males gave pant-grunts to high-ranking and some middle-ranking females and received them from low-ranking females and some other middle-ranking females. Low-ranking females rarely, if ever, received pant-grunts from any adult females but often gave them to middle- and high-ranking females. Fourteen females were assigned ranks of 1.5 or 2.5 during transitional periods.
25. Individuals are weighed by luring them up a rope attached to a spring balance by placing a piece of banana in a tin attached to the top of the rope. We fitted the natural logarithm of all weights of nonpregnant females to a curve using LOWESS. For females carrying infants, we subtracted the weight of an average infant of that age. This method makes no assumptions about the underlying shape of the curve, so the curve reflects the patterns in the data as accurately as possible. This standardizes weights by age, so we can find the residuals for a specific individual and determine whether she is bigger or smaller than average. We then regressed the average residual for each individual against dominance and found no significant relation. Thus, high-ranking individuals are not larger or smaller than lower ranking chimps.
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29. Differences in survival are not statistically significant, but there is a suggestive trend with a Breslow-Gehan test ($P = 0.07$; $X^2 = 3.22$) when females who reach age 20 are placed into two ranks at age 21, high ($n = 9$) and low ($n = 6$). This test compensates for the reduction in sample size at later ages by weighting earlier deaths more heavily. However, statistical power of the test remains low because $n = 15$.
30. There were 11 deceased females, including the sterile female, GG, who were observed from or before the estimated age of 14 years (approximate age at first birth), whose rank and lifetime reproductive success could be determined. The relation between number of offspring surviving to 5 years and average rank is not significant when GG is included ($R^2 = 0.10$, $P = 0.34$, $n = 11$) but is significant when she is excluded ($R^2 = 0.60$, $P < 0.01$, $n = 10$).
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Bypass of Senescence After Disruption of p21$^{CIP1/WAF1}$ Gene in Normal Diploid Human Fibroblasts

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Most somatic cells die after a finite number of cell divisions, a phenomenon described as senescence. The p21$^{CIP1/WAF1}$ gene encodes an inhibitor of cyclin-dependent kinases. Inactivation of p21 by two sequential rounds of targeted homologous recombination was sufficient to bypass senescence in normal diploid human fibroblasts. At the checkpoint between the prereplicative phase of growth and the phase of chromosome replication, cells lacking p21 failed to arrest the cell cycle in response to DNA damage, but their apoptotic response and genomic stability were unaltered. These results establish the feasibility of using gene targeting for genetic studies of normal human cells.

The replicative life-span of somatic cells reflects the number of cell divisions, not chronological time, and may contribute to organismic aging (*1*). Shortening of telomeres may be the molecular mechanism that triggers an irreversible arrest, referred to as senescence, of the prereplicative phase of growth in the cell cycle (G_1) (*2*). Genes that have been implicated in regulating senescence include tumor suppressors p53 (*3*) and RB1 (*4*), cyclin-dependent kinase (Cdk) inhibitors p21$^{CIP1/WAF1}$ (*5*) and p16^{INK4a} (*6*), and several currently unidentified genes (*7*). Viral oncoproteins that interfere with p53 and RB1 cause bypass of senescence and extended life-span, followed by a decline designated as crisis (*8*). Two limitations have hampered studies of human senescence. First, viral oncoproteins may not completely inactivate their targets. Second, studies in rodents cannot be extrapolated to humans because of interspecies differences in the mechanisms of senescence and immortalization (*9*).

Introduction of null mutations into a cellular gene is a direct and unambiguous way to test the function of that gene. We were concerned that normal human diploid fibroblasts (HDFs) would senesce before two sequential rounds of gene targeting could be completed. Therefore, we developed strategies for efficient gene targeting in somatic cells (*10*), established a culture system that allows high single-cell cloning efficiency (*11*), and generated a new cell strain (LF1) (Fig. 1A) (*12*).

