Physiol Genomics 17: 307–315, 2004.
First published March 23, 2004; 10.1152/physiolgenomics.00039.2004.

Additive regulation of hepatic gene expression by dwarfism and caloric restriction

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Submitted 17 February 2004; accepted in final form 10 March 2004

Tsuchiya, Tomoshi, Joseph M. Dhahbi, Xinping Cui, Patricia L. Mote, Andrzej Bartke, and Stephen R. Spindler. Additive regulation of hepatic gene expression by dwarfism and caloric restriction. *Physiol Genomics* 17: 307–315, 2004. First published March 23, 2004; 10.1152/physiolgenomics.00039.2004.—Disrupted growth hormone/insulin-like growth factor-1 signaling (DF) and caloric restriction (CR) extend life span and delay the onset of age-related diseases in rodents. In combination, these interventions additively extend life span. To investigate the molecular basis for these effects, we performed genome-wide, microarray expression analysis of liver from homozygous and heterozygous Ames dwarf mice fed ad libitum or CR. CR and DF additively affected a group of 95 genes. Individually and together, DF and CR independently affected the expression of 212 and 77 genes, respectively. These results indicate that DF and CR affect overlapping sets of genes and additively affect a subset of genes. Together, the interventions produced changes in gene expression consistent with increased insulin, glucagon and catecholamine sensitivity, gluconeogenesis, protein turnover, lipid β-oxidation, apoptosis, and xenobiotic and oxidant metabolism; and decreased cell proliferation, lipid and cholesterol synthesis, and chaperone expression. These data suggest that the additive effects of DF and CR on life span develop from their additive effects on the level of expression of some genes and from their independent effects on other genes. These results provide a novel and focused group of genes closely associated with the regulation of life span in mammals.

dwarf mutation; oligonucleotide microarray; Affymetrix

GENETIC ABLATION of growth hormone (GH) or its receptor and suppression of plasma concentration of insulin-like growth factor I (IGFI) produce a dwarf phenotype (DF) and extend the life span of rodents (45). Ames DF mice, which are homozygous for a loss of function mutation at the *Prop1* locus, exhibit a 40–70% increase in mean and maximal life span compared with their normal heterozygous siblings (8). Several lineages of anterior pituitary cells do not develop normally in these mice, leading to a combination of endocrine abnormalities, including low levels of serum GH, IGFI, thyroid-stimulating hormone, thyroid hormones, and prolactin (58). DF postpones the age-related development of neoplastic diseases, immune system decline, and collagen cross-linking, suggesting DF reduces the rate of aging (23, 34). Decreased IGFI signaling may exert the major influence on longevity. GH receptor knockout mice have significantly extended life spans, but IGFI receptor knockout mice also have extended life spans, 90% reduced levels of IGFI, and high levels of plasma GH (13, 30, 66).

Caloric restriction (CR), which is reduced caloric consumption without malnutrition, retards aging and most disease processes and increases maximum and/or mean life span in a variety of organisms (42). CR and DF together additively increase the life span of mice (3). These effects could be mediated through one pathway that is more strongly affected by the combined interventions or through distinct molecular pathways independently affected by each intervention. The shape of the life span curves suggested to Bartke et al. (3) that different molecular mechanisms were responsible for the additive life span effects of DF and CR. In contrast, Clancy and colleagues (11) suggested that overlapping mechanisms mediate these effects. In *Drosophila*, mutation of the *chico* gene, which encodes a homolog of the mammalian insulin receptor substrates 1 through 4, reduces insulin/IGFI signaling and results in a DF phenotype with extended life span. The authors of this study speculated that CR and *chico* utilize overlapping mechanisms. Shimokawa and colleagues (56) observed an additive life span effect of DF and CR in mini-rats overexpressing antisense GH RNA. They concluded that CR affects aging and longevity mostly through mechanisms other than suppression of the GH-IGFI axis. None of the studies provided strong evidence indicating whether the life-prolonging effects of DF and CR are mediated by distinct or overlapping molecular mechanisms.

To gain insight into the molecular pathways activated by DF and CR, we analyzed gene expression profiles in the liver of normal (NL) and Ames DF mice subjected to ad libitum (AL) or CR diets using Affymetrix oligonucleotide microarrays containing probes for over 12,000 transcription units. The results and the functional categories of genes affected by DF and CR suggest that their additive enhancement of life span results from the greater number of genes affected by the combined treatments and their additive effects on the expression of a subset of genes.

MATERIALS AND METHODS

Mice. Male and female mice of the Ames stock were bred and housed at Southern Illinois University. DF (*df*/*df*) and NL (+/+ or +/*df*) mice were produced by crosses between *df*/+ parents or between fertile *df*/*df* males and *df*/+ females (2). Details of the animal husbandry were as described (2). Mice had free access to tap water and standard pelleted food (LabDiet; PMI Feeds, St. Louis, MO). The cages were equipped with microisolator filter tops. The room was maintained at 22 ± 2°C. Lights were on from 0600 to 1800 h. Sentinel animals were negative for all pathogens tested.

