Up-regulation of genes for oxidative phosphorylation and protein turnover in diabetic mouse retina*

Nanami Adachi-Uehara[a,b], Masaki Kato[c], Yoshinori Nimura[c], Naohiko Seki[c],

Akinori Ishihara[a], Eriko Matsumoto[a], Katsuro Iwase[a], Satoko Ohtsuka[a],

Hiroki Kodama[d], Atsushi Mizota[b], Shuichi Yamamoto[b], Emiko Adachi-Usami[b],

Masaki Takiguchi[a,*]

[a]*Department of Biochemistry and Genetics, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

[b]*Department of Ophthalmology and Visual Science, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

[c]*Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

[d]*Department of Mathematical Sciences, University of Tokyo, Tokyo 153-8914, Japan*

* Presented in part at the 2004 annual meeting of the Association for Research in Vision and Ophthalmology

* Corresponding author. Address: Masaki Takiguchi, Department of Biochemistry and Genetics, Chiba University Graduate School of Medicine, Inohama 1-8-1, Chiba 260-8670, Japan. Tel.: +81-43-226-2035; fax: +81-43-226-2037.

E-mail address: mtak@faculty.chiba-u.jp (M. Takiguchi).

Abstract

Diabetic retinopathy is one of the most frequent complications of diabetes and is a leading cause of vision loss in adulthood. To better understand the molecular pathophysiology of diabetic retinopathy, we performed comprehensive gene expression analysis of the mouse retina under diabetic conditions with an in-house cDNA microarray system that was designed to be suitable for the small amount of RNA available from a single mouse retina. Diabetes was induced in male C57BL/6 mice by an intraperitoneal injection of streptozotocin, and the changes in retinal mRNA levels were examined in three pairs of diabetic and age-matched control mice at 1 and 3 months after the injection of streptozotocin. Northern blot analysis with amplified total cRNA confirmed the increase in mRNA levels of several selected genes. Most of the significantly up-regulated genes could be classified into two functional categories: oxidative phosphorylation and protein turnover. All mitochondrial DNA-encoded and most of the nuclear DNA-encoded genes for oxidative phosphorylation were up-regulated in the diabetic retina. This was in sharp contrast with a previous report of a down-regulation of these genes in skeletal muscles of streptozotocin-induced diabetic mice and type 2 diabetic humans. Genes for protein synthesis and ubiquitin were also up-regulated in the diabetic retina, suggesting the increase in turnover rates for at least a part of the protein population. Taken together, the diabetic retina appears to be in a state activated for intermediary metabolism, presumably because of an increase in insulin-independent glucose influx. These results provide insights into possible preventive and therapeutic intervention of diabetic retinopathy.

Keywords: microarray; retina; diabetic retinopathy; mitochondria; ubiquitin

1. Introduction

Diabetic retinopathy is one of the most frequent complications of diabetes and is a leading cause of vision loss in adulthood. Historically, ophthalmoscopically observable vascular changes of the diabetic retina have been emphasized as the cause of the retinopathy, i.e., increased vascular permeability due to breakdown of blood-retinal barrier (BRB) is manifested as macular edema followed by microaneurysms and hemorrhages, ultimately leading to vascular proliferation. Recently, several aberrations of retino-neural functions have been reported to precede the obvious vascular changes (Barber, 2003; Reiter and Gardner, 2003); e.g., abnormalities in the electroretinogram were detected in the early diabetes, and they were accompanied by the deficits in night vision and contrast sensitivity especially in color vision. It appears that all of the major retinal cell types such as neurons, macroglias and microglias, as well as vascular cells, are involved in the pathogenesis of diabetic retinopathy (Gardner et al., 2002).

Comprehensive gene expression analysis with DNA microarrays is a powerful tool to investigate the etiology and pathophysiology of diseases including diabetic retinopathy in mouse models. Attempts have been made to prepare and/or analyze DNA microarrays for the rodent retina (Cho et al., 2002; Farjo et al., 2002; Joussen et al., 2001; Sato et al., 2003). In general, the relatively small amount of RNA that can be obtained from a mouse retina hampers the construction and analysis of cDNA microarrays. Recently, we developed a system, named the widely-applicable cDNA/cRNA system, that is suitable both for the construction of a cDNA library and for the measurement of the mRNA levels with several options of hybridization analysis with cDNA and cRNA amplified from a small amount of mRNA (Ohtsuka et al., 2004). We have applied this system to the preparation of the cDNA microarray followed by its analysis and confirmation of the obtained results from a single mouse

retina.

