

Differentially abundant mRNAs in rat liver in response to diets containing soy protein isolate

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Iqbal, M. J., S. Yaegashi, R. Ahsan, D. A. Lightfoot, and W. J. Banz. Differentially abundant mRNAs in rat liver in response to diets containing soy protein isolate. *Physiol Genomics* 11: 219–226, 2002. First published September 17, 2002; 10.1152/physiolgenomics.00078.2002.—Soy diets influence cell growth, regulate lipid metabolism to lower blood cholesterol, and prevent bone losses. These biological effects are most likely due to effects of soy phytochemicals on the expression of genes. In this study, we fed 12 female obese Zucker rats (*fafa*) with a low- or a high-isoflavone soy protein diet and compared the gene expression with animals on a casein diet. Rat livers were compared by differential display of mRNA, and 62 unique sequences were identified. The change in mRNA abundance of these sequences was quantified by cDNA macroarray analysis. Thirty-three mRNAs showed more than twofold increase in abundance on soy diets compared with the control. The corresponding genes include carnitine palmitoyltransferase I, stromal cell-derived factor 1, a protein associated with MYC mRNA, basic transcription element binding protein, and expressed sequence tags (ESTs) of unknown function. Twenty-nine mRNAs showed a less than twofold change in abundance in the two diet treatments. For majority of the genes identified, there was not significant difference between the low- and high-isoflavone diet treatments. Therefore, the contrast between soy protein and casein caused the changes observed in mRNA abundance.

gene expression; soy protein diets; metabolic syndrome

SOYBEANS CONTAIN A PLETHORA of bioactive phytochemicals (such as isoflavones, saponins, oligosaccharides, peptides, phytic acids, phytosterols, trypsin inhibitors, and phenolic acids). Many of these bioactive compounds are found in the soy protein fraction, but their abundance depends on the processing method used (21). If soy protein is extracted by a water-isolation process, then many bioactive compounds remain in the soy protein isolate, resulting in a high-isoflavone soy protein (HS). In contrast, if soy protein is isolated via an alcohol-wash process, the isoflavones along with many of the other bioactive compounds are removed

from the protein resulting in low-isoflavone soy protein (LS). The isoflavone fraction contains high concentration of estrogenic analogs (phytoestrogens). Particularly abundant are genistein and daidzein (21) but also glycitin is present. If one feeds typical soy-based diets to monogastric animals, the phytoestrogens in the blood stream can exceed normal concentrations, causing physiological responses (29). It has been demonstrated that soy protein exerts specific hypocholesterolemic effects when ingested, allowing the US Food and Drug Administration to approve a health claim linking foods that are naturally rich in soy protein to a reduction in coronary heart disease (1, 2). Cardiovascular disease (CVD) is a major killer in the United States and most other industrialized nations. Hyperinsulinemia, hypertension, dyslipidemia, and android obesity are all considered risk factors for CVD. Furthermore, these risk factors have a tendency to aggregate, and this combination has been termed the metabolic syndrome. In addition, nonalcoholic steatohepatitis (NASH) and type 2 diabetes mellitus (type 2 DM) are associated with the metabolic syndrome (7, 17, 31). This group has previously demonstrated in a model of metabolic syndrome (i.e., obese Zucker rat) that soy-based diets could reverse several metabolic syndrome risk factors (29). Since these dietary supplements are known to have health-beneficial effects, there is a need to elucidate the effects of LS and HS diets on gene expression in animal model systems.

In earlier studies, soy constituents such as isoflavones have elicited regulatory effects on estrogen receptor α - and β -dependent gene expression (26). The soy isoflavone component genistein has been widely studied for its effect on specific mRNA abundance in different cell lines (37, 39) and in whole animals (9, 12). Changes in hepatic mRNA abundance in response to soy proteins were evaluated in the livers of gerbils (32). However, these studies were targeted to a few known genes and receptors and not the identification of novel genes or the detection of global regulation patterns.

Conventional and microarray measurements of mRNA abundance do not include unknown genes and expressed sequence tags (ESTs). Techniques such as serial analysis of gene expression (SAGE) (34) and differential display of mRNA (ddmRNA) avoid gene preselection and allow the comparison of mRNA abundances between cell populations (19), between organs

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(35), or between treatments (20, 33). Genes identified by ddmRNA require that the response be confirmed by independent assays (21a, 34a). Commonly used are Northern hybridization, quantitative RT-PCR, RNase protection assays, macroarrays, or microarrays. Most assays can be used with one or few genes but are labor intensive at high throughput. In cDNA macroarray, DNA fragments are spotted on a pair of identical membranes along with a panel of known genes that are likely to be constitutively expressed as references (4). The mRNA probes are labeled as cDNA by reverse transcription and hybridized to the membranes. Spot intensity data are normalized with the reference controls to measure mRNA abundance in a semiquantitative way. Most of the commercially available cDNA macroarray filters (e.g., SuperArray, Bethesda, MD) are either pathway specific or contain known genes belonging to a specific metabolic pathway. In studies with such arrays, there is a good chance of missing genes of unknown function that have altered mRNA abundance in response to a particular treatment.

In this study we employed the ddmRNA technique to identify genes/ESTs with altered mRNA abundance in the livers of female obese Zucker rats in response to casein protein or soy proteins with low and high concentrations of soy isoflavones as dietary supplements. cDNA macroarrays were used to semiquantitatively measure the change in mRNA abundance in livers caused by the different diets.