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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Study design. Starting at the age of 2 mo, 16 female Ames DF and 16 of their NL littermates were randomly assigned to two dietary regimens. For each of the two genotypes, eight mice were chosen randomly and subjected to CR while the remaining eight continued AL feeding. This genotype/diet design resulted in four experimental groups: NL genotype AL fed (NLAL); NL genotype CR (NLCR); DF genotype AL (DFAL); and DF genotype CR (DFCR). The CR regimen was introduced progressively by reducing the daily food intake of CR mice to 90% of the AL intake of animals of the same genotype for 1 wk, to 80% for the next week, and to 70% for the remainder of the study. Food consumption of the AL fed animals was monitored throughout the study, and the CR mice were fed daily, at ~1700 h, 70% of the average amount consumed daily by AL mice during the preceding week. Mice were killed at 6 mo of age, and tissues were removed, rapidly frozen on dry ice, and stored in liquid nitrogen. The average weights of the mice at the end of the experiment were as follows: NLAL, 30.1 ± 4.5 g; NLCR, 23.6 ± 1.6 g; DFAL, 15.4 ± 2.3 g; DFCR, 10.4 ± 0.6 g (SD).

Probe set expression measurement and normalization. Total liver RNA was isolated from frozen tissue as described (16). mRNA levels were measured using the Affymetrix mouse U74Av2 array according to standard protocols (10, 16). After hybridization, arrays were scanned using a Hewlett-Packard GeneArray Scanner. Image analysis was performed as described (10). Raw image files were converted to probe set data (*.CEL files) using the Affymetrix Microarray Suite software (MAS 5.0). Probe set data from all 31 arrays were simultaneously analyzed with the robust multichip average (RMA) method to generate normalized expression measures for each probe set (35). The data were further filtered to include only probe data sets that were "present" in at least 75% of the arrays per experimental group according to the MAS 5.0 detection algorithm, which uses the Wilcoxon signed rank test (1, 64). Gene names were from the LocusLink and Affymetrix databases as of November 19, 2003.

Data analysis. We performed two-way analysis of variance (two-way ANOVA) in which expression level was considered to be a function of genotype only, diet only, or a function of genotype and diet. The two-way ANOVA test is based on the following model: $y_{ijk} = \mu + G_i + D_j + (G \times D)_{ij} + \epsilon_{ijk}$, where μ is the overall mean of log-transformed intensity values of gene expression that is common to all 31 samples; G_i, is the effect of the i-th genotype ($i = 1, 2$; 1 = DF, 2 = NL); D_j is the effect of the j-th diet ($j = 1, 2$; 1 = CR, 2 = AL); $(G \times D)_{ij}$ is the interaction between genotype and diet; and ϵ_{ijk} is the stochastic error. An interaction between genotype and diet would indicate that the effect of CR on gene expression is conditional on the DF genotype. There are eight replicates in each of the NLCR, DFAL, and DFCR sample sets ($k = 1, 2, \ldots 8$) and seven replicates in the NLAL sample set ($k = 1, 2, \ldots 7$). Based on this model, y_{ijk} represents the observed log-transformed intensity value of gene expression for the k-th replicate of the i-th genotype under the j-th diet. The two-way ANOVA model was fitted to the sample data $\{y_{ijk}\}$ by the least square method.

The two-way ANOVA analysis consisted of three statistical significance tests: a test of each of the two main effects (diet and genotype) and a test of the interaction between diet and genotype. We started by testing the hypothesis of no interaction. If the hypothesis of no interaction was rejected, then we stopped further testing of the two main effects, since such a statistically significant interaction indicates that diet and genotype effects are dependent on each other. If the hypothesis of no interaction was accepted, then we continued the analysis by examining the effects of diet and genotype under the same two-way ANOVA model.

For each gene we calculated the F statistic using the linear model (LM) procedure embodied in R to test the hypothesis of no interaction (14). This method assumes normality and homoscedasticity. Our statistical significance criterion for assessing the existence of interaction was the false discovery rate (<0.05) criterion (6, 7). If the hypothesis of no interaction was accepted for a tested gene, then the F statistics corresponding to each of the two main effects and the nominal P values were calculated separately under the two-way ANOVA model. With a series of multiple simultaneous tests, the nominal P values were adjusted to reduce the type I errors.

If a gene is upregulated (or downregulated) by CR only, then the fold change of CR vs. AL was estimated by $2^{|D1-D2|}$ (or $-2^{|D1-D2|}$). Similarly, for a genotype only effect, the fold change of DF vs. NL was estimated by $2^{|D1-G2|}$ (or $-2^{|D1-G2|}$). If a gene is upregulated (or downregulated) by both CR and DF independently, then the fold change of DFCR vs. NLAL was estimated by $2^{|D1-D2+G1-G2|}$ (or $-2^{|D1-D2+G1-G2|}$). When there is an interaction between the effects of diet and genotype, the fold change of DFCR vs. NLAL was estimated by $2^{|D1-D2+G1-G2+(D\times G)11-(D\times G)22|}$ (or $-2^{|D1-D2+G1-G2+(D\times G)11-(D\times G)22|}$).

Four statistical categories of genes changed by DF, CR, or both interventions were identified. These groups were: 212 genes affected only by DF [$G_i \neq 0$, $D_j = 0$, $(G \times D)_j = 0$]; 77 genes affected only by CR [$D_j \neq 0$, $G_i = 0$, $(G \times D)_j = 0$]; 95 genes affected additively but independently by both interventions [$D_j \neq 0$, $G_i \neq 0$, $(G \times D)_j = 0$]; and 5 genes for which the effects of diet were dependent on genotype [$D_j \neq 0$, $G_i \neq 0$, $(G \times D)_j \neq 0$]; where G is genotype, D is diet, and G × D is interaction between diet and genotype.