Historically, diabetic rats produced by treating them with streptozotocin (STZ) have been used extensively for analysis of retinopathy including gene expression profiling with microarrays. A number of genes for inflammatory process were reported to be up- or down-regulated especially during the first week of STZ-induced diabetes in rat retinas (Joussen et al., 2001). Müller cells of rat retinas after 6 months of diabetes also exhibited activation of inflammation-related genes including those for acute phase response proteins (Gerhardinger et al., 2005). A recent microarray study (Knoll et al., 2005) found that the gene response in STZ-induced diabetes is largely different among rat tissues such as the renal cortex, cardiac left ventricle, skeletal muscle, and retina.

Compared to rats, studies on diabetic retinopathy of STZ-treated mice are limited, but the features of the retinopathy have been described including the vascular changes (Hammes et al., 2002), increased levels of caspase activation, a marker of apoptosis (Mohr et al., 2002), and neuronal cell loss (Martin et al., 2004). Because of its advanced genetics, the mouse provides a growing number of useful models of human diseases, as was exemplified by the Ins2[Akita] mouse with a mutation in the insulin 2 gene and exhibiting diabetic retinopathy with vascular, neural, and glial abnormalities (Barber et al., 2005).

The purpose of this study was to determine the gene expression profiles in retinas of diabetic mice 1 and 3 months after STZ injection. We found that genes involved in oxidative phosphorylation and protein turnover were activated. This was strikingly different from previous observations for gene expression profiles in the skeletal muscle of STZ-induced diabetic mice (Yechoor et al., 2002; Yechoor et al., 2004) and type 2 diabetic humans (Mootha et al., 2003; Reiter and Gardner, 2003), revealing characteristic features of gene regulation in the diabetic retina.

2. Materials and methods

2.1. Experimental animals

All experimental procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were group-housed with full access to food and water at a room temperature of 23 $\pm$ 1°C in a 12-h light/12-h dark cycle. To induce diabetes, 8-week-old male C57BL/6 mice were injected intraperitoneally with 200 mg/kg STZ (Sigma, St. Louis, MO) in 0.4 ml of 50 mM citrate (pH 4.5)/154 mM NaCl. Insulin was not administered to the mice throughout the experiment. Control mice were injected with the citrate buffer only. The glucose levels in blood samples taken from the tail vein were measured by the glucose dehydrogenase/potassium ferricyanide method with the Advantage II glucose meter (Roche Diagnostics, Tokyo, Japan). The statistical significance of differences of blood glucose levels and body weights between groups was evaluated using Student's t-test. $P < 0.05$ was considered significant. Data are shown as mean $\pm$ S.E.

Mice were killed by ether anesthesia, and the eyes were enucleated by cutting the optic nerve just behind the eyeball. Then the anterior segment of the eye was cut away, and the retina was carefully isolated from the retinal pigment epithelium layer with forceps. The isolated retina appeared clear, and no pigmentation was seen under the microscope, indicating that the retina was free from retinal pigment epithelial cells. Total RNA was prepared from the mouse retina with TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and the yield of RNA was about 4 μg from a single retina.

