late embryos. Perhaps other gene(s) function redundantly with *cki-1* during embryogenesis, or the RNAi method may not entirely eliminate *cki-1* gene activity (Fire et al., 1998). In the analysis described here, we did not exhaustively characterize *cki-1(RNAi)* phenotypes in all cell types (such as postmitotic neurons and muscles), but rather we concentrated on cases of developmental G_1 progression.

cki-1(RNAi) causes precocious VPC cell divisions

VPCs were monitored from the L1 through adult stages in *cki-1(RNAi)* animals, and up to six extra ventral hypodermal cells were observed to arise in the L2 from precocious division of P3.p-P8.p (Fig. 6A). In *cki-1(RNAi)* animals, VPCs undergo only one round of precocious division. These precocious divisions produce an apparent expanded set of VPCs that remain quiescent until the L3 when vulval development ensues as usual. In some cases the subsequent vulval morphogenesis is essentially normal.

To confirm that the precocious VPC cell divisions observed in *cki-1(RNAi)* animals produce cells with full VPC potential, we performed *cki-1(RNAi)* in a *lin-12(gf)* background. *lin-12* mediates lateral signaling among VPCs to control the pattern of vulval fates (Ferguson et al., 1987). In *lin-12(gf)* animals, the six VPCs execute secondary vulva fates independently of gonadal signals, resulting in six ventral protrusions, or pseudovulvae. If the precocious VPC daughters in *cki-1(RNAi)*

animals possess full VPC potential, then they would each have the capacity to adopt a secondary fate and generate a pseudovulva in a *lin-12(gf)* background (Fig. 6B). Indeed, *cki-1(RNAi);lin-12(gf)* animals displayed as many as 12 pseudovulvae (Fig. 6C) indicating that the precocious VPC daughters in *cki-1(RNAi)* animals each have the potential to execute vulval fates.

cki-1 mediates conditional cell cycle arrest

RNAi was used to test whether *cki-1* is required for cell cycle arrest in dauer larvae and starved L1 animals. *rnr::GFP(maIs103)* larvae were hatched in the absence of food and these starved L1s were examined for evidence of cell division by monitoring expression of *rnr::GFP*. Control *rnr::GFP(maIs103)* larvae (from uninjected parents) showed no evidence of *rnr::GFP* expression. *cki-1(RNAi);rnr::GFP(maIs103)* animals (from injected parents) showed strong *rnr::GFP* expression in hypodermal cells and the M cell, and some hypodermal cells were observed to divide (Fig. 7A). These results indicate that *cki-1* is required to maintain cells in a cytostatic state at the end of embryogenesis until the hatched L1 begins to feed.

daf-7(e1372ts) animals constitutively form dauer larvae at nonpermissive temperature, and no cell divisions occur after developmental arrest. However, in *daf-7(e1372ts); cki-1(RNAi)* dauer larvae, cell division, accompanied by *rnr::GFP* expression, was observed in the hypodermal and gonadal cell lineages (Fig. 7D,E). In these *cki-1(RNAi)* animals, formation of the lateral alae characteristic of dauer larva cuticle was blocked or severely impaired (Fig. 7B,C). Similar effects on dauer cuticle formation were observed in *daf-2(e1370ts); cki-1(RNAi)* animals (cell division and *rnr::GFP* were not monitored in this case). These results suggest that *cki-1* acts in the dauer larva to block cell division and permit dauer-specific hypodermal differentiation. Not all aspects of dauer morphology are sensitive to *cki-1(RNAi)*, as the affected animals exhibit some characteristics of normal dauer larvae, including intestinal lipid droplets, SDS-resistance and radial shrinkage.

Cell-nonautonomous effects of *cki-1(RNAi)* in the gonad

cki-1(RNAi) animals show a dramatic expansion of the gonad after the L3 compared to the wild type. The affected gonads often are morphologically abnormal and consist of one to six extra distal tip cells (DTCs) and a large excess of germ cells. DTCs signal to the germ cells via a *lag-2/glp-1* signaling pathway to promote germline proliferation (Lambie and Kimble, 1991). The *cki-1(RNAi)*-induced germline hyperplasia is blocked in a *glp-1* background (data not shown), suggesting that the extra germ cells result from ectopic *lag-2/glp-1* signaling, likely from the extra DTCs.