MATERIAL AND METHODS

Animals, diet, and experimental design. Twelve female obese Zucker rats were randomly assigned to three diets at 6 wk of age. In addition to common components, control diet (C) contained 200 g/kg casein, low-isoflavone soy protein diet (LS) contained 200 g/kg of low-isoflavone soy protein (0.2 mg isoflavone/g protein), and the high-isoflavone soy protein diet (HS) contained 200 g/kg of high-isoflavone soy protein (5.8 mg isoflavone/g protein). All soy protein used in the study, SUPRO SOY brand isolated soy protein (a Solae protein), was a gift from DuPont Protein Technologies International (St. Louis, MO). The common diet components included sucrose (300 g/kg), cornstarch (350 g/kg), corn oil (50 g/kg), cellulose (50 g/kg), vitamin mix (10 g/kg), mineral mix (35 g/kg), choline chloride (2 g/kg), DL-methionine (3 g/kg), and DL- α -tocopherol (1.2 g/kg). Experimental diets were based on the American Institute of Nutrition 93G formula for growth (30). All rats were housed in the Southern Illinois University (SIU) Small Animal Facility and maintained on their respective diet (*ad libitum*) in powdered form for 10–12 wk, then killed after an overnight fast. Livers were quickly frozen in liquid nitrogen for subsequent gene expression analysis. The SIU Animal Care and Use Committee approved all animal studies.

RNA isolation. A small piece of liver sample (30 mg) was ground to a fine powder in liquid nitrogen with a mortar and pestle. The frozen powder was transferred to a 1.5-ml Eppendorf tube, and 600 μ l of RLT buffer (RNeasy Mini Kit, catalog no. 74104; Qiagen) was added. The sample was homogenized by passing through a QIAshredder column. RNA was isolated by Qiagen RNeasy Mini Kit according to the manufacturer's instructions. RNA samples were treated with DNase in order to remove any residual DNA in the preparation. After DNase

treatment, RNA was purified by RNeasy Mini Spin column (Qiagen), and the quality was determined by running on 1.2% (wt/vol) formaldehyde agarose gel. The concentration of RNA was measured by taking absorbance at 260 nm using a Spectronic UV-visible spectrophotometer. The assays were repeated before probe synthesis.

Differential display of mRNA. For ddmRNA, equal amounts of RNA from four animals per diet were pooled together to make three samples following C, LS, or HS treatment. Total RNA (0.2 μ g) from each of the three pools was used for reverse transcription using H-T11M primers (where M was G, A, or C) as described in RNAimage kit (GenHunter, Nashville, TN). A total of 72 primer combinations (H-T11A, H-T11G, H-T11C, with HAP1–HAP24) were used for PCR amplifications after reverse transcription. After PCR, the samples from three treatments (C, LS, and HS) were electrophoresed side by side on 5% (wt/vol) acrylamide gel. The acrylamide gel was transferred on a filter paper, dried, and exposed to Biomek MR films (Kodak).

Cloning of differentially expressed sequences. After visual comparisons of amplification profiles of cDNAs, the differentially expressed fragments were excised from the gel and reamplified by the original primer combination. The PCR products were resolved on an agarose gel, and amplified fragments were purified by Qiagen gel purification kit, ligated in pGEM-T vector (Promega, Madison, WI) and transformed in to DH10B electrocompetent cells (GIBCO-BRL; Life Technologies, Grand Island, NY) by electroporation. The recombinant clones were picked after blue/white screening and stored in freeze culture media. After plasmid isolation, the inserts were sequenced by ABI Big Dye Terminator chemistry on an ABI377 automated DNA sequencer. Clones were sequenced by one pass using M13 forward or reverse primer. Vector and primer sequences were removed, and the remaining sequences were used for the BLAST search of the GenBank or rat, mouse, or human gene index at <http://www.tigr.org/> for homology to known genes/proteins and ESTs.

Amplification of cDNA inserts for macroarray analysis. Inserts of cDNA clones were amplified using M13 forward and reverse primers in 50 μ l reaction volume containing 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dATP, dGTP, dCTP, and dTTP, 5 μ M of M13 forward and reverse primers, 1 U *Taq* DNA polymerase, and 10 ng of plasmid DNA. Amplification was carried out in a PerkinElmer 9700 thermal cycler. After initial denaturation of 94°C for 3 min, the amplification was carried out for 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The reactions were incubated for 4 min at 72°C for final extension and followed by a hold at 4°C until removed from the thermal cycler. An aliquot (5 μ l) of each reaction was electrophoresed on 1.2% (wt/vol) agarose gel to check the quality and quantity of the amplified inserts relative to one another. The PCR products were purified by ethanol precipitation, washed once with 70% (vol/vol) ethanol, and vacuum dried.

Spotting of cDNA fragments. The vacuum-dried PCR products were resuspended in 45 μ l of 0.4 M NaOH, 10 mM EDTA. Four PCR reactions of each cDNA were combined together to make a uniform concentration of amplified fragment for all the filters. The samples were denatured at 95°C for 5 min and cooled to room temp. MilliBlot-D (Millipore) was used to transfer 20 μ l of each sample onto Hybond-N⁺ (Amersham Pharmacia Biotech, Piscataway, NJ) nylon membrane. The membrane were washed with 2 \times SSC, air dried, and stored at room temperature under vacuum.

Synthesis of α -³²P-labeled cDNA probe. RNA from individual animals in a treatment was pooled to eliminate any

variability due to uniqueness of individual animals. Total RNA (15 μ g) was used to synthesize α - 33 P-labeled cDNA probe for each sample. Reverse transcription of poly-A mRNA was carried out in a 60- μ l reaction containing 1 \times first-strand cDNA synthesis buffer [50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂], 1.5 μ g oligo(dT)₁₂₋₁₈, 0.05 mM of each dATP, dGTP, and dTTP, 7.5 μ l of [α - 33 P]dCTP (3,000 Ci/mmol), 0.01 M DTT, and 15 μ g of total RNA. The reaction mixture was heated at 65°C for 5 min and then cooled to 44°C. Two microliters of SuperScript II (SS II, GIBCO-BRL; Life Technologies) RNase H⁻ reverse transcriptase (RT) was added and incubated. After 25 min, another 2 μ l of SS II RT was added, and the reaction was incubated at 44°C for an additional 35 min. The reaction was terminated by adding 7.5 μ l of 500 mM EDTA. To hydrolyze residual RNA, 6 μ l of 2.5 M NaOH was added and the reaction was incubated at 65°C for 1 h. After cooling to room temperature, 12.5 μ l of 2 M Tris·HCl (pH 7.5) was added. To remove unincorporated nucleotides, the probe was passed through a Sephadex G-50 column.

