Mutations in the clk-2 gene of the nematode Caenorhabditis elegans affect organismal features such as developmental, behavior, reproduction, and aging as well as cellular features such as the cell cycle, apoptosis, the DNA replication checkpoint, and telomere length. clk-2 encodes a novel protein (CLK-2) with a unique homologue in each of the sequenced eukaryotic genomes. We have studied the human homologue of CLK-2 (hCLK2) to determine whether it affects the same set of cellular features as CLK-2. We find that overexpression of hCLK2 decreases cell cycle length and that inhibition of hCLK2 expression arrests the cell cycle reversibly. Overexpression of hCLK2, however, renders the cell hypersensitive to apoptosis triggered by oxidative stress or DNA replication block and gradually increases telomere length. The evolutionary conservation of the pattern of cellular functions affected by CLK-2 suggests that the function of hCLK2 in humans might also affect the same organisinal features as in worms, including lifespan. Surprisingly, we find that hCLK2 is present in all cellular compartments and exists as a membrane-associated as well as a soluble form.

Identifying and studying the processes and the genes that are involved in determining the rate of aging is a challenging area of modern genetics. In particular, it would be of interest to determine whether the activity of specific genes limits human life span. Several epidemiological studies of centenarians are being carried out with this goal in mind (1). However, given the pervasive evolutionary conservation of physiological processes among organisms, a practical approach to find genes that might be involved in human aging is to first investigate the genetic basis of aging in lower organisms. The nematode genetic model system, Caenorhabditis elegans, is being extensively used to test this end, and a number of genes that have been identified in this organism for their effect on aging are now also being studied in vertebrates (2, 3).

The clk-2 mutants of C. elegans display a pleiotropic phenotype (reviewed in Ref. 4) that includes a slowing down of numerous physiological processes, including embryonic and postembryonic development, behavioral rates, and reproduction (5). clk-2 mutants also show an increase in life span that is particularly dramatic in combination with mutations in other genes, such as clk-1 and daf-2 (2, 6). The clk-2 mutants are temperature-sensitive (5, 7) and at 25 °C produce a lethal embryonic phenotype resulting in differentiated but highly disorganized embryos (5). This is likely to be the null phenotype, since it is also produced by RNA interference at all temperatures. Extensive temperature shift experiments have demonstrated that clk-2 is required for embryonic development only during a narrow time window in which oocyte maturation, fertilization, the completion of meiosis, and the initiation of embryonic development occurs (5). However, these events as well as subsequent embryonic development appear to proceed entirely normally until the 100-cell stage, after which aberrant development becomes apparent. Surprisingly, all clk-2 phenotypes, including the phenotypes observed in adults that are ~1000 times larger than the eggs produced by the mother, are rescued by a maternal effect; i.e. homozygous mutant animals, issued from a heterozygous mother, appear wild type. This maternal rescue effect suggests that the presence of maternally provided clk-2 product might induce a self-maintained epigenetic state, although the possibility that the maternally provided clk-2 product can still function efficiently after extreme dilution cannot be excluded.

A number of cellular phenotypes of clk-2 mutants have also been identified in addition to the organisinal phenotypes described above (7, 8). For example, the germ lines of clk-2 mutants do not respond normally to ionizing radiation. In the wild type, irradiation leads to cell cycle arrest in the mitotic phase of the germline and to apoptotic cell death in the meiotic phase of the germ line. Both of these responses are abolished in clk-2 mutants. In addition, clk-2 mutants fail to respond with cell cycle arrest to treatment with hydroxyurea (HU), a drug that blocks DNA replication, suggesting a defect in the S-phase replication checkpoint. Taken together, these cellular phenotypes suggest that clk-2 mutants are defective in important aspects of the normal cellular response to DNA damage.