LF1 cells (5×10^7 cells) at passage 7 were electroporated with a targeting vector containing a neomycin (*neo*)-resistance gene (Fig. 2A) (*13*). Twenty colonies were obtained and expanded into clonal cell strains. Southern (DNA) blotting analysis showed that three clones (HE1.2-1, HE1.3-2, HE3.2-1) (*14*) contained one targeted p21 gene copy (Fig. 2B). HE1.3-2 cells (5×10^7 cells) were electroporated with a vector containing a hygromycin (*hyg*)-resistance gene (Fig. 2A), yielding 24 clones, one of which (HO7.2-1) had targeted the second p21 gene copy (Fig. 2B), and two of which had retargeted the *neo*-targeted gene copy (*15*, *16*). Protein immunoblotting analysis confirmed that the HO7.2-1 clone did not express p21 protein (Fig. 2C).

The hygromycin-resistant colonies were expanded into cell strains and passaged until senescence (Fig. 1C) (*17*). The 21 nontargeted (p21 +/−) strains senesced between passages 2 and 10 (mean passage 6.76 ± 2.55 SD) (*18*), whereas the HO7.2-1 strain did not cease proliferation until passage 19, when it displayed signs of crisis (*19*). During the period of extended life-span, no cell death was evident in HO7.2-1 cultures. Because in our experimental regimen one passage is equivalent to a minimum of two population doublings (PD) (*11*), loss of p21 resulted in quantitatively the same life-span extension (20

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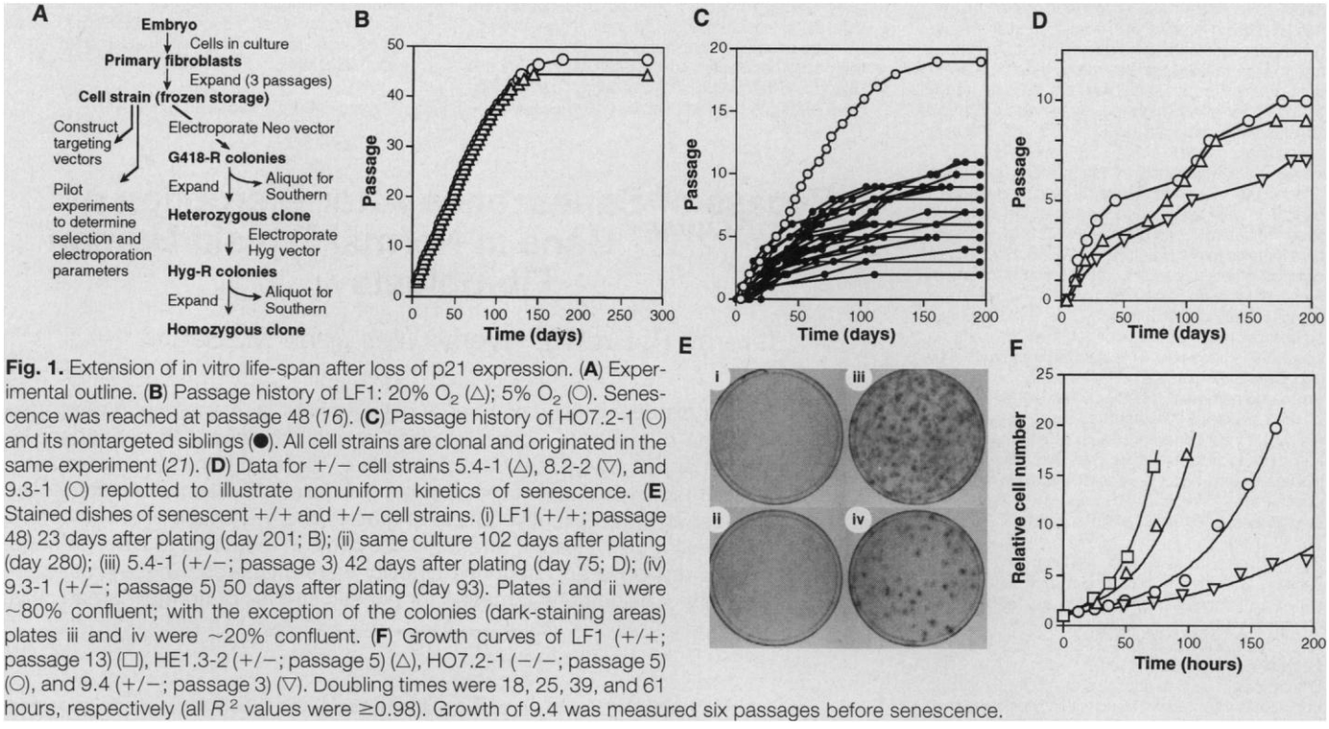