Hybridization and post hybridization processing. Prehybridization and hybridization were carried out in 6 \times SSC (1 \times SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 \times Denhardt's reagent [1 \times Denhardt's reagent consists of 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone, and 0.02% (wt/vol) bovine serum albumin], 1% (wt/vol) SDS, and 100 μ g/ml sheared salmon sperm DNA. Filters were briefly rinsed with 2 \times SSC and prehybridized for 1 h at 65°C. Probe was denatured in boiling water for 5 min, chilled on ice, and added to the hybridization bottles. Hybridization was carried out at 65°C for 16 h. After hybridization, filters were briefly rinsed with 2 \times SSC, 0.1% (wt/vol) SDS and then washed twice for 5 min at 55°C with 1 \times SSC, 0.1% (wt/vol) SDS. If the filters were still hot, then the wash step was repeated at 65°C for 5 min. In all cases, filters hybridized with probe from C, LS, and HS treatments were subjected to identical conditions. The filters were wrapped in plastic wrap and used to expose a PhosphorImager cassette. The three filters representing C, LS, and HS diet treatments were used to expose the same cassette and were scanned using PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA). The spot finding, quantification, and background subtraction was done using ImageQuant software (Molecular Dynamics).

Data analysis. Spot intensities were normalized with reference to the intensity of β -actin gene present on each filter after background subtraction. Changes in gene expression were calculated by dividing the spot intensity in LS diet by control diet (LS/C), and HS diet by control (HS/C), and LS diet by HS diet (LS/HS). The ratios were calculated independently for each experimental replication, and the data were subjected to analysis of variance using the general linear model. Orthogonal contrasts (27) were conducted to compare and contrast changes in mRNA abundance of LS/C vs. HS/C, LS/C vs. LS/HS, and HS/C vs. LS/HS. Means were generated from ANOVA, and the standard error of means was calculated from the standard deviation.

RESULTS AND DISCUSSION

In the rats in this study, the soy phytochemical diets (LS and HS) elicited an attenuation of fatty liver, an improvement in glucose tolerance, a correction of dyslipidemia, and a marked increase in body weight (results not shown). In previous studies (29), the metabolic and physiological consequences were similar to those in the male and female rats of this study. The addition of soy protein or dietary supplement contain-

ing specific soy compounds reduced insulin resistance and decreased the incidence of the metabolic syndrome, as well as CVD.

Genes and ESTs identified by differential display of mRNA. ddmRNA amplified around 3,500 cDNA fragments with the 72 primer combinations used in the PCR stage of analysis. Amplified cDNA fragments with differential abundance in LS and HS diet compared with the control diet were selected for further analysis. A total of 110 cDNA fragments from the acrylamide gel (Fig. 1) were isolated, cloned in pGEM-T vector and sequenced. The cDNAs identified had higher intensities in LS and/or HS dietary treatments. After sequence alignment, 62 fragments had unique sequences. These sequences were BLAST searched to identify homologies to the known genes/ESTs and submitted in the GenBank (Tables 1 and 2). Over one-half

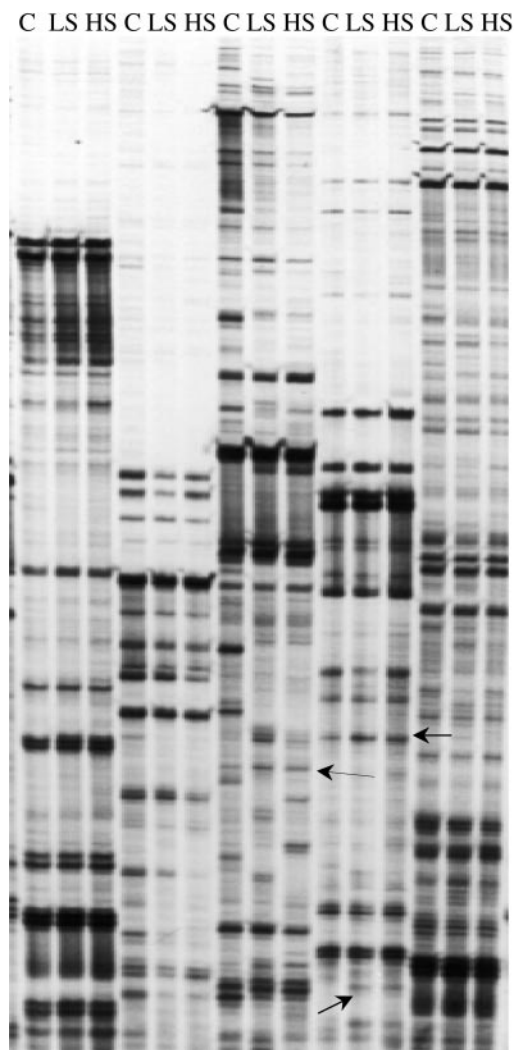


Fig. 1. A comparison of cDNA profiles of control (C), low-isoflavone soy protein (LS) diet, and high-isoflavone soy protein (HS) diet obtained after running PCR product obtained from differential display of mRNA (ddmRNA) on a 5% (wt/vol) denaturing acrylamide gel. Equal amounts of RNA from four liver samples of each treatment were pooled together for comparison by ddmRNA. Some of the selected bands are indicated by arrows.