The C. elegans clk-2 gene encodes a protein of 877 amino acids that is similar to Saccharomyces cerevisiae Tel2p and has a unique homologue in every eukaryotic genome sequenced to date (5). Yeast cells carrying the hypomorphic tel2-1 mutation grow slowly and have short telomeres (9). The telomeres shorten gradually in the tel2 cells, reaching their shortest lengths only after ~150 generations. In addition to affecting the length of telomeres, Tel2p has also been shown to be involved in DNA replication checkpoint, telomere length checkpoint, and cell cycle arrest to treatment with HU, apoptosis during DNA damage, meiosis, and cell cycle arrest to treatment with HU.
hCLK2 Function in Mammalian Cells

**TABLE 1**

| Name (ATCC No) | Tissue derivation | Hygromycin units/ml |
|----------------|-------------------|---------------------|
| C2C12 (CRL-1772) | Mouse myoblast | 400                 |
| Rat-1 R12 (CRL-2210) | Rat fibroblast | 200                 |
| A549 (CCL-188) | Human lung carcinoma | 400             |
| SK-N-AS | Human neuroblastoma | 400               |
| SK-HEP-1 | Human liver adenocarcinoma | 400          |
| HT-1080 | Human fibrosarcoma | 400               |
| 293 | Human kidney carcinoma | 400            |
| MCH58 | Human fibroblast | 100                 |

Preparation of Antibodies Directed against hCLK2 Protein—Two separate antibodies were used to develop anti-hCLK2 polyclonal antibodies. The first antigen was generated as follows. A PCR fragment corresponding to bases 1516–1929 of the hclk2 clone hko2952, encoding amino acids 414–551 of hCLK2, was cloned into the pGEX-3X expression vector (Amersham Biosciences). A GST-hCLK2 (414–551) protein of the expected size (≈46 kDa) was expressed in DH10b bacteria and purified by affinity chromatography on a GST slurry. This recombinant protein was injected into two rabbits (2779 and 2780) to obtain polyclonal antibodies. To generate the second antigen, a PCR fragment corresponding to bases 279–1519 of the hclk2 clone hko2952, encoding amino acids 2–415 of hclk2, was cloned into the pGEX-3X expression vector. A GST-hCLK2 (2–415) protein of the expected size (~78 kDa) was expressed in DH10b bacteria and was purified from bacterial inclusion bodies. This recombinant protein was injected into two rabbits (2838 and 2839) to obtain polyclonal antibodies. All four sera specifically react to hCLK2 by the following criteria. The terminal bleb of each rabbit recognizes the corresponding bacterial antigen, in vitro translated hCLK2, a band at the expected size of ~100 kDa in cell extracts, and a strong band of the same size in cells over-expressing hCLK2 (see Table 1). This ~100-kDa band is not detected by any of the preimmune sera. Moreover, this band disappears upon preabsorption of the antibody with the corresponding purified GST-hCLK2 protein, but not upon preabsorption with other unrelated bacterially expressed proteins, including GST fusions. Also, the intensity of this band is drastically reduced in hclk2-siRNA-treated cells as compared with controls. The serum from rabbit 2780 gave the strongest reaction and was used for immunoblot analyses throughout this study.

Immunoblot Analysis— Cultured cells were trypsinized and pelleted and then resuspended in 5× volumes of extraction buffer (500 mM NaCl, 20 mM Tris, pH 8.0, 1% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors (Roche Applied Science)). The resuspended cells were submitted to five freeze-thaw cycles (frozen in liquid nitrogen and thawed at 37 °C). Cell debris were removed by centrifugation, and the quantity of protein was measured (Bio-Rad protein assay). 50 µg of protein were separated on 7.5 or 12% polyacrylamide gels and transferred to polyvinylidene difluoride cellulose. The membranes were preincubated in blocking solution (TBS-T plus 5% nonfat milk) at room temperature for 1 h and then incubated with the primary antibody at 4 °C overnight at the following concentrations: rabbit anti-hCLK2 antibody (1:500 to 1:1000), mouse anti-a-tubulin antibody (1:10000; Sigma), rabbit anti-actin antibody (1:500; Sigma), mouse anti-cytocrome c (1–2 µg/ml; Molecular Probes, Eugene, OR), and mouse anti-p300 (2 µg/ml; Upstate Biotechnology, Inc., Lake Placid, NY). After 3 × 15 min TBS-T washes, the membranes were incubated in blocking solution at room temperature for 2 h. The membranes were then incubated with donkey anti-rabbit IgG secondary antibody (1:3000; Jackson Immunoresearch Laboratories) or goat anti-mouse IgG (1:20000; Pierce) at room temperature for 1 h, followed by three 15-min TBS-T washes. Finally, the signal was detected by chemiluminescence (Amersham Biosciences).