Validation of microarray results. The expression of a total of 16 genes was examined by quantitative PCR (qPCR) (49). Primers were designed using the NetAffx analysis center and PCR products sequenced and verified against the public database (**http://www.affymetrix.com/analysis/index.affx** and **http://www.ncbi.nlm.nih.gov**; Supplemental Table S1, available at the *Physiological Genomics* web site).[1] Primers for transcription elongation factor A1 (SII) were amplified in parallel with the genes of interest as a control. SII mRNA is unaffected by CR (60) and DF (data not shown). Real-time, two-step RT-PCR was performed with a QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) and an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Amplification specificity was confirmed by melting curve analysis and agarose gel electrophoresis using standard techniques.

RESULTS AND DISCUSSION

Data analysis. Four statistical categories of genes changed by DF, CR, or both interventions were identified by two-way ANOVA (Fig. 1*A*). These groups were as follows: 212 genes affected only by DF; 77 genes affected only by CR; 95 genes affected additively but independently by both interventions; and 5 genes for which the effects of diet were dependent on genotype. Since only 5 of 389 changed genes were conditional on genotype, CR and DF work mostly independently to regulate gene expression. However, this does not necessarily imply that CR and DF work through completely independent pathways. CR and DF might independently and additively regulate gene expression by changing the activity of discrete transcription factors (Fig. 1*B*). Alternatively, coregulation could be mediated by cross talk between signal transduction systems or effects on different steps in gene expression.

Validation by qPCR. To ensure the analysis resulted in a low false discovery rate, the expression of 16 randomly chosen genes was reanalyzed using qPCR (Fig. 2). The primers used for these studies are shown in Supplemental Table S1. Changes in the expression of all 16 genes were verified as to direction and magnitude. Thus the methods used are reliable. In general,

[1]The Supplementary Material for this article (Supplemental Tables S1 and S2) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00039.2004/DC1**.

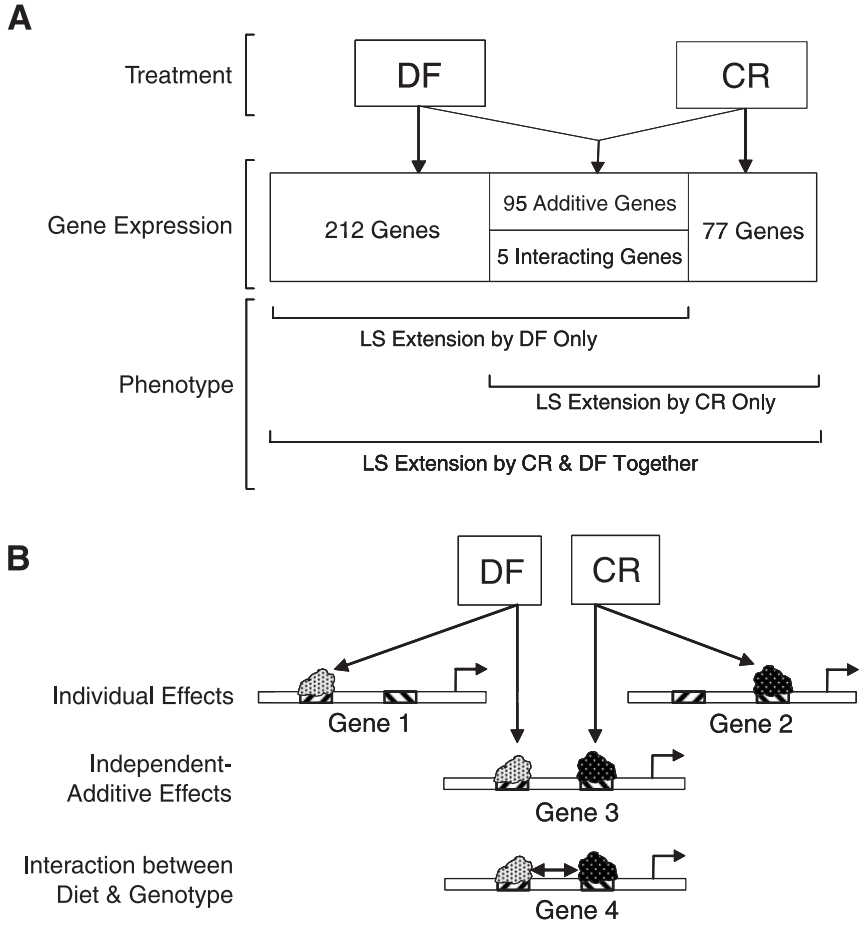

Fig. 1. A numerical summary of hepatic gene expression profiling of normal and DF mice fed ad libitum (AL) or a calorically restricted diet (CR). *A*: dwarfism (DF) changed the expression of 312 genes (212 + 100 genes), CR changed the expression of 177 genes (77 + 100), and DF and CR together changed the expression of 389 genes (212 + 100 + 77 genes), 100 of which were additively changed in expression. Of the additively changed genes, 95 showed no statistical evidence of interaction between DF and CR, while 5 showed evidence of interaction. *B*: a model for the regulation of 212 genes by DF (hypothetical *gene 1*), 77 genes by CR (hypothetical *gene 2*), 95 genes independently and additively by CR and DF (hypothetical *gene 3*), and 5 genes for which diet and genotype interact to regulate expression (hypothetical *gene 4*). LS, life span.

qPCR found a greater change in gene expression than was found by microarrays.

Functional classification of genes. To explore the molecular basis for the effects of DF and CR, we functionally classified the changed genes (see the table in Fig. 3). A complete list of changed genes is given in Supplemental Table S2. DF and CR alone and in combination had major effects on genes associated with energy metabolism (18, 18, and 11%), transcription (10, 7, and 4%), signal transduction (10, 8, and 11%), and xenobiotic and oxidant metabolism (5, 5, and 11%).