Table 1. Homology of cDNA fragments identified from ddmRNA after BLAST search to known genes

Sr. No.	SIU Clone ID	GenBank Accession no.	Size, bp	Homology to Known Sequences after BLAST	Identity, %	TC/THC	P
1	CAP17-13B	BM414888	275	<i>Mus musculus</i> retinal short-chain dehydrogenase/reductase I	90	TC270321	5.0e-55
2	AAP13-97B	BM414860	116	<i>Rattus norvegicus</i> thrombin mRNA, 3' end	99	TC242385	1.0e-20
3	GAP16-44A	BM414911	89	Rat mRNA for contraspain-like protease inhibitor related protein (Cpi-26)	98	TC241168	4.1e-14
4	CAP15-67A	BM423316	118	<i>M. musculus</i> WAC mRNA	96	TC270718	4.6e-22
5	CAP10-46A	BM414868	148	<i>R. norvegicus</i> cytochrome-c oxidase III	100	TC249306	7.4e-29
6	GAP13-33A	BM414901	83	<i>Homo sapiens</i> protein associated with Myc mRNA	97	THC752723	2.3e-11
7	GAP4-3A	BM414910	190	h-lamp-2-lysosome-associated membrane protein-2	86	TC270175	2.1e-38
8	GAP8-12B	BM414915	128	Rat carnitine palmitoyltransferase I	97	TC250171	8.5e-23
9	CAP18-17A	BM414883	131	<i>R. norvegicus</i> Sialoprotein (osteopontin) (Spp1)	99	TC259284	5.0e-24
10	GAP11-25B	BM414897	404	<i>R. norvegicus</i> mRNA for serum amyloid P (SAP)	99	TC260022	2.7e-85
11	CAP16-69A	BM414881	105	<i>R. norvegicus</i> deiodinase, iodothyronine type III (Dio3)	100	TC254938	3.6e-16
12	CAP11-51A	BM423317	152	Rat metallothionein-2 and metallothionein-1 genes	100	TC268860	2.4e-29
13	CAP11-50A	BM423318	122	<i>R. norvegicus</i> mRNA for BTE binding protein	100	TC269076	5.6e-22
14	CAP19-20B	BM414890	111	<i>R. norvegicus</i> mitochondrial cytochrome oxidase subunits I, II, III genes, ATPase subunit 6 gene	98	TC249470	2.7e-19
15	AAP14-101A	BM414862	80	<i>M. musculus</i> , Similar to procollagen, type VI, alpha 3	100	TC240400	4.7e-11
16	AAP16-106A	BM414870	98	<i>R. norvegicus</i> BHE/Cdbb tRNA-Lys gene	100	TC249436	1.9e-15
17	AAP14-102A	BM414864	96	<i>R. norvegicus</i> stromal cell derived factor 1 (SDF-1)	100	TC260008	3.6e-16
18	AAP16-105A	BM414859	159	<i>R. norvegicus</i> Aminolevulinatase synthase 2 delta (Alas2)	100	TC250785	1.1e-30
19	GAP4-1A	BM414908	587	<i>M. musculus</i> signal recognition particle 54 kDa (Srp54)	98	TC389642	1.2e-104
20	GAP5-6A	BM414916	429	<i>M. musculus</i> AU-binding enoyl-CoA hydratase	90	TC393639	1.5e-73
21	GAP16-43A	BM414900	194	<i>R. norvegicus</i> nonmuscle caldesmon mRNA	98	TC240000	9.8e-38
22	AAP6-96A	BM414867	204	<i>R. norvegicus</i> mitochondrial genome	100	TC239942	1.6e-41
23	AAP16-104A	BM414863	339	<i>R. norvegicus</i> EGR 1 and NGF-1A	97	TC260956	5.4e-72
24	CAP4-71A	BM414892	223	<i>R. norvegicus</i> mRNA for p47	100	TC269365	4.7e-47
25	GAP8-11B	BM414914	160	<i>R. norvegicus</i> cDNA similar to human mRNA for semaphorin E	94	TC253762	6.9e-21
26	CAP15-65A	BM414878	309	Rat mRNA for ribosomal protein S20	100	TC258665	4.2e-65
27	GAP4-4A	BM414906	131	<i>M. musculus</i> Nbr1 (Nbr1) mRNA	97	TC382616	6.0e-20
28	CAP9-45A	BM414896	129	<i>M. musculus</i> bromodomain-containing protein BP75	96	TC425115	4.7e-21
29	CAP13-55A	BM414873	204	<i>R. norvegicus</i> mRNA for ATP synthase subunit e	100	TC250729	5.9e-41
30	CAP7-78A	BM414899	165	<i>R. norvegicus</i> testosterone-repressed prostate message 2	98	TC259505	3.0e-31
31	GAP23-110A	BM414907	227	Rat mRNA for cytochrome P-450 (CYP2C23)	100	TC249600	3.1e-46
32	CAP4-73xA	BM414889	179	<i>H. sapiens</i> mitogen inducible 2 (MIG2) mRNA	88	TC240149	3.7e-3

TC, tentative consensus identified from TIGR rat or mouse gene index; THC, tentative human consensus identified from TIGR human gene index; SIU, Southern Illinois University; ddmRNA, differential display of mRNA. Accession numbers are obtained after submitting the sequences to GenBank.

of the cDNA fragments (32 of 62) showed a significant homology (over 85% identity) to the known genes and proteins (Tables 1 and 2). The sequence homology ranged from 86% to 100% (Table 1). The sequences having significant homology to the known genes were further BLAST searched in The Institute of Genome Research rat, mouse, and human gene index database (TIGR, <http://www.tigr.org>), and tentative consensus (TC) sequences (from rat or mouse gene index) and tentative human consensus (THC) sequences (from human gene index) were identified (Table 1). The probability values for the TC and THC search ranged from $3.7e-3$ to $1.2e-104$. Size of the identified cDNA fragments ranged from 70 bp (CAP13-58A) to 587 bp (GAP4-1A).