Unlike the *cki-1(RNAi)*-induced extra VPCs, ectopic distal tip cells in *cki-1(RNAi)* animals do not necessarily arise from duplications of differentiated distal tip cells, as might have been expected from the expression of *cki-1::GFP* in the DTCs. When we removed all the DTCs from *cki-1(RNAi)* L2 larvae by laser ablation, new DTCs were observed by the L4. Thus, extra DTCs can arise from as yet unidentified somatic gonadal precursors - suggesting that *cki-1* normally acts to restrict the potential of those other cells, perhaps by inhibiting cell division during a critical developmental period.

DISCUSSION

cki-1 acts as a G_1/S regulator and is developmentally expressed in *C. elegans*

cki-1 shares sequence identity with mammalian and

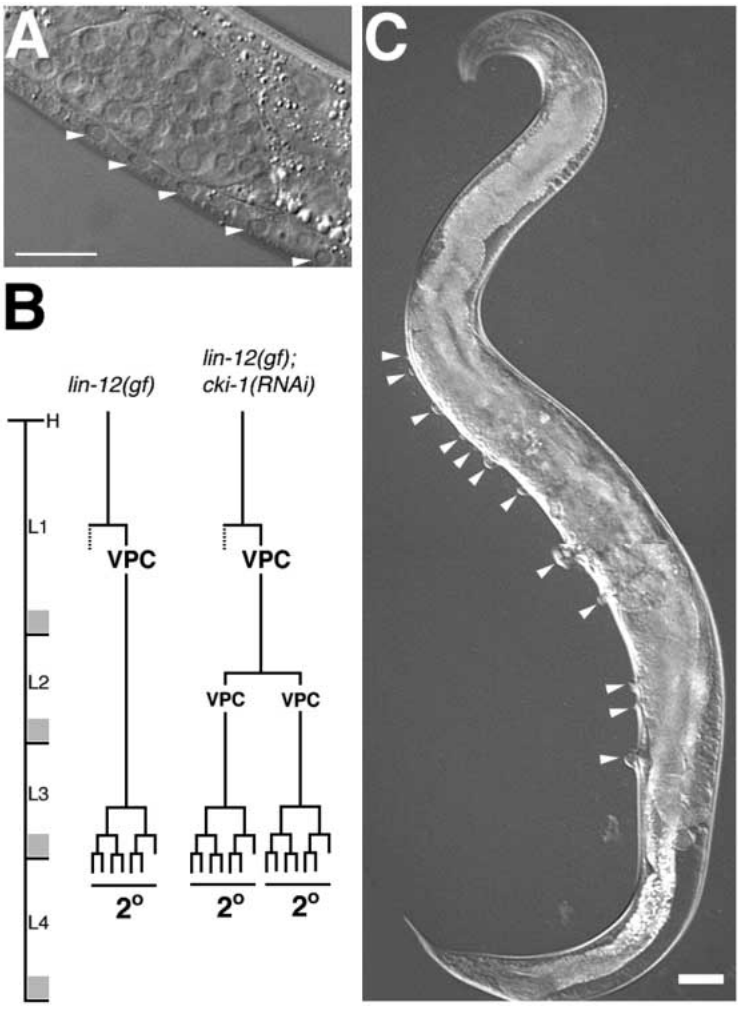

Fig. 6. *cki-1(RNAi)* developmental defects. (A) An animal in L2 lethargus with extra hypodermal cells (arrowheads), in the P6.p/P8.p region. Precocious divisions were never observed outside the P3.p-P8.p equivalence group. Note the abnormal morphology of the gonad typical of *cki-1(RNAi)* animals. (B) In a *lin-12(gf)* mutant, every VPC adopts the secondary fate. In *lin-12(gf);cki-1(RNAi)* animals extra VPCs generated via precocious divisions are fully competent to develop secondary fates. (C) *lin-12(gf);cki-1(RNAi)* animals possess pseudovulvae in numbers greater than the maximum of six found in *lin-12(gf)*. This *lin-12(gf);cki-1(RNAi)* animal has twelve pseudovulvae (arrowheads) owing to the duplication of all six original VPCs. Scale bar, 5 μm (A); 25 μm (B).