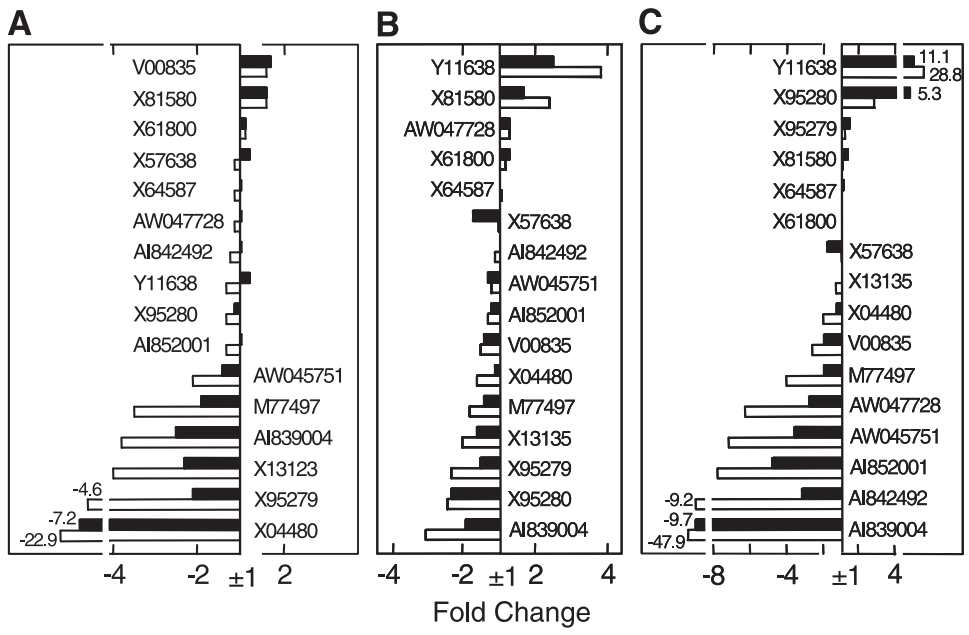

Fig. 2. Expression analysis of 16 genes measured using Affymetrix microarrays and quantitative PCR (qPCR). Solid and open bars represent microarray or qPCR data, respectively. The fold change for microarray and qPCR were calculated as described (MATERIALS AND METHODS). Genes are identified by their GenBank numbers.

Category/GenBank	Gene Symbol	DF	CR	DFCR
Energy Metabolism				
Glycolysis				
L41631	Gck	-1.3	-1.1	-1.5
D63764	Pklr	-1.5	-1.2	-1.9
Gluconeogenesis Related				
AF009605	Pck1	1.3	1.2	1.6
U09114	Glul	-1.4	1.0	-1.4
M14220	Gpi1	1.1	1.2	1.4
AF080469	G6pt1	1.0	1.3	1.3
Lipid Uptake				
Z22216	Apoc2	1.4	1.3	1.8
X58426	Lipc	-1.6	-1.2	-2.1
AI846600	Mgll	-1.4	-1.1	-1.6
Lipid Transport				
M64248	Apoa4	-4.3	1.3	-3.6
U28960	Pltp	-1.7	1.0	-1.7
AF015790	Plscr2	-1.4	1.0	-1.4
Y14004	Cte1	-1.4	1.3	-1.1
M65034	Fabp2	-1.8	-1.1	-2.0
X61431	Dbi	-1.2	-1.1	-1.4
Fatty Acid Synthesis (Lipogenesis)				
AI839004	Elovl6	-2.5	-2.3	-4.8
AW121639	Acly	-1.7	-1.2	-2
J02652	Mod1	-2.0	-1.1	-2.3
X13135	Fasn	-2.3	-1.6	-3.6
X95279	Thrsp	-2.1	-1.5	-3.2
Cholesterol Synthesis				
X97755	Ebp	-1.4	-1.2	-1.6
AW045533	Fdps	-1.9	-1.2	-2.3
D42048	Sqle	-1.6	-1.2	-2
AW122260	Cyp51	-1.6	1.0	-1.6
AW106745	Nsdhl	-1.7	-1.2	-2.2
AF057368	Dhcr7	-1.6	1.0	-1.7
Beta-oxidation				
U96116	Hadh2	1.2	1.2	1.5
Y11638	Cyp4a14	1.2	2.5	11.1
U89906	Amacr	1.2	1.2	1.4
U07159	Acadm	1.2	1.1	1.4
AB018421	Cyp4a10	1.1	1.8	2.4
AI840013	Peci	1.1	1.3	1.5
AW122615	Hadhb	-1.1	1.3	1.2
AF017175	Cpt1a	1.1	1.3	1.4
Xenobiotic and Oxidant Metabolism				
Phase I				
M27168	Cyp2d9	1.5	1.1	1.6
D26137	Cyp3a16	1.3	1.2	1.6
U90535	Fmo5	1.3	1.3	1.7
Y11995	Cyp3a25	1.6	1.2	1.9
AI839690	Cyb5r1-pending	1.1	1.3	1.4
Phase II				
U89491	Ephx1	1.2	1.2	1.5
Z37107	Ephx2	1.3	1.2	1.6
J03952	Gstm1	1.2	1.1	1.4
J03953	Gstm3	1.5	1.3	2.0
X53451	Gstp2	1.6	1.4	2.6
J03958	Gsta2	2.3	1.1	2.8
L06047	Gsta4	1.5	1.1	1.8
X98056	Gstt2	1.1	1.3	1.5
Anti-oxidant				
U85414	Gclc	1.2	1.2	1.6
D87896	Gpx4	1.2	1.1	1.4
AF032714	Tdpx-ps1	1.2	1.1	1.4
AI851983	Gsr	1.1	1.2	1.4
Other				
L11333	Es31	-5.3	-1.3	-6.4
Signal Transduction				
Growth Related				
M22957	Prlr	-3.0	-1.3	-4.5
X04480	Igf1	-7.2	-1.1	-9.7
X81580	Igfbp2	1.6	1.7	3.0
D17444	Lifr	-3.5	-1.7	-5.0
AI853531	Mig6	-1.6	1.2	-1.3
U88327	Socs2	-2.7	1.0	-2.8
X81579	Igfbp1	7.2	1.5	11.6
D50086	Nrp	-1.4	-1.1	-1.6
X61597	Serpina3c	-1.6	1.1	-1.5
L19932	Tgfbi	1.5	1.1	1.6
U92437	Pten	1.1	-1.7	-1.6
U50413	Pik3r1	-1.1	-1.6	-1.7
AW124627	Prkcn	1.1	1.2	1.4
Cytokine and Others				
M93422	Adcy6	1.3	1.1	1.6
Z50190	Adcy9	1.3	1.1	1.4
Transcription				
L10409	Foxa2	-1.2	-1.2	-1.5
X74938	Foxa3	1.2	1.3	1.6
AF084524	Creg	1.2	1.2	1.5
Transport and Trafficking				
Membrane Transport				
AI173996	Abcc2	1.4	1.2	1.7
AA833514	Abcc3	1.3	1.1	1.4
AF103875	Abcg2	1.8	1.3	2.3
Intracellular Transport				
L03290	Slc7a2	1.3	1.1	1.5
U95132	Slc10a1	-1.4	1.1	-1.2
AF058054	Slc16a7	1.5	1.1	1.7
U38652	Slc22a1	-1.6	-1.2	-2.0
AI844736	Slc23a1	1.4	1.1	1.6
AI838274	Slc29a1	-1.3	1.0	-1.4