2.2. Preparation of mouse retina cDNA library

The cDNA library was constructed from the mouse retina as schematically represented in Steps 1-8 of Fig.1. Detailed procedures for Steps 1, 2, and 4-8 were described previously (Ohtsuka et al., 2004). Briefly, poly(A)$^+$ RNA derived from 1 μg of total RNA was adsorbed onto 50 μg of oligo(dT)-paramagnetic beads, Dynabeads Oligo(dT)$_{25}$ (Dynal, Oslo, Norway) (Step 1), and was subjected to antisense-strand cDNA synthesis with reverse transcriptase, followed by sense-strand cDNA synthesis-coupled replacement reaction with *Escherichia coli* DNA polymerase I, DNA ligase, and RNase H (Step 2). The resulting bead-fixed double-stranded cDNA was divided into three aliquots, which were each digested with one of three restriction enzymes *Ban*I, *Eco*O109I and *Hinc*II (Step 3). Each of these enzymes cuts random DNA sequences once on an average of every 1,024 bp length, preventing the loss of long cDNA species in the following PCR steps. The bead-fixed 3'-trimmed cDNAs were mixed, blunt-ended, and ligated with 1 μg of the linker containing the T7 promoter sequence (Step 4). The sense-strand cDNA was liberated by heat-denaturation (Step 5), and was again converted to the double-stranded form with priming from the oligo(dT) primer containing the SP6 promoter sequence (Step 6). The double-stranded cDNA was amplified with PCR using known sequences at both ends as primers (Step 7). Restriction ends for *Ava*I 5'-CCGG-3' and *Acc*I 5'-CG-3' were constructed on 5' and 3' terminals, respectively, of the amplified cDNA with exonuclease activity of T4 DNA polymerase in the presence of dATP and dTTP but in the absence of dCTP and dGTP. This allowed a unidirectional ligation with *Ava*I/*Acc*I-cut pUC19 (Step 8) after gel-purification of cDNA fragments longer than 800 bp with glass powder Glassmilk (Qbiogene, Montreal, Canada). The ligation products were subjected to transformation of *E. coli* strain DH10B (Invitrogen). Nucleotide-sequencing from the 5' end of 2,880 cDNA

clones allowed them to be classified into about 1,900 distinct genes.

2.3. Preparation of cDNA microarray

A microarray chip harboring the 2,880 retinal cDNAs was prepared (Step 9), essentially as described (Yoshikawa et al., 2000). Briefly, PCR-amplified cDNAs were mixed with nitrocellulose, and spotted onto carbodiimide-coated glass slides (Nisshinbo Industries, Inc., Chiba, Japan) with a robotics SPBIO-2000 (Hitachi Software Engineering Co., Yokohama, Japan).

2.4. Microarray analysis

Target mixtures were prepared, and hybridization was carried out as follows (Fig. 1, Steps 1, 2, and A to D). The bead-fixed double-stranded cDNA derived from 2 μg of total RNA was synthesized as described (Steps 1 and 2), and ligated with 1 μg of the T7 promoter-linker (Step A). In vitro transcription of sense-strand cRNA (Step B) was carried out with a kit for T7 RNA polymerase AmpliScribe T7 (Epicentre, Madison, WI) in 20 μl of the reaction mixture at 37°C for 2 hr. The cDNA-beads were separated magnetically, and the synthesized cRNA in the supernatant was collected by ethanol precipitation. Typically, 10 μg of sense-strand cRNA were obtained, and 0.6 μg of the cRNA was used as a template for reverse transcription-coupled incorporation of 5-(3-aminoallyl)-dUTP (Ambion Inc., Austin, TX) into antisense-strand cDNA, which was in turn postlabeled with Cy3 or Cy5 with the Mono-Reactive Dye Pack (Amersham Biosciences, Tokyo, Japan) using standard procedures according to manufacturer's' protocols. The Cy3- and Cy5-labeled cDNAs (each 9 μl) were mixed and made up to 40 μl of the solution containing 1.25 μg/μl of yeast tRNA, 1.25 μg/μl of poly(A), 25 ng/μl of the upper

7

strand of the T7 promoter-linker, 25 ng/μl of the SP6 promoter/oligo(dT)-primer, 3.4 x SSC [1 x SSC: 0.15 M NaCl/15 mM sodium citrate], and 0.3% SDS. The mixture was hybridized with the microarray at 65°C overnight under humidified conditions. The array was washed twice for 10 min with 2 x SSC/0.1% SDS at room temperature, and twice for 10 min with 0.2 x SSC/0.1% SDS at 40°C. After centrifugation at 2,000 rpm for 1 min and air-drying, the array was scanned with a fluorescence laser-scanning device (ScanArray4000; GSI Lumonics, Bedford, MA).

2.5. Data analysis

Microarray images were analyzed with the Quant Array software (GSI Lumonics). The median for each of Cy3 and Cy5 fluorescence intensities of 656 blank spots in the array was used as the background value. Spots with Cy3 or Cy5 intensities greater than the median of the background-subtracted intensities at least once in the whole set of array analyses were subjected to significance analysis of microarrays (SAM) (Tusher et al., 2001) for the detection of significant changes in mRNA levels. The significance for each gene of functionally-classified categories was evaluated by the test of population means.