Immunostaining— Cells were transiently transfected with a plasmid, and 24 h later, they were seeded on coverslips. Forty-eight hours later, the coverslips were fixed in 4% paraformaldehyde/TBS for 10 min, washed 3× in acetone at 4 °C, then incubated at room temperature for 1 h with rabbit polyclonal anti-hCLK2 (2780, 2838, 1:100–1000), followed by biotinylated goat anti-rabbit or mouse IgG (1:5000) for 1 h. Finally, the cells were incubated with fluorescein-conjugated streptavidin (10 µg/ml) for 30 min and viewed under a Leitz fluorescence microscope. Similar results were obtained with 2780 and 2838 antisera. The pattern observed for the secondary antibody alone is not detected by the primary or the secondary antibody alone. In addition, the observed pattern does not appear upon preabsorption of the antibody with the corresponding purified GST-hCLK2 protein but not upon preabsorption with other unrelated bacterially expressed proteins, including GST fusions.
**TABLE II**

| Treatment          | Working concentration | Time of treatment |
|--------------------|-----------------------|-------------------|
| Etoposide          | 100 μM                | 24 h              |
| Sodium azide       | 15 μM                 | 48 h              |
| Menadione          | 12 μM                 | 24 h              |
| Anisomycin         | 2 μM                  | 16 h              |
| t-Butyl hydroperoxide | 40 μM               | 48 h              |
| Staurosporine      | 2 μM                  | 24 h              |
| All-trans-retinoic acid | 4 μM               | 96 h              |
| Hydrogen peroxide  | 0.5 μM                | 24 h              |
| Juglone            | 0.5 μM                | 24 h              |
| Hydroxyurea        | 0.6 mM                | 96 h              |
| Tunicamycin        | 5 μg/ml               | 24 h              |

**Growth Rate Assay**—Cells were seeded in six-well dishes at 1 × 10^5/well. At the times indicated, the cells were trypsinized and counted with a hemocytometer.

**Cell Death Assay**—Cells were seeded at 1 × 10^5 in six-well dishes. The next day, the cells were treated by γ-ray (20 gray) and counted 72 h later. A series of different apoptosis-inducing agents was also investigated, and the cells were analyzed at various times following treatment (see Table II). Cell viability was measured by the trypan blue exclusion method, by counting with a hemocytometer.

**Measuring the Length of Telomeres**—Genomic DNA from cultured cells was recovered by phenol-chloroform extraction and ethanol precipitation. 10 μg of DNA was digested by HindIII and RsaI (10 units/μg DNA) at 37°C overnight. The completely digested DNA was separated on 0.7% agarose gel at 23 V for 24 h and transferred by capillary transfer to a positively charged nylon membrane (Amersham Biosciences) overnight. The telomere-specific sequence (5′-TTAGGGTTAGGG-3′) was used as a probe to detect telomeric repeats. The membrane was incubated in prehybridization solution (5× SSC, 0.1% SDS, and 5′-32P-end-labeled probe) at 37°C. The membrane was then washed in 3× SSC, 0.1% SDS at 42°C for 3 × 10 min and exposed at room temperature overnight.