Fig. 3. Representative list of the effects of DF, CR, and DF and CR (DFCR) together on hepatic gene expression. Different intervention groups are identified by color: DFCR, red; DF, green; CR, blue; interaction between DF and CR, magenta; and no significant change, black.

Category/GenBank	Gene Symbol	DF	CR	DFCR
Cell Proliferation (Cell Cycle and DNA Replication)				
X59846	Gas6	-1.5	-1.4	-2.0
AI849928	Ccnd1	-1.6	1.2	-1.3
AW060791	Pole4	-1.3	-1.1	-1.5
AL021127	Cetn2	-1.7	-1.1	-2.1
AA913994	Shmt1	-1.4	-1.2	-1.7
AF064088	Tieg1	-1.3	1.4	1.1
X95280	G0s2	-1.1	-1.9	-2.0
Apoptosis				
Y13087	Casp6	1.2	1.2	1.4
U57325	Psen2	-1.3	-1.3	-1.6
M21828	Gas2	1.7	1.0	1.8
U32170	Rgn	-1.2	-1.1	-1.4
Chaperone (Protein Folding)				
AI846938	Herpud1	1.2	1.3	1.6
AF055664	Dnaja1	-1.2	-1.2	-1.5
AA615831	Hspa4	-1.4	-1.2	-1.7
L40406	Hsp105	-1.6	-1.1	-1.7
J04633	Hspca	-1.5	-1.1	-1.7
AW122022	Ppid	-1.2	-1.2	-1.4
AI842377	P5-pending	-1.4	1.0	-1.4
AV373612	Bag3	-1.3	1.0	-1.4
D17666	Hspa9a	1.1	1.3	1.4
AF109906	Hspa1b	1.1	1.5	1.8
AA879709	Ssr1	-1.1	-1.3	-1.5
Pheromone				
M17818	Mup1	-6.7	-1.6	-10.2
M16357	Mup3	-3.8	-1.2	-5.0
M16358	Mup4	-4.6	-1.3	-6.0
M16360	Mup5	-3.6	-1.2	-4.7

Fig. 3—*Continued*.

Gluconeogenesis. Separately and in combination, DF and CR enhanced gene expression associated with gluconeogenesis (table in Fig. 3; Pck1, Glul, Gpi1, and G6pt1). Individually, and additively in combination, they enhanced expression of the key gating enzyme of gluconeogenesis, Pck1. DF decreased Glul expression, which may reduce the rate of glutamine synthesis in the liver, sparing glutamate for gluconeogenesis. CR upregulated Gpi1 and G6pt1, genes important for gluconeogenesis. These results and our previous studies of CR suggest that DF and CR individually enhance the enzymatic capacity for the turnover and renewal of hepatic and extrahepatic protein, and these effects are additively enhanced in DFCR mice (10, 18).

Glycolysis. Several key enzymes involved in liver glycolysis were underexpressed in DF mice (Gck and Pklr). We previously showed that CR decreases glucokinase, pyruvate kinase, and acetyl-CoA carboxykinase expression (17). Together these results suggest that DF and CR decrease substrate availability for de novo lipogenesis in the liver.