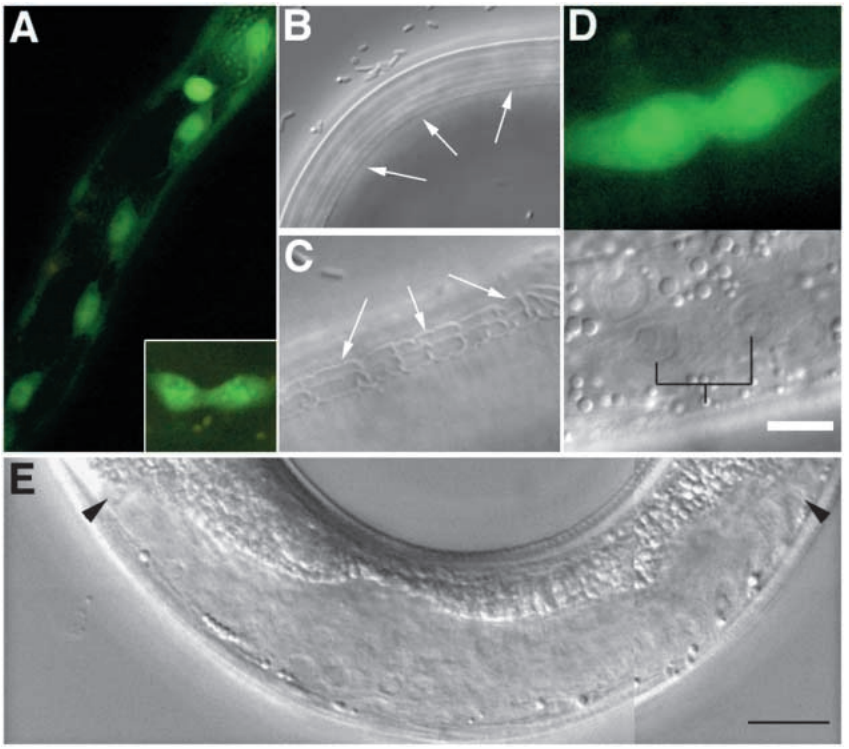

Fig. 7. *cki-1* mediates conditional cell cycle arrest. (A) *cki-1(RNAi)* L1 larva hatched in the absence of food expresses an S phase reporter *rnr::GFP*. *rnr::GFP* is not expressed in untreated animals (see Fig. 3H). Some cells of starved L1 *cki-1(RNAi)* undergo cytokinesis (inset). (B,C) Lateral alae (arrows) in *daf-7(e1372ts)* maintained at 25°C (B), and lateral cuticle of a *daf-7(e1372ts);cki-1(RNAi)* animal maintained at 25°C (C). Note the disorganized alae pattern in C. (D) Cell divisions continue in *cki-1(RNAi)* dauer larva. *daf-7(e1372ts); rnr::GFP; cki-1(RNAi)* animals were maintained at 25°C, and *rnr::GFP* expression was monitored (upper). The corresponding DIC image indicates sister cell nuclei. (E) Enlarged gonad of a *daf-7(e1372ts);cki-1 (RNAi)* animals maintained at 25°C (arrowheads indicate distal tip cells) due to ongoing somatic and germ cell divisions. Scale bar, 5 μm (A-C,E); 2.5 μm (D).