**Preparation of Subcellular Fractions**—Subcellular fractionation was performed as described (18). From 1 to 10^7 cells, the cells were washed twice with ice-cold PBS and resuspended in buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, protease inhibitors (Roche Applied Sciences)) at a concentration of 2 × 10^7 cells/ml. Cells were homogenized on ice (10–20 strokes at 1000 rpm; Potter-Elvehjem) until 95% of the cells were lysed based on trypan blue dye uptake. The samples were transferred to 1.5-mL Eppendorf centrifuge tubes (1 mL/tube) and centrifuged at 500 × g for 5 min to pellet the nuclei. The nuclear pellet was then resuspended in 0.5–2 mL of 1.6 M sucrose containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂. After underlayering with 1–2 mL of 2.0–2.3 M sucrose containing the same buffer and centrifugation at 150,000 × g for 60 min, the resulting nuclear pellets were resuspended in 0.1–0.3 mL of 1% Triton X-100-containing buffer (0.15 M NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA, 1% Triton X-100). The supernatant resulting from the initial low speed centrifugation was subjected to centrifugation at 10,000 × g for 15 min at 4°C to obtain the heavy membrane fraction (a pellet that should include mitochondria, lysosomes, Golgi, and rough endoplasmic reticulum). The supernatant was centrifuged for 60 min at 15,000 × g to obtain the light membrane fraction (a pellet that should include the smooth and rough endoplasmic reticulum) and the cytosolic fraction (supernatant). The heavy membrane and light membrane fractions were resuspended in 1% Triton-containing lysis buffer. An equal amount of protein (50 μg) from each fraction was analyzed by immunoblot.

**RESULTS**

**Growth Stimulation by Overexpression of hCLK2 in SK-HEP-1 Cells**—To achieve high levels of hCLK2 expression in cultured cells, we used a retroviral vector expressing hCLK2 to infect a panel of cell lines (see “Experimental Procedures”) and established stable cell lines, derived from pools of cells infected with the vector expressing hCLK2 or the empty vector control. A high level of hCLK2 expression was detected in all of the established cell lines (Fig. 1A and data not shown). The next day, the cells were treated by siRNA directed against firefly luciferase or by buffer alone. The immunoblots were probed with an anti-actin antibody to control for equal loading of total protein (50 μg in each lane).

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**Reducing the Level of hCLK2 Expression Causes Reversible Growth Arrest**—To investigate the consequences of a loss of function of hclk2, we used the siRNA technique (17). SK-HEP-1 cells were treated with either 1) hclk2-specific siRNA, 2) siRNA for luciferase, a gene that is not normally found in human cells, or 3) the same volume of siRNA annealing buffer. The level of hCLK2 and the cell number were determined daily for several days following siRNA treatment (Figs. 1B and 2B). The immunoblots demonstrate that when the cells were treated with hclk2-specific siRNA, the level of hCLK2 was significantly decreased by day 2 and remained low until at least day 6. As expected, neither luciferase siRNA nor siRNA annealing buffer alone resulted in a decrease of the expression of hCLK2. In addition, the expression of actin was not affected by hclk2-specific siRNA, luciferase siRNA, or siRNA annealing buffer alone (Fig. 1B). hclk2 siRNA treatment dramatically slowed cellular growth rate, in contrast to treatment with luciferase siRNA, which had only a minor effect (Fig. 2B). The effect on growth rate lasted until day 7, after which time the cells appeared to recover from the treatment and resumed growth. No increase in cell death or...
TUNEL-positive nuclei was observed in cells treated with the means and S.E. of triplicate experiments are shown.

1.5 treated by siRNA or buffer the next day (day 1) at a density of about hCLK2 and control cells were plated at a density of 1/H11003 level of expression of hCLK2. A highly toxic hydroxyl radical (21) (Fig. 3B). The cells were also hypersensitive to two methods of increas-

other obvious changes were observed, indicating that the arrest was not the consequence of major damage to the cells. Treated cells were also sorted by fluorescence-activated cell sorting according to DNA content (data not shown). The arrested cells treated with hclk2 siRNA did not appear to have arrested in any particular phase of the cell cycle.

Overexpression of hCLK2 Produces Hypersensitivity to Apoptosis Triggered by Oxidative Stress or DNA Replication Block—Prompted by the findings in the germ line of C. elegans, where clk-2 mutations affect the response to ionizing radiation and to DNA replication block induced by HU, we investigated the response of SK-HEP-1 cells overexpressing hCLK2 to 10 different agents capable of inducing apoptotic cell death as well as to HU and γ-rays. The cells overexpressing hCLK2 did not show any general increase in sensitivity to apoptotic stimuli but were specifically hypersensitive to two methods of increasing oxidative stress: menadione treatment, which leads to intracellular overproduction of superoxide (20), and t-butyl hydroperoxide treatment, which leads to the production of the highly toxic hydroxyl radical (21) (Fig. 3A). The cells were also hypersensitive to the DNA synthesis inhibitor HU (Fig. 3A). To verify that the cell death observed was indeed apoptotic, we stained the cells using the TUNEL method (22), which consists of in situ labeling of the 3′-OH ends of the cleaved DNA typical of apoptotic cells. A significant increase in the number of TUNEL-positive nuclei was observed in cells treated with the compounds that produced increased cell death compared with controls, namely menadione, t-butyl hydroperoxide, and hydroxyurea (Fig. 3B).