2.6. Northern analysis

Sense-strand cRNAs (0.25 μg per lane) transcribed from the bead-fixed cDNAs (Fig. 1, Step B) were electrophoresed in denaturing formaldehyde-agarose (1%) gels. RNAs were made visible by ethidium bromide staining and blotted onto nylon membranes. The cDNA-harboring plasmids were linearized by digestion with a restriction enzyme *Xba*I or *Xho*I whose site resides in the T7 promoter-linker (Step 10). They were then subjected to synthesis of DIG-cRNA probes by in vitro

transcription with SP6 RNA polymerase (Step 11), using DIG RNA Labeling Kit (Roche Diagnostics). Northern hybridization of the blotted membrane (Step E) and following chemiluminescent detection on X-ray films were done as recommended by Roche Diagnostics. Densitometric quantification was performed by Personal Scanning Image PDSI (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Construction of cDNA microarray from mouse retina

Our system enabled both the preparation (Fig. 1, Steps 1-9) and analyses (Steps A-D) of the in-house cDNA microarray with a small amount of RNA derived from a single mouse retina. In addition, the results obtained from the array analysis could be confirmed by Northern analysis (Steps 10, 11, and E) with the same cDNA clones and the amplified cRNAs used in the array experiments. A cDNA library was constructed from the mouse retina, and the nucleotide sequences of 2,880 clones at the 5' end of cDNA inserts were determined, leading to the classification of the clones into about 1,900 gene clusters. Categories of the 2,880 clones are shown in Fig. 2. A previous study using the serial analysis of gene expression (SAGE) method identified a number of genes specifically expressed in the retina (Blackshaw et al., 2001), among which most frequently expressed were genes for rhodopsin, rod arrestin (S-antigen), phosducin, transducin G proteins, cGMP-phosphodiesterase subunits, and rod outer segment membrane protein 1. The cDNA clones for these genes in the present study accounted for 4.4% of the total sequenced clones (Fig.2, Category 2). The percentage was approximately the same as that obtained in previous studies on expression profiles of the retinas of mice (Blackshaw et al.,

9

2001) and humans (Sharon et al., 2002; Shimizu-Matsumoto et al., 1997). This was concordant with the notion that our cDNA library and microarray are representative of the mRNA population of the mouse retina. Previous studies also reported that genes involved in energy production and protein synthesis were highly expressed in the retina (Sharon et al., 2002; Shimizu-Matsumoto et al., 1997), reflecting the high energy consumption and protein turnover of the retina, respectively. Consistent with this view, cDNA clones for mitochondrial DNA-encoded proteins and for protein synthesis made up a substantial portion of the total clones, 13.2% and 2.5%, respectively (Categories 1 and 3). A microarray that harbors these 2,880 retinal cDNAs and other 192 cDNAs was then prepared.

3.2. General features of cDNA microarray analysis for diabetic mouse retina

Each of 3 pairs of a diabetic and age-matched control mouse at 1 and 3 months after STZ injection was subjected to cDNA microarray analysis for a total of 6 hybridization experiments. The time points of 1 and 3 months were selected based on previous studies describing the onsets of diabetes-induced morphological and functional abnormalities such as a significant increase in the frequency of apoptosis in rat retinas at 1 and 3 months after STZ injection (Barber, 1998), and in mouse retinas 10 weeks after the injection (Martin et al., 2004).

Blood glucose levels of STZ-treated mice were increased compared to saline-injected control mice significantly at 1 month (472.3 $\pm$ 103.0 mg/dl vs 151.7 $\pm$ 10.0 mg/dl, $P < 0.05$), and apparently at 3 months (> 600, > 600, and 569 mg/dl for each of STZ-treated mice vs 153.3 $\pm$ 6.4 mg/dl for control mice). Body weights of STZ-treated mice were reduced compared to control mice significantly both at 1 month (19.8 $\pm$ 3.9 g vs 29.5 $\pm$ 1.0 g, $P < 0.05$), and at 3 months (18.2 $\pm$ 1.9 g vs 36.1 $\pm$ 3.3 g, $P < 0.01$).