Genes/ESTs with twofold change in mRNA abundance. Of 62 genes and ESTs spotted for macroarray analysis, 33 showed a twofold or higher increase in mRNA abundance in livers of animals fed with LS diet compared with control (Table 3). GAP15-37A and CAP24-24A had a significant difference of change in expression as measured by mRNA abundance ($P < 0.05$) between LS and HS soy diets compared with the

control by the orthogonal contrast analysis. GAP15-37A and CAP24-24A had 2.53 ± 0.42 -fold and 2.93 ± 0.33 -fold increases in abundance in the livers of rats on LS diet compared with the livers of rats on control diet. The changes in mRNA abundance of these two ESTs when HS diet was compared with the control diet were 1.37 ± 0.07 and 1.27 ± 0.16 , respectively. However, so far no function has been assigned to these ESTs. Because of the significantly higher accumulation of mRNA of these ESTs in LS diet animals, it will be important to identify full-length cDNA for gene identification and to correlate their function in the effects of soy dietary supplements. The mRNAs having twofold or higher change in abundance in livers under LS supplement in diet are listed in Table 3. A three-way orthogonal contrast analysis of changes in gene expression (LS/C vs. HS/C, LS/C vs. LS/HS, HS/C vs. LS/HS) revealed that *Rattus norvegicus* EST identified by CAP14-60A has a significant difference ($P < 0.05$) between HS/C vs. LS/HS contrast (Table 3). Rat metallothionein gene identified by CAP11-51A also has a significant difference ($P < 0.05$) in LS/C vs. LS/HS and HS/C vs. LS/HS contrasts. The transcript of this gene

Table 2. List of cDNA sequences identified from *ddmRNA* that do not have a good homology to known genes

Sr. No.	SIU Clone ID	GenBank Accession no.	Size, bp	BLAST Result
1	CAP19-19B	BM414894	138	<i>R. norvegicus</i> EST
2	CAP141-60A	BM414871	215	<i>R. norvegicus</i> EST
3	CAP17-14B	BM414884	165	<i>R. norvegicus</i> EST
4	GAP14-35A	BM414902	163	<i>R. norvegicus</i> EST
5	AAP17-22A	BM414866	180	<i>R. norvegicus</i> EST
6	GAP11-27B	BM414898	179	<i>R. norvegicus</i> EST
7	CAP11-47B	BM414869	268	<i>R. norvegicus</i> EST
8	GAP15-40A	BM423319	182	<i>R. norvegicus</i> EST
9	GAP15-37A	BM414903	266	<i>R. norvegicus</i> EST
10	CAP14-59A	BM414875	222	<i>R. norvegicus</i> EST
11	CAP16-68B	BM414880	322	<i>R. norvegicus</i> EST
12	CAP11-49A	BM423314	211	<i>R. norvegicus</i> EST
13	GAP13-32B	BM414905	117	<i>R. norvegicus</i> EST
14	CAP24-24A	BM414891	79	<i>R. norvegicus</i> EST
15	CAP19-21A	BM423315	99	<i>R. norvegicus</i> EST
16	CAP13-58A	BM414874	70	<i>R. norvegicus</i> EST
17	GAP8-10B	BM414913	386	<i>R. norvegicus</i> EST
18	GAP5-7B	BM414912	334	<i>R. norvegicus</i> EST
19	CAP13-54B	BM414872	297	<i>M. musculus</i> EST
20	AAP14-100A	BM414861	143	<i>R. norvegicus</i> EST
21	GAP4-2A	BM414909	190	<i>R. norvegicus</i> EST
22	CAP17-16A	BM414886	149	<i>R. norvegicus</i> EST
23	CAP17-15A	BM414885	146	<i>R. norvegicus</i> EST
24	CAP15-66A	BM414879	256	<i>R. norvegicus</i> EST
25	CAP17-13A	BM414877	267	<i>R. norvegicus</i> EST
26	CAP11-51B	BM414865	154	<i>R. norvegicus</i> EST
27	CAP14-61A	BM414882	159	<i>R. norvegicus</i> EST
28	CAP11-52A	BM414876	144	<i>R. norvegicus</i> EST
29	GAP15-41A	BM414904	127	<i>R. norvegicus</i> EST
30	CAP4-73A	BM414893	202	<i>R. norvegicus</i> EST

EST, expressed sequence tag.

as measured by cDNA macroarray was more than twofold higher in LS and HS diets compared with control diet-fed animals. The maximum change in abundance in response to LS diet was observed in a transcript showing 93% homology to TPAR1 (12-*O*-tetradecanoylphorbol 13-acetate repressed gene 1) or SDF-1 (stromal cell-derived factor 1) gene. The TPAR1 has been known to be repressed in regenerating rat livers compared with normal adult livers (16). Bleul et al. (6) and Oberlin et al. (25) independently showed that SDF-1 is a potent in vitro inhibitor of infection by lymphocyte-trophic HIV-1 strain. It has also been speculated to play a role in immune surveillance and in basal extravasation of lymphocytes and monocytes. SDF-1 maps with a region encompassing a quantitative trait locus associated with susceptibility to lung cancer (*Sluc3*) on mouse chromosome 6 (5, 8) and is abundantly expressed in breast cancer-derived metastasis. Further studies in cancer models are required to confirm the association of SDF-1 mRNA abundance and its beneficial effects with soy dietary supplements. cDNA clone GAP13-33A, having a homology of 97% (1e-34) with human protein associated with protooncogene MYC, was expressed approximately two times more abundant in livers of rats on LS and HS diet compared with the control diet. This protein interacts directly with transcriptional activation domain of

MYC, a gene that plays an important role in the regulation of cellular proliferation, differentiation, and apoptosis and has been implicated in a variety of human tumors (11). The effect of the two dietary supplements was not significantly different (gene number 6, Fig. 2).