We have also investigated the response of siRNA-treated SK-HEP-1 cells and found that the cells depleted for hCLK2 did not show any general increase in sensitivity to apoptotic stimuli (data not shown). It is unclear whether a reduction in hCLK2 levels has no effect on the sensitivity of the cells to the agents used or whether the arrest produced by siRNA treatment prevents the detection of any effect.

Overexpression of hCLK2 Gradually Lengthens Telomeres—To investigate whether hclk2 affects telomere length in human cells, as it does in S. cerevisiae and in C. elegans, we determined the telomere length of SK-HEP-1 cells overexpressing hCLK2 and of SK-HEP-1 control cells by Southern blot analysis. We examined the telomere length at regular intervals during prolonged culturing (138 population doublings) (Fig. 4). The telomere length of the cells overexpressing hCLK2 gradually grew longer at an average rate of ~15 bp/population doubling, whereas it remained absolutely stable in the control cells (Fig. 4). Additional population doublings do not appear to increase telomere length further (data not shown).

hCLK2 Is Present in Most Compartments of the Cell—To determine the subcellular localization of hCLK2, we used im-
municytochemistry to detect native and overexpressed hCLK2 in SK-HEP-1 cells. The level of native hCLK2 appeared to be too low to be detectable by this method with our antisera directed against hCLK2 (see “Experimental Procedures”). However, in cells overexpressing hCLK2, the signal appeared to be everywhere in the cell, filling both the cytoplasm and the nucleus (Fig. 5A). The same distribution was also observed in another overexpressing cell line HT-1080 (Fig. 5B). Controls included immucytochemistry using the preimmune sera, the secondary antibody alone, and sera preabsorbed with a number of bacterial antigens. We determined the subcellular distribution of hCLK2 by immucytochemistry following treatment with etoposide and menadione, two apoptotic triggering agents, which result in DNA replication inhibition and oxidative stress, respectively (see above). No changes in the subcellular distribution of hCLK2 were observed (data not shown).

To clarify whether this ubiquitous distribution of hCLK2 was a nonspecific result of overexpression, we expressed hCLK2 in the HT-1080 cell line (23) under an inducible promoter. Expression in these cells produced the same ubiquitous expression at all levels of induction, over a 20-fold range (data not shown).

As an independent test of subcellular distribution of hCLK2, we carried out subcellular fractionation and immunoblot analysis. We found that both native and overexpressed hCLK2 in SK-HEP-1 cells were present in all subcellular fractions, including in the nuclear, heavy membrane (which includes mitochondria), light membrane, and cytosolic fractions (Fig. 6A). Surprisingly, the levels of hCLK2 in each fraction were almost identical. This was true for both the low levels of native hCLK2 and the higher levels found in cells that overexpressed hCLK2. Control proteins showed the expected distributions (Fig. 6A).

Since an identical amount of protein is loaded on the gel for each fraction, this demonstrates that the concentration of hCLK2 relative to other proteins is very similar in all compartments.

hCLK2 can be both soluble and membrane-associated—At least one form of the hCLK2 protein is clearly soluble, since it is present in the cytosolic fraction. To characterize hCLK2 in the membrane fractions, we used alkaline sodium carbonate to treat the nuclear, heavy membrane, and light membrane fractions from overexpressing SK-HEP-1 cells. For the membrane fractions, most of the hCLK2 cannot be extracted by sodium carbonate and is detected in the pellets (Fig. 6B). However, for the nuclear fraction, almost equal amounts of hCLK2 were found in the pellet and supernatant, which is consistent with the immucytochemical observation of hCLK2 in the nucleoplasm (Fig. 5). Since sodium carbonate treatment is capable of solubilizing peripheral membrane proteins, these observations also indicate that hCLK2 can be relatively tightly associated with the membrane in all three types of subcellular fractions. As is also evident from Fig. 6B, there is no substantial difference in molecular size between soluble and membrane-associated hCLK2.
hCLK2 Function in Mammalian Cells