Drosophila members of the CIP/KIP family of cyclin-dependent kinase inhibitors. Using an S phase-specific reporter, *rnr::GFP*, we have shown that expression of CKI-1 in worms results in a cell-autonomous G_1 arrest, consistent with its role as a G_1/S regulator. Like other members of this family of CKIs, CKI-1 may function by inhibiting cyclin/cdk complexes responsible for the G_1/S transition (Sherr and Roberts, 1995).

cki-1::GFP is expressed strongly in differentiating cells, a pattern shared by mammalian p21CIP, p27KIP1 and p57KIP2. *cki-1::GFP* is also expressed dynamically in non-differentiating progenitor cells in the larva, suggesting a role for *cki-1* in coupling cell cycle progression to developmental signals. DNA quantitation has been previously used to determine the timing of S phase in certain lineages (Hedgecock and White, 1985; Euling and Ambros, 1996), wherein *cki-1::GFP* expression occurs clearly during a predicted G_1 phase while *rnr::GFP* is expressed coincident with the predicted S phase. For other cells, cell cycle phases had not been previously determined, but the reciprocal pattern of *cki-1::GFP* and *rnr::GFP* expression suggests that G_1/S is also developmentally regulated in these cells. Overall, the postembryonic pattern of *cki-1::GFP* expression correlates with developmentally regulated cell cycle progression in diverse cell lineages. This result implicates *cki-1* as a downstream effector of developmental pathways controlling postembryonic larval arrest in the absence of food, dauer larva arrest, and developmental timing during continuous development.

The continuous *cki-1::GFP* expression in intestinal cells was somewhat unexpected, since these cells undergo endoreduplicative cell cycles. A similar expression in certain endoreduplicating cells was reported for *Drosophila* Dacapo (de Nooij et al., 1996). This observation suggests that CKI-1 may play a role during endoreduplicating cell cycles, although that role is likely to be distinct from its activity in mitotic cell cycles.

cki-1 functions in developmental G_1 progression and differentiation

Since no *cki-1* mutations were available, we used RNA-mediated interference (RNAi) to analyze *cki-1* function. The most striking *cki-1(RNAi)* phenotype that we observed was the inappropriate cell cycle progression by progenitor cells in the developing larva. This RNAi phenotype is consistent with the expression of *cki-1::GFP* in numerous postembryonic lineages at times when cells are temporarily or conditionally quiescent. For example, *cki-1::GFP* expression in starved L1s and dauer larvae is consistent with the abnormal cell cycle entry at these stages in *cki-1(RNAi)* animals; similarly, *cki-1::GFP* expression in lateral hypodermal cells between molts, and in VPCs, is consistent with the extra cell divisions observed in these lineages in *cki-1(RNAi)* larvae. These results suggest a major developmental role for *cki-1* in holding progenitor cells in G_1 prior to later proliferation.

cki-1::GFP was strongly expressed in certain neurons, muscles and hypodermal cells at the terminus of cell lineages, and in dauer larva hypodermal cells, which produce a dauer-specific cuticular structure called lateral alae. We did not exhaustively monitor the effects of *cki-1(RNAi)* in all these differentiated cell types, but for specific cases, namely the differentiation of hypodermal cells in dauer larvae and at the L4 molt, *cki-1(RNAi)* had a clear effect. In *cki-1(RNAi)* animals, some hypodermal cells fail to form adult-specific alae (data not shown), and hypodermal cells in *cki-1(RNAi)* dauer larvae fail to withdraw from the cell cycle and differentiate.

The inability of cells to exit the cell cycle during differentiation likely contributes to the extensive hyperplasia in some organs in p27 or p57 deficient mice (Fero et al., 1996; Kiyokawa et al., 1996; Zhang et al., 1997), supernumerary rounds of epidermal cell division in Dacapo mutant embryos, and the inability of p27–/– oligodendrocytes to efficiently differentiate into dendrocytes (Cassaccia-Bonnefil et al., 1997).