Lipid and cholesterol metabolism. DF and CR decreased the expression of genes key to hepatic lipogenesis (table in Fig. 3). Hepatic expression of 16 lipid- and cholesterol-related genes were underexpressed in DF mice. These genes are involved in lipid transport (Apoa4, Pltp, Plscr2, Cte1, Fabp2, and Dbi) and uptake (Mgll), fatty acid synthesis (Acly, Mod1, Fasn, and Thrsp), and cholesterol biosynthesis (Fdps, Sqle, Cyp51, Nsdhl, and Dhcr7). DF and CR alone and additively in combination downregulated Lipc, an important enzyme in HDL metabolism, Elovl6, a key enzyme of lipid synthesis, and Ebp, a key enzyme of cholesterol synthesis (table in Fig. 3). The expression of Apoc2 was additively upregulated by DF and CR. The product of this gene is a potent activator of lipoprotein lipase and plays an important role in the catabolism of triglyceride-rich lipoproteins. DF and CR individually and additively together enhanced the expression of key enzymes involved in β-oxidation of fatty acids [Hadh2 and Cyp4a14 (by 11.1-fold)]. Separately, DF and CR upregulated the expression of Amacr, Acadm, Cyp4a10, Peci, Hadhb, and Cpt1a. Taken together, these results suggest that DF and CR individually and additively enhance the enzymatic capacity for gluconeogenesis and lipid utilization for energy production, and suppress the capacity for glycolysis and de novo lipogenesis. The association of microsomal transfer protein and lipoprotein particle size with extreme longevity in humans suggests that these effects may be important to the longevity of DF and CR mice (4, 27).

Eight of the 16 lipid-related genes are transcriptionally regulated by sterol response element binding proteins (SREBPs; table in Fig. 3; Refs. 24, 31). Thus disrupted GH signaling in DF mice may reduce lipid and cholesterol metabolism by modulating the activity of SREBPs. A similar mechanism has been proposed to explain the underexpression of fatty acid- and cholesterol synthesis-related genes in the liver of hypophysectomized rats (25).

Young adult dwarf mice have more body fat than normal mice. But, with age, normal mice from this line accumulate fat at a higher rate, and the percent body fat in old DF mice does not differ from that of normal mice, as measured by dual energy X-ray absorptiometry (DEXA) (29). Downregulation of lipid biosynthetic genes and upregulation of β-oxidation-related genes in the liver of DF mice may explain this slower rate of fat deposition.

Regulation of the phosphatidylinositol 3-kinase (PI3K)-IGFI cascade by hormones. GH induces PI3K activation in hepatocytes via the Janus kinase 2-insulin receptor substrate (IRS)-1/IRS-2 pathway (65). PI3K activation induces expression of IGFI, which affects the growth, survival, transformation, and differentiation of other cells, and perhaps growth and apoptosis of hepatocellular carcinomas (57, 61).

The most likely source of the longevity effects of DF is suppression of GH/IGFI signaling (45). As expected, DF alone (−7.2-fold) and in combination with CR (−9.7-fold) strongly downregulated Igf1 mRNA. Consistent with these observations, DF repressed the expression of Mup1, Mup3, Mup4, and Mup5, which are repressed by low GH levels (38). CR downregulated the mRNA for the PI3K regulatory subunit (Pik3r1), consistent with the 70% reduction in serum IGFI levels in CR mice (10, 26). DF and CR also additively induced the expression of Igfbp2 3-fold, and DF upregulated Igfbp1 mRNA 7.2-fold. Previously, we found that CR induces the expression of IGF binding protein 7 (10). IGF binding proteins are regarded as general inhibitors of the growth promoting effects of the IGFs, suggesting that both DF and CR may further inhibit IGFI signaling through overexpression of these proteins.

Prolactin (PRL), acting through the prolactin receptor, can also stimulate the Janus kinase 2/PI3K signaling cascade,

suggesting that it may also regulate IGFI production (65). Interestingly, Prlr was negatively regulated by 3.0- and 1.3-fold in DF and CR mice, and in DFCR mice the receptor mRNA was reduced by 4.5-fold. This downregulation should exacerbate the already reduced prolactin signaling in DF mice. Serum insulin levels are reduced in both DF and CR mice (17, 19). Therefore, reduced serum GH, PRL, and insulin signaling may converge to suppress IGFI production in DF and CR mice.

Forkhead transcription factor. Downregulation of GH/IGFI signaling results in upregulation of forkhead transcription factors and thereby upregulation of genes coding for stress resistance, including antioxidant enzymes (45). In this regard, the forkhead transcription factors Foxa2 and Foxa3 were underexpressed and overexpressed, respectively, in CR and DF mice, and the interventions additively regulated these genes in DFCR mice (table in Fig. 3). Foxa-binding sites exist upstream of more than 100 genes that are expressed in the liver, pancreas, intestine, and lung (39). Foxa isoforms regulate liver genes including phospho*enol*pyruvate carboxykinase (PEPCK; Pck1), glucose-6-phosphatase, fructose-2,6-bisphosphatase, catalase, and IGF-binding protein 1 (Igfbp1; Refs. 40, 54). Overexpression of Foxa2 is associated with steatosis and mitochondrial damage (32). Foxa3 is central to the maintenance of differentiated functions in hepatocytes. Daf-16, a factor that regulates life span in *Caenorhabditis elegans*, has been found to be homologous to members of the forkhead transcription factor family (44). Foxa3 may enhance stress resistance through induction of catalase and the repression of cell proliferation (47). Thus the additive switch from Foxa2 to Foxa3 expression in DFCR mice may lead to the additive induction of gluconeogenesis and stress resistance and reduced cell proliferation. Their influence may be key to the additive effects of DF and CR on life span.