To prepare the target mixtures (Fig. 1, Steps 1, 2, and A to C), sense-strand cRNA was subjected to Cy3- or Cy5-labeling of antisense-strand cDNA. For two pairs of mice, these target cDNAs derived from diabetic mice were Cy5-labeled, and those from control mice Cy3-labeled. For another pair of mice, labeling with Cy5 and Cy3 was interconverted to compensate for possible biases such as intensity-dependent fluorescence variations of the two dyes (Quackenbush, 2002). The Cy3- and Cy5-labeled target cDNAs were mixed and hybridized with the microarrays.

There were 2,446 spots with Cy3 or Cy5 fluorescence intensities higher than the median at least once in a total of 6-pair array analyses. For these spots, a statistical program called significance analysis of microarrays (SAM) (Tusher et al., 2001) was used to identify genes with significant changes in expression levels in the diabetic state. Genes for 13 and 57 spots were significantly up-regulated at 1 and 3 months, respectively, after STZ injection, with a false discovery rate (FDR) of 23.1 and 9.3%. No significantly down-regulated genes were detected at both time points.

When recurrent genes were integrated, the up-regulated genes were assembled into 5 and 15 species at 1 and 3 months, respectively (Tables 1 and 2). Interestingly, a number of genes could be classified into a limited number of functional categories: mitochondrial DNA-encoded proteins (Table 1, Nos. 1, 4, 5; Table 2, Nos. 2, 5, 6, 8); nuclear DNA-encoded mitochondrial proteins (Table 2, Nos. 4, 9, 13); ribosomal proteins (Table 2, Nos. 12, 14); and ubiquitin (Table 1, No. 3; Table 2, No. 10). All of the mitochondrial DNA- and nuclear DNA-encoded mitochondrial proteins are involved in oxidative phosphorylation, i.e., electron transfer and following ATP synthesis. Ribosomal proteins and ubiquitin are involved in protein synthesis and degradation, respectively.

To examine the validity of the microarray analysis, we performed Northern analysis (Fig. 3) for the mRNA levels of cytochrome c oxidase subunit (COX) 1,

COX3, ATPase subunits 6 and 8, ribosomal protein S6 (see Fig. 5A), and ubiquitin, using mRNA-derived amplified sense-strand cRNAs (Fig. 1, Steps 10, 11, and E). The mRNA levels of these proteins at 3 months after STZ injection were elevated in the retina of diabetic mice compared to control mice in all 3 pairs. The increases in these mRNA levels were specific, because no apparent change was observed in the mRNA levels for rhodopsin. Therefore, the results of microarray analysis were validated. We then focused on the regulation of genes for oxidative phosphorylation and protein turnover.

3.3. Regulation of genes for oxidative phosphorylation

Because a number of genes involved in mitochondrial oxidative phosphorylation was determined to be significantly up-regulated by the SAM method, and because recent studies of diabetic muscle (Mootha et al., 2003; Patti et al., 2003) demonstrated that modest but coordinated changes in the expression of functionally-related genes can be associated with disease phenotypes, we examined the changes in the expression levels for all of the mitochondrial DNA-encoded (Fig. 4A) and nuclear DNA-encoded (Fig. 4B) oxidative phosphorylation-related genes contained in our in-house microarray.

Mitochondrial DNA encodes 13 genes for mitochondrial proteins, all of which are components of the oxidative phosphorylation pathway, while more than 60 other components of the pathway are encoded by the nuclear DNA, synthesized in the cytosol, and post-translationally imported into the mitochondria (Maechler and Wollheim, 2001; Wallace, 1999). All of the 13 mitochondrial DNA-encoded genes were found in our array (Fig. 4A), and were assembled into 10 mRNA/cDNA species because more than one gene shared an identical transcript. The mRNA levels of all the 13 genes were elevated, mostly significantly, in the retina of diabetic mice

compared to control mice at 1 and 3 months after STZ injection, with generally higher elevation at 3 months (Fig. 4A). Thus, these genes appeared to be regulated in a coordinated manner.

For nuclear DNA-encoded mitochondrial protein genes, we found 13 spots for oxidative phosphorylation genes with adequate fluorescence intensities in our array (Fig. 4B). A number of these genes were up-regulated in the diabetic retina at 1 and/or 3 months after STZ treatment. Therefore, both mitochondrial DNA- and nuclear DNA-encoded genes involved in oxidative phosphorylation were up-regulated under diabetic conditions, suggesting that this pathway is potentially activated in the diabetic retina. Because glycolysis and the citric acid cycle are in close relationship with oxidative phosphorylation in the context of glucose metabolism, we also examined the expression of genes for these pathways (Fig. 4C). The gene for pyruvate kinase M was significantly up-regulated at 3 months, while the gene for phosphoglycerate mutase was slightly but significantly down-regulated at 1 month.