Fig. 1. Extension of in vitro life-span after loss of p21 expression. (**A**) Experimental outline. (**B**) Passage history of LF1: 20% O_2 (△); 5% O_2 (○). Senescence was reached at passage 48 (*16*). (**C**) Passage history of HO7.2-1 (○) and its nontargeted siblings (●). All cell strains are clonal and originated in the same experiment (*21*). (**D**) Data for +/− cell strains 5.4-1 (△), 8.2-2 (▽), and 9.3-1 (○) replotted to illustrate nonuniform kinetics of senescence. (**E**) Stained dishes of senescent +/+ and +/− cell strains. (i) LF1 (+/+; passage 48) 23 days after plating (day 201; B); (ii) same culture 102 days after plating (day 280); (iii) 5.4-1 (+/−; passage 3) 42 days after plating (day 75; D); (iv) 9.3-1 (+/−; passage 5) 50 days after plating (day 93). Plates i and ii were ~80% confluent; with the exception of the colonies (dark-staining areas) plates iii and iv were ~20% confluent. (**F**) Growth curves of LF1 (+/+; passage 13) (□), HE1.3-2 (+/−; passage 5) (△), HO7.2-1 (−/−; passage 5) (○), and 9.4 (+/−; passage 3) (▽). Doubling times were 18, 25, 39, and 61 hours, respectively (all R^2 values were ≥0.98). Growth of 9.4 was measured six passages before senescence.

to 30 PD) as the introduction of SV40 large tumor antigen (T-Ag) (*20*).

Most p21 +/− strains senesced with unusual kinetics (Fig. 1D). Rather than the rapid and irreversible decline in growth characteristic of normal HDFs (Fig. 1B) (*21*) many +/− strains resumed growth after an initial decline in proliferation. Cultures with a high proportion of senescent cells spontaneously gave rise to colonies of healthy cells (Fig. 1E); it was possible to clone some of those colonies and propagate them for several passages. The same phenomenon occurred with an independently *neo*-targeted p21 +/− strain (HE3.2-1) that had been transfected and selected for hygromycin resistance (*15*). Spontaneous life-span extension was not observed with the parental LF1 strain, nor has it been reported to occur spontaneously in cultures of normal HDFs.

Southern (DNA) hybridization revealed that the apparent extension of life-span was accompanied by loss of heterozygosity (LOH) at the p21 locus (Fig. 2B). The intensity of the wild-type band diminished gradually with passage. Protein immunoblotting analysis confirmed loss of p21 expression (Fig. 2C). In contrast, p21 expression persisted at late passage in p21 +/− strains displaying kinetics of senescence indistinguishable from normal HDFs. p21 was lost in 12 of 12 cultures displaying extended life-span but persisted in 3 of 3 cultures with normal kinetics of senescence.

Cells expressing SV40 T-Ag continue to shorten their telomeres during the extended life-span phase (*22*). Early-passage HO7.2-1 cells had shorter telomeres than senescent LF1 cells, and telomeres continued to shorten as HO7.2-1 cells approached crisis (Fig. 3A). Both the reduction of telomere length and the weak hybridization signal are indicative of cells in crisis. Neither presenescent LF1 cells nor HO7.2-1 cells during their extended life-span phase expressed telomerase activity (*23*, *24*).

The Cdk inhibitor p16 is up-regulated in senescent cells (6) and thus has been linked