Insulin sensitivity. DF and CR caused underexpression of Gas6, a growth factor ligand for the Axl tyrosine kinase receptor. Axl interacts with the product of the Pten gene, and reduced Pten signaling improves insulin sensitivity and normalizes glucose concentration in genetically diabetic mice. Thus reduced Gas6 and Pten signaling may result in the enhancement of insulin sensitivity in DF and CR mice. (17, 19). Ames dwarf mice are known to have increased insulin receptor content, phosphorylation of IRS-1 and -2, association of the p85 regulatory subunit of PI3K with IRS-1 after insulin stimulation, and enhanced activation of insulin-stimulated protein kinase B (17, 19).

Glucagon and epinephrine sensitivity. DF upregulated adcy6 and adcy9 (table in Fig. 3). These plasma membrane-bound proteins catalyze the formation of cAMP in hepatocytes. Adcy6 is activated by forskolin and glucagon while Adcy9 is stimulated by β-adrenergic receptor agonists but is insensitive to Ca^{2+}/calmodulin, forskolin, and somatostatin. Upregulation of these enzymes should enhance hepatic sensitivity to glucagon and epinephrine and increase glycogenolysis and glucose output during fasting.

Carcinogenesis in DF and CR rodents. The DF mutations reduce the incidence and growth of spontaneous and transplanted tumors in mice (34). Igf1, which is negatively regulated by DF, is a key regulator of mitogenesis and tumorigenicity and plays a crucial role in the survival of transformed cells in vivo (52). In tumor cells, IGFI acts as an autocrine/paracrine growth factor as well as an inhibitor of apoptosis (52). IGFI receptor is emerging as a critical factor in hepatocarcinogenesis (53). Defects in IGFI receptor expression and/or activation inhibit tumorigenicity, reverse the transformed phenotype, and cause massive apoptosis in vitro and in vivo (9, 52). CR also has a well-described anticarcinogenic effect on spontaneous and chemically induced tumors (33). Reduction of cell proliferation and induction of apoptosis are thought to be the mechanisms for these effects of CR in liver (33). In addition, downregulation of Fasn and the fatty acid-synthesis pathway by DF and CR may have anticancer effects (table in Fig. 3; Ref. 10). Both are required for the survival of many human cancer cell lines, and inhibition of Fasn leads to apoptosis in cancer cells (43, 48).

Cell proliferation. DF and CR produced changes in gene expression consistent with reduced cellular growth and cellular stress. DF and CR additively induced expression of Creg, an inhibitor of cell growth (62). DF downregulated Socs2, which is part of a classic negative feedback system that downregulates GH signaling. It may be underexpressed in response to reduced GH signaling in DF mice.

DF and CR led to substantial downregulation of Lifr. This cytokine receptor affects the proliferation of a wide variety of cells and affects other signaling systems, including those for GH and prolactin. Mig6/Gene 33, an adapter protein that is induced by diabetes and persistent stress was downregulated in DF and DFCR mice. DF mice underexpressed Ccnd1, Pole4, Cetn2 (which is essential for centriole duplication), Nrp, and Serpina3c (which may be involved in inflammation and cell growth). DF resulted in overexpression of Tgfbi, a putative mediator of the growth inhibitory effects of TGFβ. CR downregulated G0s2, which is upregulated following receipt of mitogenic stimuli, and upregulated Tieg1, a putative tumor suppressor-like transcriptional repressor, and Prkcn/pkd3, a diacylglycerol-responsive, serine-threonine kinase that activates mitogen-activated protein kinase. DF also suppressed the expression of Shmt1, which generates single carbon units for purine, thymidine, and methionine biosynthesis.

Apoptosis. Hepatocytes from DF mice have enhanced rates of apoptosis in response to oxidative insult (41). Short- and long-term reductions in caloric intake are correlated with increased programmed cell death in mitotically competent tissues (33). Globally active, circulating factors, especially IGFI, are thought to regulate mitogenic signaling and apoptosis in many types of normal and cancer cells (33). Suppression of IGFI signaling appears to be responsible for the protective effect of CR against neoplastic progression (22). Defects in IGFI receptor expression and/or activation inhibit tumorigenicity, reverse the transformed phenotype, and cause massive apoptosis in tumor cells in vitro and in vivo (33). However, the mechanism for the additive delay of tumor formation and/or progression in DFCR mice is unclear, since hepatocytes do not express significant levels of IGFI receptor (61). It is possible that activation of IGFI receptor expression is an obligatory step in hepatocarcinogenesis in mice. DF, alone and in combination with CR, induced a pattern of gene expression consistent with increased apoptotic potential (table in Fig. 3). DF and CR additively induced the expression of the apoptosis-mediator Casp6 and repressed the expression of Psen2, the familial Alzheimer disease gene. Psen2 is both required for apoptosis and is processed by caspase 3 into an anti-apoptotic COOH-terminal polypeptide that antagonizes the progression of cell

death (63). DF induced the expression of Gas2, which is highly expressed in growth-arrested cells and induces rearrangement of the actin cytoskeleton during apoptosis (5). DF led to underexpression of Rgn (SMP30), which protects cells from apoptosis (36). CR induced overexpression of Tieg1, which can induce apoptosis in a pancreas-derived cell line, as can TGFβ (51).

Other molecular pathways also may contribute to the effects of CR and DF on apoptosis. DF alone and additively in combination with CR decreased the expression of eight chaperone genes (table in Fig. 3). We have previously shown that the mRNA and protein levels of most hepatic endoplasmic reticulum (ER) chaperones increase with age and decrease with CR and fasting, most likely in response to changes in the insulin to glucagon ratio (15). Reduced chaperone expression increases apoptotic responsiveness to genotoxic stress through both the endoplasmic stress and mitochondrial apoptosis signaling pathways (50, 59). Thus DF, CR, and fasting reduce ER chaperone levels and thereby enhance apoptosis in liver, perhaps accounting for a part of their anticancer benefits (28, 37).