We are confident that the *cki-1(RNAi)* phenotypes described here reflect *cki-1* loss of function. RNAi is a convenient method for reducing the level of activity of a gene for which exon sequence is available (Rocheleau et at, 1997; Fire et al., 1998). The method appears to be specific to the targeted gene, likely reflecting a mechanism involving base-pairing. In cases where genomic mutations were available for test genes targeted by RNAi, the RNAi phenotype accurately reflected the known loss-of-function phenotype, with the possible exception of members of gene families that are highly conserved in DNA sequence (Fire et al., 1998). However, *cki-1* and *cki-2* are not well conserved at the DNA sequence level, and we observed that the *cki-1(RNAi)* phenotype was the same whether the injected RNA corresponded to the entire *cki-1* coding sequence, or only a carboxy-terminal segment unrelated to *cki-2* (data not shown). It should be noted that *cki-1(RNAi)* did not lead to uniformly expressed or completely penetrant defects. For example, some cells that expressed *cki-1::GFP* either were not affected, or the effects were weak or variable. We cannot say whether this incomplete expressivity and penetrance of *cki-1(RNAi)* reflects functional redundancy with other CKIs such as *cki-2*, epistatic effects of positive effectors of cell cycle, or simply an incomplete loss of *cki-1* function. A critical test of the null phenotype of *cki-1* will require isolation of the appropriate mutant alleles.

cki-1 regulates vulva precursor cell G_1 progression

cki-1(RNAi) caused one round of precocious vulva precursor cell (VPC) divisions in the L2. The precocious VPC daughters in *cki-1(RNAi)* animals exhibited characteristics of VPCs with full competence for vulval development, suggesting that loss of *cki-1* causes VPCs to enter the cell cycle, undergo one round of division, but retain VPC developmental potential. We did not observe more than one round of precocious VPC cell division, suggesting that *cki-1* may act in parallel with other factors that control G_1 progression in VPCs. Cullins such as CUL-1 might play roles in VPC G_1 progression by regulating the degradation of G_1 cyclins (Mathias et al., 1996; King et al., 1996; Kipreos et al., 1996). However, although *cul-1* mutants display supernumerary cell divisions in the vulval cell lineages (Kipreos et al., 1996), extra VPCs were not observed (E. Kipreos, personal communication), suggesting that *cki-1* and *cul-1* are not functioning equivalently in VPCs during the L2.

The precocious VPC divisions in *cki-1(RNAi)* animals are reminiscent of the precocious vulval development of *lin-14(lf)* or *lin-28(lf)* mutants, which also commences in the L2, but with a distinct difference: in *lin-14(lf)* or *lin-28(lf)* mutants, multiple rounds of precocious divisions produce precocious differentiated vulval cells. The fact that vulval fates are not expressed until the normal time in the L3 of *cki-1(RNAi)* animals indicates that the VPCs are still subject to the normal temporal control on VPC competence and vulval cell fate determination, presumably via *lin-14* activity (Euling and Ambros, 1996).

Our analysis of the effects of *cki-(RNAi)* on VPCs clarifies the interpretation of defects observed in p27–/– mice. In the mouse mutants, organs such as the spleen and thymus were observed to be abnormally large without any obvious defects in differentiation or morphogenesis. One inference from those results was that loss of p27 caused over-proliferation of early progenitor cells in certain organs (Fero et al., 1996). The VPC duplication observed in *cki-1(RNAi)* animals validates this particular interpretation of the p27 phenotype, and suggests a general role for *cki-1/p27* in the control of organ size by regulating G_1 progression in progenitor cells. Defects in progenitor cell G_1 progression were not observed in the postembryonic development of Dacapo mutants, consistent with the observation that Dacapo expression was limited to differentiating cells (de Nooij et al., 1996; Lane et al., 1996).

cki-1 mediates a developmental checkpoint

In larvae that hatch in the absence of food, cells are arrested in G_1, and elevated levels of *cki-1::GFP* expression persist until animals begin feeding and cells reenter the cell cycle. In starved *cki-1(RNAi)* L1 larvae, certain postembryonic blast cells escape the starvation-induced G_1 arrest and enter the cell cycle prematurely. Perhaps the transition between embryonic and larval development coincides with a reduction in maternally supplied growth factors, causing an increase in *cki-1* expression and consequent cell cycle arrest. Development in the absence of food may be detrimental to the larva. It should be noted that cells in *cki-1(RNAi)* starved L1 larvae undergo only limited rounds of division, suggesting additional constraints, besides the CKI-1 level, on cell cycle progression in the absence of food.