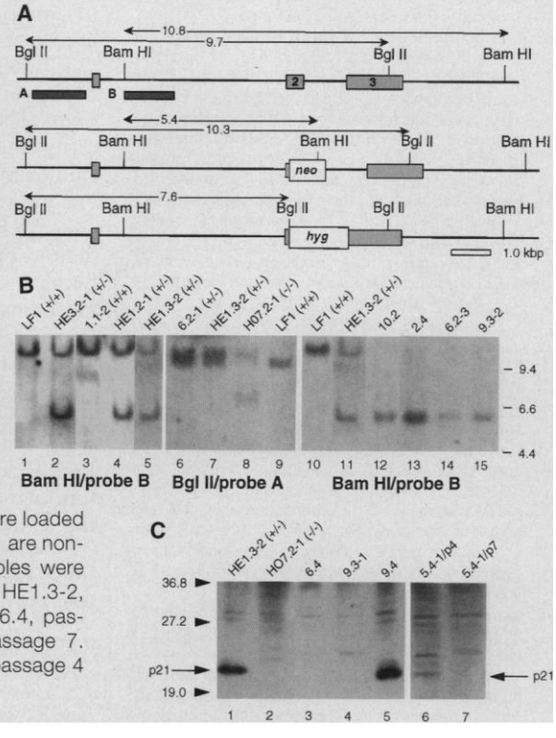

Fig. 2. Gene targeting of p21 locus. (**A**) Schematic representation of wild-type (top), *neo*-targeted (middle), and *hyg*-targeted (bottom) gene copies. Exons, filled boxes; Southern (DNA) hybridization probes, solid bars. (**B**) Southern (DNA) hybridization analysis. Cell strains are listed on top. Size markers (kbp) are on the right. Lanes 1 to 5 (Bam HI), first round of targeting; 1.1-2 is a nontargeted sibling. Lanes 6 to 9 (Bgl II), second round of targeting; 6.2-1 is a nontargeted sibling. Lanes 10 to 15 (Bam HI), LOH in +/− cell strains; 10.2, 2.4, 6.2-3, and 9.3-2 are nontargeted siblings of HO7.2-1. (**C**) Protein immunoblotting analysis. Cell strains are listed on top. Size markers (kD) are on the left. Equal amounts of protein were loaded in all lanes. 6.4, 9.3-1, 9.4, and 5.4-1 are nontargeted siblings of HO7.2-1. Samples were harvested at the following passages: HE1.3-2, passage 9; HO7.2-1, passage 13; 6.4, passage 8; 9.3-1, passage 7; 9.4, passage 7. Lanes 6 and 7, 5.4-1 harvested at passage 4 and passage 7, respectively.

with entry into senescence. Expression of p16 increased with age in LF1 cells and continued to increase in HO7.2-1 cells during their extended life-span (Fig. 3B). Thus, loss of p21 allows cells to bypass senescence in spite of expression of p16.

There was no correlation between expression of p21 and growth rate: the +/− strain 9.4 (which expressed p21) (Fig. 2C) grew more slowly than the −/− strain HO7.2-1, which in turn grew more slowly than its +/− parent HE1.3-2 (Fig. 1F). Furthermore, there was little difference in growth between strain 9.4 and several of its siblings that lacked p21. Thus p21 is not a growth-rate determinant in normal HDFs.

Cell cycle profiles were identical in exponentially growing LF1 and HO7.2-1 cultures (Fig. 4A), but LF1 cells responded more completely to serum withdrawal. Therefore, although p21 evidently contributes to the ability of normal human cells to become quiescent, additional factors also must participate. Consistent with the phenotype of murine p21 null cells (*25*), HO7.2-1 cells did not arrest at the DNA damage-induced G_1 checkpoint (Fig. 4B). Loss of p21 in a human adenocarcinoma cell line enhances apoptosis (*26*). HO7.2-1 cells grew more slowly but became apoptotic at the same rate as LF1 cells (Fig. 4, C and D) (*27*).

To investigate the role of p21 in genomic instability, amplification of the CAD (trifunctional enzyme carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase) gene was selected by using three times and nine times the median lethal dose of the drug *N*-(phosphonacetyl)-L-aspartate (*27*). Treatment of HO7.2-1 cells (1×10^6 cells at each concentration of drug) over a 3-month period produced no colonies (*15*).

Our results clearly establish the importance of p21 as a key regulator of senescence. We find that of the three major functions of p53—checkpoint control, apoptosis, and genome stability—in normal human cells, p21 has a role in only the first one. It is highly probable that the ability of p21 null cells to become apoptotic may prevent them from developing into cancers. The methods we have developed allow direct manipulation of genes in normal human cells.