In contrast to the results above, DF and CR upregulated Hspa9a, Hspa1b, and Herpud1. Hspa9a and Hspa1b, homologs of the hsp70 family that differ by only two amino acids, were induced by CR in NL and DF mice. Hspa9a (mortalin-1), is antiproliferative in normal cells and may be a chaperone in mitochondria and the ER. Hspa1b (mortalin-2) has proliferative functions, can repress p53-mediated transcriptional transactivation via a nuclear-exclusion mechanism, and may be a chaperone involved in intracellular trafficking and mitochondrial import. Herpud1 is an ER-resident chaperone thought to regulate ER-associated protein degradation. It also represses transcription as a heterodimer with other factors. Thus induction of these multifunctional chaperones may contribute to the growth-repressive molecular environment in DF and CR liver.

Oxidant and toxin defense. Oxidative and other genotoxic damage to DNA has been implicated in tumor formation (12). We found additive induction of eight phase I and II xenobiotic metabolism-related genes by DF and CR (Cyp2d9, Cyp3a16, Fmo5, Ephx1, Ephx2, Gstm1, Gstm3, and Gstp2). In addition, DF upregulated three such genes (Cyp3a25, Gsta2, and Gsta4), and CR upregulated two of these genes (Cyb5r1-pending and Gstt2). We also found that DF and CR alone and in combination upregulated Gclc expression, a rate-limiting enzyme in the synthesis of glutathione, which plays a crucial role in the intracellular antioxidant defense systems. In addition, DF upregulated Gpx4 and Tdpx-ps1, and CR upregulated Gsr expression. The function of this gene is presently unknown. The upregulation of genes for xenobiotic and antioxidant metabolism might enhance life span through their anticarcinogenic effects (55). Interestingly, Es31, a carboxylesterase with uncharacterized substrate specificity, was five- to sixfold downregulated by DF.

DF induced three members of the ATP-binding cassette membrane multidrug resistance transporters (Abcc3, Abcc2, and Abcg2). Abcc3, multidrug resistance transporter 3, exports a wide range of organic anions back to the blood, thereby decreasing exposure and toxicity to the liver. Abcc2 mediates ATP-dependent transport of various amphipathic endogenous and xenobiotic compounds across the canalicular membrane into the bile and is a major driving force for bile flow. Abcg2, which codes for a transmembrane transporter localized in the liver bile canaliculi, protects the organism from potentially harmful xenobiotics. These results suggest that DF mice have enhanced protection from potentially harmful endogenous and xenobiotic toxins.

DF induced intracellular solute carrier transporters for cationic amino acids (Slc7a2); vitamin C (Slc23a1); many monocarboxylates, such as lactate, pyruvate, branched-chain oxo acids derived from leucine, valine, and isoleucine, and α-ketoacids (Slc16a7). Together these data are consistent with the evidence for enhanced gluconeogenic activity and protein turnover in DF mice discussed above.

DF repressed the expression of Slc10a1, which encodes a hepatocyte-specific transporter for the uptake of taurocholate and other bile salts; Slc22a1, which encodes the main receptor for uptake of a variety of structurally diverse organic cations and toxins in hepatocytes; and Slc29a1, an equilibrative nucleoside transporter that plays an important role in adenosine-mediated regulation of physiological processes and the uptake of cytotoxic nucleosides. Downregulation of these genes should decrease the uptake of potentially toxic xenobiotics and endogenous substances.

Published studies of DF gene expression. Several cDNA array studies of gene expression in DF and CR mice have been published. One study of Ames DF mice found 13 possible changed genes in liver, although only IGFI was significant after adjustment for multiple comparisons (20). Our results confirm three of these changes (Igfbp2, Igf1a, and Hspca). In a study of 6-mo-old Snell DF mice, 17 changed genes met the Bonferroni-adjusted significance criterion (21, 21). Three of these changes were confirmed in our study (Igf1, Igfbp2, Mup1). A simple comparison between lists of found genes from different CR and Snell DF studies found 29 overlapping genes (46). Our results confirm five of these (Igfbp2, Mup1, Mbl1, Hsd17b2, and Tfpi2). The scarcity of cDNA-array-found genes common to our studies may reflect the relative stability of the two array platforms, study design, the relatively small number of probes common to both arrays (1,411 of 12,000 Affymetrix probe sets), and differences in analytical and statistical methods.

How do DF and CR additively extend life span? Here we found that the vast majority of the effects of DF and CR on gene expression fall into two general categories. The first category of genes changed expression in response to only one intervention. The other category of genes was additively affected by the combination of the interventions. The genes in each of these categories were spread throughout the functional categories of genes affected by the interventions (Supplemental Table S2). For example, the number of DF-, CR- and additively DFCR-responsive genes in the xenobiotic and oxidant metabolism, signal transduction, transcription, and chaperone functional categories was approximately proportional to the total number of the DF-, CR-, and additively DFCR-responsive genes (Fig. 1*A*; and Supplemental Table S2). In contrast, the DF-responsive genes were dominant in the categories of nucleotide metabolism, glycolysis, fatty acid synthesis, lipid transport, cholesterol synthesis, and immune system. Together, these results suggest that genes which are additively and individually affected by the interventions contribute to the additive effects of DF and CR on life span.

ACKNOWLEDGMENTS

We thank Hyon J. Kim for helpful discussions and advice, Glenn S. Shea for excellent technical support, and BioMarker Pharmaceuticals and the Life Extension Foundation for unrestricted gifts in support of this research.

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