The other genes with twofold or higher increase in mRNA abundance in rat livers under LS diet include mRNA for protein (CAP11-50A with 1e-61) that binds to basic transcription element binding protein (13) and WAC mRNA (CAP15-67A with 5e-50), a novel member of WW-domain-containing proteins for RNA processing (38). A gene with increase in expression in response to phagocytic or immunologic response (18), i.e., h-lamp-2 (1e-33), identified by GAP4-3A was also expressed at significantly higher level in livers of rats with LS diet. The change in mRNA abundance was higher in LS diets compared with HS diets for AAP13-97B (rat thrombin mRNA), CAP15-67A (mouse WAC mRNA), CAP10-46A (cytochrome-c oxidase III), GAP3-4A (h-lamp-2), GAP8-12B (rat CPT I), GAP11-25B (rat SAP), CAP11-51A (rat metallothionein 2),

Table 3. List of cDNA fragments that have a twofold or higher increase in mRNA abundance in livers of rats fed with LS diet compared with the control as determined by macroarray analysis

Sr. No.	SIU Clone ID	LS/C	HS/C	LS/HS	P
1	CAP19-19B	1.97 ± 0.59	1.58 ± 0.43	1.24 ± 0.04	
2	CAP17-13B	1.99 ± 0.57	1.17 ± 0.28	1.68 ± 0.07	
3	CAP14-60A	2.00 ± 0.16	1.60 ± 0.02	1.26 ± 0.12	†
4	CAP17-14B	2.01 ± 0.56	0.89 ± 0.38	2.44 ± 0.43	
5	GAP14-35A	2.05 ± 0.11	1.33 ± 0.20	1.58 ± 0.32	
6	AAP17-22A	2.07 ± 0.51	1.83 ± 0.80	1.25 ± 0.26	
7	AAP13-97B	2.07 ± 0.58	1.05 ± 0.12	1.94 ± 0.33	
8	GAP16-44A	2.11 ± 0.52	1.67 ± 0.12	1.25 ± 0.22	
9	GAP11-27B	2.13 ± 0.59	1.21 ± 0.48	1.87 ± 0.25	
10	CAP15-67A	2.23 ± 0.33	0.98 ± 0.31	2.42 ± 0.44	
11	CAP10-46A	2.23 ± 0.28	1.15 ± 0.11	1.99 ± 0.44	
12	GAP13-33A	2.27 ± 0.42	1.93 ± 0.16	1.20 ± 0.31	
13	GAP4-3A	2.29 ± 0.69	1.28 ± 0.20	1.75 ± 0.26	
14	CAP11-47B	2.33 ± 0.55	1.93 ± 0.29	1.19 ± 0.11	
15	GAP8-12B	2.35 ± 0.44	1.57 ± 0.09	1.48 ± 0.19	
16	GAP15-40A	2.50 ± 0.93	1.39 ± 0.29	1.73 ± 0.30	
18	GAP11-25B	2.51 ± 0.63	1.35 ± 0.36	1.87 ± 0.02	
19	GAP15-37A	2.53 ± 0.42	1.37 ± 0.07	1.84 ± 0.21	<0.05
20	CAP16-69A	2.55 ± 0.58	1.72 ± 0.18	1.46 ± 0.18	
21	CAP14-59A	2.58 ± 0.48	1.93 ± 0.55	1.38 ± 0.14	
22	CAP16-68B	2.58 ± 0.87	1.74 ± 0.51	1.47 ± 0.07	
23	CAP11-49A	2.66 ± 0.63	1.58 ± 0.24	1.66 ± 0.14	
24	CAP11-51A	2.75 ± 0.24	2.17 ± 0.21	1.27 ± 0.01	
25	CAP11-50A	2.79 ± 0.82	2.16 ± 0.61	1.29 ± 0.01	
26	CAP19-20B	2.84 ± 0.99	1.90 ± 0.64	1.49 ± 0.02	
27	GAP13-32B	2.89 ± 0.55	1.50 ± 0.02	1.94 ± 0.39	
28	CAP24-24A	2.93 ± 0.33	1.27 ± 0.16	2.31 ± 0.03	<0.05
29	AAP14-101A	2.97 ± 0.66	1.74 ± 0.21	1.68 ± 0.18	
30	CAP19-21A	3.02 ± 1.56	1.82 ± 0.70	1.55 ± 0.26	
31	AAP16-106A	3.07 ± 0.39	1.62 ± 0.38	1.94 ± 0.01	
32	CAP13-58A	3.77 ± 1.12	1.69 ± 0.83	2.50 ± 0.55	
33	AAP14-102A	3.97 ± 1.28	2.25 ± 0.60	1.74 ± 0.10	

Values are means ± SE. LS, low-isoflavone soy protein; HS, high-isoflavone soy protein; C, control. P values of significant difference (P < 0.05) of change in mRNA abundance in LS/C compared with HS/C contrast are indicated. †Probability of HS/C vs. LS/HS ratios contrast analysis was P < 0.05.