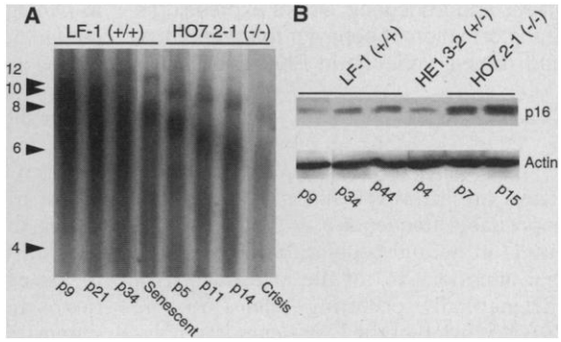

Fig. 3. Telomere length (**A**) and p16 expression (**B**) during aging of p21 +/+ and −/− cells. Cell strains are listed on top. Passage number (p) is indicated below each lane. Telomere length was measured by the terminal restriction fragment assay (*22*). One microgram of DNA was loaded in each lane. Size markers (kbp) are on the left. Expression of p16 protein was measured by immunoblotting. Equal amounts of protein were loaded in all lanes.

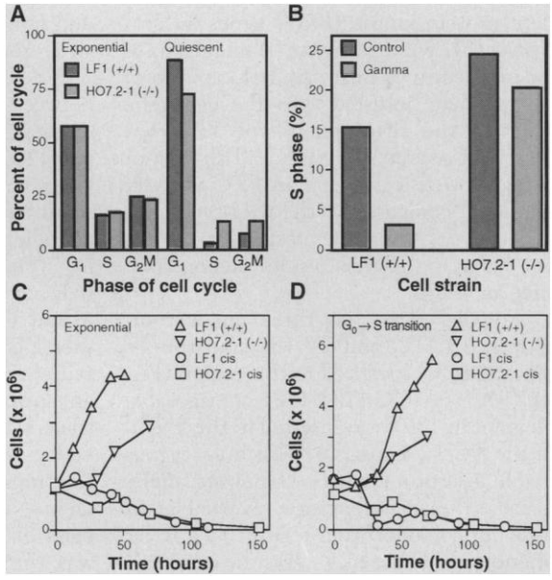

Fig. 4. Phenotypes of p21 +/+ and −/− cells. (**A**) Cell cycle distribution. Exponential cells: cultures were passaged 1:4 twice at <50% confluence. Quiescent cells: cultures at 80% confluence were incubated in 0.25% serum for 60 hours. Cells were stained with propidium iodide for flow cytometry. S, phase of chromosome replication; M, mitosis; G_2, period between S phase and onset of M. (**B**) S-phase entry. Quiescent cells were stimulated (15% FBS) and after 2 hours were irradiated with 12 gray of gamma rays. LF1 and HO7.2-1 samples were collected for flow cytometry 16.5 and 21.5 hours after stimulation, respectively. Analogous results were obtained when cisplatin was used to induce DNA damage (*15*). (**C** and **D**) Apoptosis. Cisplatin (cis; 10 μg/ml) was added at 50% confluence (C) or 2 hours after serum stimulation (D). Total cells (adherent plus floating cells) were counted with a Coulter counter. Analogous results were obtained with etoposide (*15*).

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14. HE and HO indicate heterozygous and homozygous derivatives, respectively. Nontargeted siblings are identified by clone number only. LF1 and HO7.2-1 cells tested negative for mycoplasma, human immunodeficiency virus, and HPV, and their karyotypes were normal.
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cell strain–specific.
17. Cultures were considered senescent when 1 PD took longer than 2 weeks. Senescent cultures were >95% β-galactosidase–positive [G. P. Dimri *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9363 (1995)] and could be maintained for over 2 months without a significant decrease in cell number (*15*).
18. Passage number after gene targeting was arbitrarily set to 0 for each cell strain when the culture was first expanded into a 10-cm culture dish.
19. Low-level mitotic activity coexisted with cell death; cells of variable size, irregular shape, and a significant number of large multinucleated cells were observed; cultures gradually declined over 6 to 8 weeks (*15*).
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Natural Behavior Polymorphism Due to a cGMP-Dependent Protein Kinase of *Drosophila*

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Naturally occuring polymorphisms in behavior are difficult to map genetically and thus are refractory to molecular characterization. An exception is the *foraging* gene (*for*), a gene that has two naturally occurring variants in *Drosophila melanogaster* food-search behavior: rover and sitter. Molecular mapping placed *for* mutations in the *dg2* gene, which encodes a cyclic guanosine monophosphate (cGMP)–dependent protein kinase (PKG). Rovers had higher PKG activity than sitters, and transgenic sitters expressing a *dg2* complementary DNA from rover showed transformation of behavior to rover. Thus, PKG levels affected food-search behavior, and natural variation in PKG activity accounted for a behavioral polymorphism.