DISCUSSION

CLK-2 Function Is Conserved from Yeast and Worms to Humans—The study of mutants of tel2, the S. cerevisiae homologue of hclk2, have implicated the gene product Tel2p in the regulation of telomere length and subtelomeric silencing as well as in an undefined function necessary for cell viability (9). In worms, clk-2 mutations have been shown to affect numerous processes, including organismal features such as organized embryonic development, developmental rate, behavioral rates, and reproduction (6, 24) as well as cellular features such as the apoptotic death and mitotic arrest responses to irradiation and DNA replication block (5, 7, 8) (reviewed in Ref. 4).

The broad pleiotropy observed in clk-2 mutants suggests that, in worms, the function of CLK-2 links important cellular processes such as cell cycle control, apoptosis, and telomere length regulation. Alternatively, the pattern of effects observed in C. elegans might result from some difficult-to-disentangle series of secondary effects specific to this organism. To study this further, we have investigated the function of the human clk-2 homologue (hclk2) in SK-HEP-1 human hepatoma cells. We find that overexpression of the hCLK2 protein decreases the population doubling time (Fig. 2A) and that knocking down the expression of hCLK2 with siRNAs produces reversible growth arrest (Fig. 2B). We also find that overexpression of hCLK2 results in an increased apoptotic response to oxidative stress and HU treatment but not to other treatments that induce apoptosis (Fig. 3). Finally, we find that overexpression of hCLK2 gradually, but dramatically, increases telomere length (Fig. 4). These findings indicate that CLK-2 and its homologues affect the same set of cellular processes in yeast, worms, and humans and suggest the possibility that it could also affect in humans the same set of organismal processes that are affected in worms, including life span.

hclk2 and Regulation of Telomere Length—In yeast, the tel2-1 mutation produces a gradual decrease in telomere length (9). In worms, however, the partial loss-of-function clk-2(qm37) mutation produces an overall lengthening of telomeres (5). In the SK-HEP-1 hepatoma cells, we now find that overexpression of hCLK2 clearly increases telomere length (Fig. 4). This suggests that a loss of function of the gene hclk2 would shorten telomeres, since it is the case in the yeast tel2-1 mutant, but contrary to the clk-2(qm37) mutant in the worm. Note that the effect of overexpression of Tel2p in yeast has not been reported. How can we reconcile these findings?

One relatively unlikely possibility is that the clk-2(qm37) mutation is not a simple loss-of-function mutation but instead produces also a recessive gain of function that results in telomere lengthening. Another possibility, suggested by the observation made on individual worm telomeres, is that the effect of clk-2 on telomeres is context dependent. Indeed, upon examination of individual worm telomeres, which can be visualized with probes that are specific to the non-repeat parts of terminal restriction fragments (5), it was observed that some individual telomeres, but not all, were shortened by overexpression of wild-type clk-2. This indicates that different telomeres react differently, indicating that the effect of clk-2 on telomere length is context dependent. Furthermore, when telomere length is examined in worms, the DNA is extracted from whole worms at a variety of developmental stages. Overall, a lengthening of telomeres is observed, but, if the telomeres of a minor cell type were affected differently, this would probably not be detected. On the other hand, the SK-HEP-1 hepatoma cells represent a single cell type. One view, therefore, is that the Tel2p/CLK-2/hCLK2 protein is involved in a relatively complex network of processes that ultimately impinge on telomere length and that this network’s reaction to perturbation might depend on the organism or cell type. Note that the telomere lengthening is very gradual, which suggests that telomerase is the ultimate effector of length changes and not other mechanisms such as alternative lengthening of telomeres (25).

hclk2 and Apoptosis—After ionizing irradiation treatment, a sharp increase in apoptosis is observed in the meiotic phase of the germ line of wild-type worms but not in clk-2 mutants (7). We find that hCLK2 overexpressing cells are hypersensitive to HU and undergo apoptotic death in response to treatment with this compound. It should be noted, however, that HU is also an oxidating agent (26) and that its effect in our system might be similar to that of other compounds that generate reactive oxygen species.