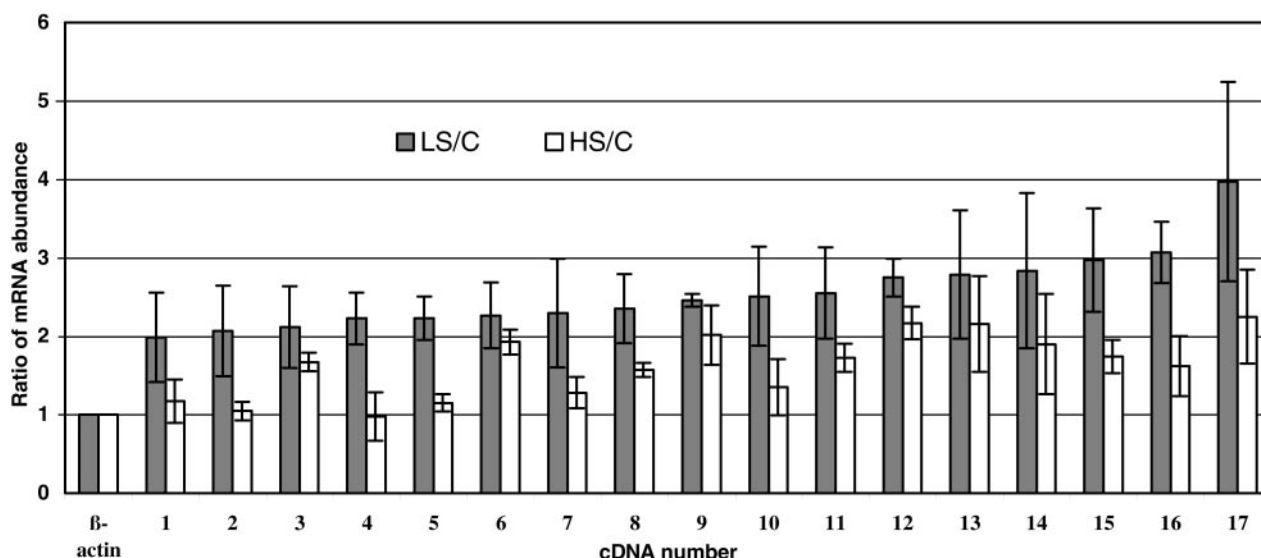


Fig. 2. A comparison of change in mRNA abundance in low-isoflavone soy protein diet (LS) and high-isoflavone soy protein diet (HS) compared with control. cDNA fragments having high homology to known genes and a twofold increase in mRNA abundance are presented. Raw spot intensities were normalized to the β -actin present on each array. Intensity values from the treatment were divided by the control, and the means of the ratios are plotted. The SIU clone IDs for the genes are 1 = CAP17-13B, 2 = AAP13-97B, 3 = GAP16-44A, 4 = CAP15-67A, 5 = CAP10-46A, 6 = GAP13-33A, 7 = GAP4-3A, 8 = GAP8-12B, 9 = CAP18-17A, 10 = GAP11-25B, 11 = CAP16-69A, 12 = CAP11-51A, 13 = CAP11-50A, 14 = CAP19-20B, 15 = AAP14-101A, 16 = AAP16-106A, and 17 = AAP14-102A. Detailed description is in Table 1. Bars show standard error of means; for the solid bars, the descending portion of the error bar is obscured, but this is equal in size to the ascending portion.

AAP14-101A (mouse procollagen), and AAP16-106A (rat BHE) cDNAs (Fig. 2). The difference of change in mRNA abundance (LS/C compared with HS/C) for these genes was more than the standard error of means (Fig. 2), but the three-way orthogonal contrast analysis (LS/C vs. HS/C, LS/C vs. LS/HS, HS/C vs. LS/HS) indicated that the difference of change was not significant ($P > 0.05$).

Dietary soy proteins have been found to have implications to obesity (14), and isoflavones appear to influence lipid metabolism by altering gene expression for lipid-related genes (32). A cDNA clone identified by ddmRNA in this study, GAP8-12B (1e-54), representing rat carnitine palmitoyltransferase I (CPT I) mRNA, was expressed at significantly higher level in rat livers fed with LS and HS soy diet supplements. In mitochondria, the oxidation of fatty acids is an important source of energy for ATP synthesis. However, long-chain fatty acids do not diffuse into mitochondria. The transport of long-chain fatty acids into mitochondria for oxidation is accomplished by the CPT systems (CPT I and CPT II). Therefore, the higher expression of CPT I may help in reducing obesity and in lowering the risks of type II diabetes thought to be associated with high levels of fatty acids in the cells (10, 22, 36). Therefore, we hypothesize that the increase in mRNA abundance of CPT I by soy dietary supplements may have an indirect effect on controlling obesity. This hypothesis needs to be further tested. The other genes related to metabolism with a twofold or higher upregulation in livers with soy diet supplement include CAP17-13B (mouse retinal short-chain dehydrogenase/reductase I), AAP16-106A (rat BHE/Cdbb tRNA-Lys), and CAP19-20B (a rat mitochondrial cytochrome

Table 4. List of cDNA fragments that have a less than twofold increase in mRNA abundance in livers of rats fed with LS diet compared with the control as determined by macroarray analysis