Our observations implicating *cki-1* in a larval cell division checkpoint are analogous to the stimulation of p27KIP levels observed after serum withdrawal in fibroblasts (Coats et al., 1996), and the reduced dependence on growth factors observed in p27–/– mammalian cells (Fero et al., 1996; Coats et al., 1996). These results suggest a general role for CKIs in mediating growth factor responses.

Transcriptional control of *cki-1* expression

The developmental patterns of mRNA accumulation for *Drosophila* Dacapo and mouse p21 and p57 have suggested that CIP/KIP CKIs are transcriptionally controlled (Parker et al., 1995; de Nooij et al., 1996; Lane et al., 1996), but did not provide direct evidence of promoter-dependent expression. Our experiments demonstrate that the pattern of *cki-1::GFP* expression in *C. elegans* larvae is governed by 5′ upstream sequences, and hence suggest that *cki-1* transcription is developmentally regulated.

The spatial pattern of expression of *cki-1::GFP* depended on the length of the *cki-1* 5′ upstream sequence included in the fusion gene. The 2 kb of upstream sequence contained in *maIs109* was sufficient for expression in certain differentiated cells, while expression in specific lateral and ventral hypodermal progenitor cells required additional upstream sequences contained in *maIs113*. It should be noted that even *maIs113*, with 8 kb of upstream sequence, was not expressed

in all resting cells, particularly the P cells and some somatic gonadal progenitors. If endogenous *cki-1* is expressed in these cells, then additional sequence may be required for the full range of *cki-1* developmental expression.

The temporal dynamics of *cki-1* expression within a cell lineage may also depend on promoter sequences. For example, in the M lineage, both *maIs113* and *maIs109* were expressed in the M cell and its descendant vulval muscles, while only *maIs113* was expressed in SM, the immediate precursor to the vulval muscles. Thus, the observed dynamics of *cki-1::GFP* expression in other lineages, such as the vulva, could also be transcriptionally programmed. Substituting the *unc-54* 3′UTR for the *cki-1* 3′UTR had no discernible effect on *cki-1::GFP* expression, so mRNA translation or stability does not contribute significantly to the observed temporal patterns of *cki-1::GFP* expression. However, these results with a GFP reporter do not necessarily reflect the endogenous levels of *cki-1* mRNA, nor do they rule out the possibility that posttranscriptional regulation may be significant to the expression of endogenous CKI-1 protein. Furthermore, *cki-1* protein sequences are not included in the *maIs113* or *maIs109* transgenes, so our results do not rule out the possibility that regulated protein degradation contributes to the cell cycle dynamics of endogenous CKI-1 expression, as is the case for p27KIP1 (Hengst and Reed, 1996; Sheaff et al., 1997; reviewed by King et al., 1996).

Developmental pathways controlling cell cycle via *cki-1*

Although developmental control of the cell cycle is universal, few genetic pathways have been directly implicated in controlling the developmental signals that influence cell cycle progression. These pathways include the *anachronism* and *roughex* genes of *Drosophila* which regulate cell cycle entry in certain neuroblasts, and in the eye, respectively (Ebens et al., 1993; Thomas et al., 1994). In *Drosophila* embryogenesis, certain blastoderm cell cycles occur in spatial patterns owing to the regulation of *string* transcription by spatial patterning genes (Edgar et al., 1994).

In *C. elegans*, a genetic pathway that includes *gon-2* (Sun and Lambie, 1997) mediates feeding-dependent postembryonic cell cycling in somatic gonadal lineages. *gon-2* encodes a TRP channel protein that may mediate the transduction of signals for gonadal growth (R. Biron, Y. Sun, D. Church and E. Lambie, personal communication), resulting directly or indirectly in *cki-1* down-regulation (Fig. 8A). *cki-1* appears to be activated in dauer larvae to affect cell cycle arrest, as one consequence of a complex signaling cascade that includes *daf-7* (TGF-β; Ren et al., 1996), *daf-2* (insulin-related receptor; Kimura et al., 1997), and *daf-12* (nuclear hormone receptor; Riddle et al., 1997). Our experiments do not distinguish whether *cki-1* is regulated directly or indirectly by specific components of the dauer regulatory pathway (Fig. 8B). Interestingly, the possible role of *cki-1* as a downstream effector of the TGF-β (*daf-7*) signaling is analogous to the role of p27KIP as a mediator of TGF-β-induced epithelial cell division arrest (Polyak et al., 1994). However, *daf-7* does not activate, but rather antagonizes, dauer arrest suggesting that the effect of TGF-β/*daf-7* on downstream CKIs may be cell-type specific (Ravitz et al., 1996).