Since we have not found evidence suggesting that an increased sensitivity of the overexpressing cells to agents or treatments that can damage DNA directly, such as etoposide (an inhibitor of topoisomerase) and irradiation, it is possible that the failure to respond appropriately to irradiation and HU in worms does not reveal a specific defect in a DNA damage checkpoint but is the result of a decreased sensitivity to oxidative stress and/or a failure to respond appropriately to oxidative injury.

hclk2 and Cell Cycle Progression—We found that knocking down hCLK2 levels with siRNA treatment almost completely arrests the cell cycle and that overexpressing hCLK2 shortens cell doubling time. This finding indicates that the activity of hCLK2 is necessary for cell cycle progression and that the level of hCLK2 is limiting for cell cycle progression, at least in SK-HEP-1 cells. Since the cells do not appear to arrest in any particular phase of the cycle, hCLK2 is probably not associated with any of the particular mechanisms that allow cells to pass from one phase to the next, such as DNA damage checkpoints. However, one cannot exclude the possibility that the activity of hCLK2 links DNA damage to progression of the cell cycle as a whole.

In worms, somewhat paradoxically in view of the results just described with hCLK2, partial loss of function of clk-2 leads to a failure to arrest the cell cycle in response to irradiation and HU injury. However, in the absence of an understanding of the molecular function of hCLK2/CLK-2/Tel2p, it is difficult to speculate on the significance of these differences, since we cannot know which aspects of the function of CLK-2 have been lost and which have been retained in the two temperature-sensitive point mutants that have been characterized.

hCLK2 Is Present in All Cellular Compartments—In order to understand how hCLK2 can affect diverse processes, we have determined its cellular location, using immunocytochemistry and subcellular fractionation. Surprisingly, we find that hCLK2 is present in most, and maybe all, subcellular compartments. Furthermore, hCLK2 is present both as a soluble and a membrane-associated form. Many proteins can have unexpected multiple cellular locations. For example, Bcl-2 is localized in the outer mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum membrane (27). Also, yeast major adenylate kinase (Adk1p/Aky2p) is both mitochon-
drial and cytosolic (28). Moreover, some proteins can shuttle between different locations depending on signaling events. For example, catenin and associated proteins can be cytoskeletal, cytoplasmic, or nuclear (29). However, to our knowledge there is no previous example of a protein present in so many different cellular compartments at the same time and in similar amounts.

In its membrane-bound form, hCLK2 is tightly associated with the membrane, since it cannot be extracted by alkaline sodium carbonate treatment, which extracts peripheral membrane proteins. Furthermore, the molecular weight of membrane-associated hCLK2 is indistinguishable from that of the soluble form. Since hCLK2 has no predicted transmembrane domain, it is possible that it associates tightly with an integral membrane protein or, maybe covalently, with a lipid.

**What Is the Molecular Function of hCLK2/CLK-2Tel2p—** To speculate what the molecular function of hCLK2 and its homologues might be, we have to take into account the pleiotropy of its action and its unusually broad cellular distribution. One should also note that the amino acid sequence of hCLK2 is not evolutionarily well conserved. This suggests that this protein does not enter into high specificity protein-protein interactions, which might be expected to constrain protein evolution. What sort of function is carried out everywhere in the cell, but does not involve very specific interactions with several other proteins? One possibility is that hCLK2 participates in a form of membrane homeostasis. The exact composition of each membrane leaflet determines structural properties of membranes as well as the function of membrane proteins. Both soluble and membrane-associated hCLK2 could bind a membrane lipid, aid its integration into the membrane, and regulate its abundance in the membrane. Membrane lipids are small and relatively abundant compared with proteins, which would help to explain the relatively large pool of soluble hCLK2, which would bind the nonmembrane pool of the lipid.

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