Sr. No.	SIU Clone ID	LS/C	HS/C	LS/HS	P
1	AAP16-105A	1.15 ± 0.31	0.81 ± 0.09	1.40 ± 0.22	
2	GAP4-1A	1.16 ± 0.02	0.93 ± 0.32	1.42 ± 0.50	
3	GAP5-6A	1.23 ± 0.53	0.96 ± 0.31	1.23 ± 0.17	
4	GAP8-10B	1.29 ± 0.48	0.90 ± 0.43	1.53 ± 0.21	
5	GAP5-7B	1.32 ± 0.41	1.23 ± 0.19	1.05 ± 0.17	
6	GAP16-43A	1.35 ± 0.71	1.11 ± 0.27	1.13 ± 0.36	
7	CAP13-54B	1.39 ± 0.15	1.05 ± 0.17	1.39 ± 0.36	
8	AAP6-96A	1.40 ± 0.42	0.97 ± 0.09	1.42 ± 0.29	
9	AAP16-104A	1.41 ± 0.33	1.41 ± 0.26	0.99 ± 0.05	
10	CAP4-71A	1.42 ± 0.51	1.54 ± 0.32	0.89 ± 0.14	
11	AAP14-100A	1.46 ± 0.43	0.83 ± 0.20	1.73 ± 0.10	
12	GAP8-11B	1.52 ± 0.11	1.02 ± 0.15	1.50 ± 0.11	
13	GAP4-2A	1.53 ± 0.24	1.17 ± 0.16	1.30 ± 0.03	
14	CAP17-16A	1.53 ± 0.13	0.60 ± 0.22	2.87 ± 0.84	
15	CAP15-65A	1.58 ± 0.18	1.18 ± 0.00	1.34 ± 0.15	
16	CAP17-15A	1.65 ± 0.26	0.93 ± 0.38	1.98 ± 0.52	
17	CAP15-66A	1.66 ± 0.49	1.01 ± 0.29	1.63 ± 0.02	
18	GAP4-4A	1.66 ± 0.32	0.96 ± 0.28	1.79 ± 0.19	
19	CAP9-45A	1.67 ± 0.17	1.59 ± 0.12	1.04 ± 0.02	*
20	CAP17-13A	1.68 ± 0.35	1.29 ± 0.27	1.30 ± 0.00	
21	CAP13-55A	1.69 ± 0.47	0.90 ± 0.18	1.85 ± 0.13	
22	CAP7-78A	1.70 ± 0.48	1.20 ± 0.07	1.40 ± 0.31	
23	CAP11-51B	1.72 ± 0.20	1.48 ± 0.09	1.16 ± 0.05	†
24	CAP14-61A	1.73 ± 0.29	1.27 ± 0.08	1.35 ± 0.15	
25	CAP11-52A	1.75 ± 0.08	1.01 ± 0.02	1.73 ± 0.04	
26	GAP23-110A	1.77 ± 0.18	1.21 ± 0.14	1.46 ± 0.01	
27	GAP15-41A	1.79 ± 0.67	1.31 ± 0.32	1.32 ± 0.18	
28	CAP4-73A	1.82 ± 0.74	1.56 ± 0.24	1.12 ± 0.29	
29	CAP4-73xA	1.85 ± 0.44	1.42 ± 0.04	1.30 ± 0.27	

Values are means ± SE. There was no significant difference of change in mRNA abundance in LS/C compared with HS/C contrast ($P > 0.05$). *Probability of LS/C vs. LS/HS ratios contrast analysis was $P < 0.05$. †Probability of HS/C vs. LS/HS ratios contrast analysis was $P < 0.05$.

oxidase subunit I, II, III genes, ATPase subunit 6 gene). There was no difference in the treatment effect of LS and HS diets for CAP17-13B and CAP19-20B. The change in mRNA abundance for AAP16-106A was higher in LS/C diet compared with HS/C analysis (Fig. 2); however, the three-way orthogonal contrast analysis predicted it was not significant ($P = 0.09$).

One-half of the cDNA fragments identified from ddmRNA and having more than twofold upregulation in livers of rats on diet with LS supplement were ESTs with unknown function. These ESTs are potentially new targets for research, and the identification of their corresponding full-length cDNAs will be helpful in finding the proteins they code and their role in rats response to soy dietary supplements.

Genes with less than twofold change in expression. The macroarray analysis of cDNA fragments obtained from ddmRNA identified 29 genes and ESTs having less than twofold change in expression in LS diet treatment (Table 4). Thirteen of these sequences were ESTs, and 16 had good homology to known genes. An EST identified by CAP11-52A had upregulation by 1.75 ± 0.08 in LS diet treatment and no change (1.01 ± 0.02) in HS diet treatment. The statistical analysis revealed that there was a significant ($P < 0.05$) dietary supplement effect (LS/C vs. HS/C) on its abundance in liver. Other genes with less than twofold but more than 1.5-fold increase in mRNA abundance include cytochrome P-450 (GAP23-110A), a human mitogen inducible 2 (MIG2) (CAP4-73xA) mRNA, rat testosterone-repressed prostate message 2 (TRPM2) (CAP7-78A), mRNA for ATP synthase subunit e (CAP13-55A), mouse bromodomain-containing protein BP75 (CAP9-45A), and a mouse Nbr1 mRNA (GAP4-4A). These genes have higher expression in both dietary treatments but did not show a significant difference between the two dietary supplements.

Alterations of key metabolic enzymes/pathways in the obese (*falpa*) Zucker rat are similar to humans exhibiting type 2 DM, NASH, and the insulin-resistant metabolic syndrome. This model has been well established for testing diet and disease relationships (29); thus they were used as our in vivo model of insulin resistance and the diabetic phenotype. When Wistar fatty rats were fed with soybean protein/partially beef tallow, the insulin receptor mRNA concentrations were higher in the livers compared with those fed with any other protein/fat combination (15).

In this study we used ddmRNA to randomly screen more than 3,500 unknown transcripts and then spotted the differentially expressed cDNA fragments identified by visual comparison on nylon membranes and quantitatively confirmed the changes in expression under different dietary supplements in rat livers. We identify genes and ESTs that increased in response to soy dietary supplements. Soy proteins and isoflavones/phytoestrogens are known to lower blood cholesterol (28) and to reduce the risks of various types of cancers (3, 23). By combining ddmRNA and macroarray analysis, genes involved in lipid metabolism, regulation of transcription and translation, protease inhibition, apop-

osis, and cell proliferation regulation were found to be expressed at higher levels in rat livers fed with the LS and HS diet compared with the livers of control diet animals. The coordinated regulation of these genes in livers further supports the hypothesis of multiple health benefits of soy supplements (1–3, 23). In addition to the known genes, the ESTs with potential involvement in the response to soy isoflavone or soy protein diets were identified. Since some of these may derive from the effects of genetic differences of individual rats, a larger population should be evaluated for the gene expression analysis and EST annotation. For the identification of onset of these changes in expression/mRNA abundance, different developmental/growth stages of animals on experimental diets should be evaluated. Moreover, full-length genes for the ESTs should be identified to elucidate their role in the animal's response to these soy dietary supplements.

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