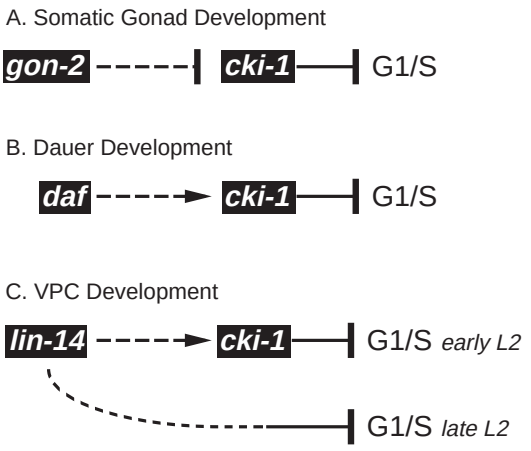

Fig. 8. Models for the role of *cki-1* in developmental cell cycle control. Proposed regulatory interactions (which could be direct or indirect) control expression of *cki-1*. (A) When larvae feed after hatching, a *gon-2*-mediated signal represses *cki-1* expression in G_1-arrested somatic gonadal cells, resulting in cell cycle entry. (B) Dauer development involves global cell cycle arrest, mediated by a pathway of regulatory genes (*daf*) that activate *cki-1* activity and lead to G_1 arrest. (C) In wild-type animals, *lin-14* activates *cki-1* to prevent the VPC G_1/S transition in the early L2. *lin-14* also functions (likely independently of *cki-1*) to block VPC G_1/S during the late L2.

A pathway of heterochronic genes, including *lin-14* and *lin-28* and their repressor, *lin-4* (Lee et al., 1993; Moss et al., 1997), controls the timing of G_1 progression and developmental competence in VPCs (Euling and Ambros, 1996). Our observation that *cki-1::GFP* expression is reduced specifically in the VPCs of *lin-14(0)* animals strongly suggests that *cki-1* is activated in VPCs by *lin-14*. *lin-14* and *lin-28* expression are mutually interdependent (Moss et al., 1997), so we cannot say whether *cki-1* is a direct target of either (or both) of *lin-14* or *lin-28*. Since the *cki-1(RNAi)* phenotype results in precocious VPC division, but is not identical to the *lin-14(0)* VPC defect, we conclude that *lin-14* acts via *cki-1* to keep VPCs in G_1 during the first half of the L2 stage, but acts independently of *cki-1* to maintain VPC G_1 through the end of L2 (Fig. 8C). Further molecular and biochemical analysis are required to determine precisely how *cki-1* is targeted for regulation by the heterochronic pathway in VPCs. *lin-31* and *lin-25* mutants also display precocious VPC divisions, although the *lin-25* defect is of low penetrance (Ferguson et al., 1987), and in the case of *lin-31*, the precocious divisions can sometimes produce neuroblasts (Miller et al., 1993). Despite these differences between *lin-25*, *lin-31* and *cki-1(RNAi)* VPC phenotypes, these observations suggest that *lin-31 and lin-25* may function with *lin-14* to potentiate *cki-1* activity in VPCs.

We are grateful to Rebecca Burdine and Michael Stern for *egl-17* plasmids and worm strains, the Sanger Center for providing cosmid T05A6, Andy Fire for plasmids, Eric Lambie for worm strains, and to the Stern, Kipreos and Rothman labs for sharing information prior to publication. We also thank Eric Moss, Philip Olsen, Edward Kipreos and Eric Lambie for discussion, and Rosalind Lee for assistance. R. R. was supported by a Human Frontiers Science Program fellowship. This work was supported by a U. S. Public

Health Service research grant GM34208 (V. A.). Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by NIH National Center for Research Resources (NCRR).

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