The molecular identities of genetic polymorphisms in behavior have been difficult to establish because these traits are usually inherited polygenically. One example of a single gene underlying a naturally occurring polymorphism is the *foraging* gene (*for*), which is involved in food-search behavior in the fruit fly *Drosophila melanogaster* (*1*, *2*). Individuals with a rover allele for^R move greater distances while feeding than do those homozygous for sitter alleles for^s (*3*). This difference in foraging behavior is observed during both the larval and adult stages (*4*). Rovers and sitters do not differ in general activity in the absence of food (*4*). Both rovers and sitters are wild-type forms that exist at appreciable frequencies (70% rover; 30% sitter) in natural populations (*1*, *5*). Several mutations (*6*) of the locus map with the naturally occurring alleles in the 24A3-5 region of the *D. melanogaster* polytene chromosomes (*2*, *7*). This region contains *dg2*, one of two cGMP-dependent protein kinase (PKG) genes in *Drosophila* (*8*). We report that (i) mutations in *for* mapped in or near *dg2*, (ii) excision of a P-element inserted into the *dg2* gene reverted the sitter phenotype to rover, (iii) wild-type for^s flies and all sitter mutants showed a decrease in PKG activity and level compared to the wild-type for^R, and (iv) *dg2* transgenes rescued rover larval behavior. These results demonstrate that *for* is *dg2*.

The *dg2* gene has three major transcripts, T1, T2, and T3 (*8*), and the *for* mutations are localized to this region (Fig. 1) (*9*). The P[GAL4] (*10*) transposable element in 189Y was inserted in the 5′ end of the *dg2* T2 transcript. This homozygous viable insertion identified a new *for* allele, because P-element excision reverted larval foraging behavior from a sitter to a rover phenotype (Table 1). As was the case with other sitter alleles, locomotion of the 189Y larvae was not reduced in the absence of food, indicating that the change in behavior was foraging-specific.

PKG enzyme assays were performed on adult heads of wild-type for^R and for^s, and mutant for^{s1} and for^{s2} strains (Table 2). for^R flies had significantly higher amounts of PKG enzyme activity than did for^s flies. Even greater reductions in enzymatic activity were seen in the mutants for^{s1} and for^{s2}. The amount of PKG in adult heads correlated with the adult foraging phenotypes of these strains (*4*).

To determine whether PKG is directly responsible for the foraging polymorphism in *Drosophila*, we overexpressed *dg2* in sitter larvae. This resulted in a change of behavior to the rover phenotype. The transgenic strain contained four copies of a heat shock– driven *dg2*-cDNA (*11*). The basal level of PKG expression in this transgenic strain (Fig. 2) was sufficient to rescue rover larval behavior, thus eliminating the lethal and sublethal effects of heat on the *dg2*-transgenic larvae (Table 3). As expected, the PKG enzyme activities of the dissected larval central nervous systems (CNSs) showed that without heat shock, the *dg2*-cDNA transgenic strain had levels of PKG similar to those of for^R and significantly higher than those of the sitter control strain (Table 3).

The basis for the *dg2* activity difference between for^R and for^s was further addressed by measurement of RNA levels and PKG protein. Northern (RNA) analysis revealed that for^s and for^{s2} showed a small but consistent (*12*) reduction in the abundance of T1 RNA relative to that in for^R (Fig. 3A). T2 and T3 RNA were also reduced in these strains, but to a lesser extent (*12*). To assess protein levels, we subjected extracts of adult heads to protein immunoblot analysis by probing with an antibody to bovine PKG, or the extracts were affinity-purified by chromatography on cGMP-sepharose, labeled, and electrophoresed (*13*). In both experiments, a prominent band at a molecular mass of 80,000 Daltons was found. This was the only band strongly induced by heat shock in the *dg2*-cDNA transgenic strain, and it was less intense in for^s than

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