3.4. Regulation of genes for protein turnover

The genes for both ribosomal proteins and ubiquitin were significantly up-regulated in the analysis by the SAM method (Tables 1 and 2). This prompted us to examine other spots on the array for genes involved in protein synthesis and degradation. Figure 5A shows the results for ribosomal proteins and translational initiation and elongation factors. A number of genes were up-regulated at 1 and/or 3 months after STZ injection, suggesting an activation of protein synthesis in the diabetic retina. Figure 5B shows the results for the ubiquitin-proteasome system, while the number of spots for this system with adequate intensities in our array was limited. The gene for ubiquitin specific protease 20, in addition to the ubiquitin gene,

was significantly up-regulated in the diabetic retina at 1 month after STZ injection. On the other hand, the gene for proteasome subunit β type 6 was significantly down-regulated at 3 months.

Taken together with the up-regulation of genes for protein synthesis, the turnover of at least a part of the protein population appears to be activated in the retina under the diabetic condition.

4. Discussion

We have found that the genes for oxidative phosphorylation are up-regulated in the diabetic retina. This was in sharp contrast with the previous reports for a down-regulation of these genes in the skeletal muscle of STZ-induced diabetic mice (Yechoor et al., 2002; Yechoor et al., 2004) and type 2 diabetic humans (Mootha et al., 2003; Patti et al., 2003). It was also reported that mitochondrial proteins for electron transfer were decreased in the heart of STZ-treated rats (Turko and Murad, 2003). Coincidently, insulin treatment of the diabetic mice corrected the repression of genes for oxidative phosphorylation in the skeletal muscle (Yechoor et al., 2002; Yechoor et al., 2004), and hyperinsulinemic euglycemic clamp of healthy humans increased the expression of those genes (Rome et al., 2003).

What is the cause of these differences between the retina and muscle in the regulation of genes for oxidative phosphorylation under diabetic conditions? A highly likely explanation for this is the difference of insulin dependency of glucose transporters between the retina and muscle. While the glucose transporter GLUT4 of the skeletal muscle and myocardium requires insulin for its activity, the retinal transporters GLUT1 and GLUT3 are insulin-independent (Kumagai, 1999). GLUT1 and GLUT3 are major components of the transporters in BRB and the neural retina.

14

Insulin independency of these transporters causes hyperglycemia-facilitated influx of glucose and a resultant increase in glucose concentration in the neural retina under diabetic conditions. In turn, the increase in intracellular glucose concentration appears to activate genes for oxidative phosphorylation as an adaptive response to consume glucose. On the other hand, glucose import into the muscle under the diabetic condition is severely impaired because of the insulin dependency of GLUT4, even in the hyperglycemic state. Down-regulation of genes for oxidative phosphorylation in the diabetic muscle appears to be an adaptive response in the direction opposite to that of the retina, while the down-regulation can also be a causative factor of type 2 diabetes (Mootha et al., 2003). Possible differential effects of insulin on the retina and muscle in the diabetic state remain to be examined.

The up-regulation of genes for oxidative phosphorylation in the diabetic retina is remarkable from the viewpoint of possible preventive and therapeutic interventions of diabetic retinopathy. The critical role of superoxide overproduced by excess glucose loading has been remarked in triggering diabetic complications in a number of cell types through several mechanisms such as increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C, and increased hexosamine pathway flux (Brownlee, 2001). The possible activation of the pathway for oxidative phosphorylation revealed in the present study is a double-edged sword for superoxide production: the first half of the pathway, i.e., the electron transfer system generates, while the latter half, i.e., ATP synthase, consumes the mitochondrial membrane potential, the elevation of which enforces production of superoxide. Despite this ambivalence, the augmented capacity for oxidative phosphorylation can be utilized to degrade excess intracellular glucose and its metabolites responsible for diabetic complications, by supplying oxygen appropriately. Elimination of the excess metabolites might also ameliorate retinal edema by reducing the osmotic pressure of intra- and/or intercellular fluid in the

retina.

Our results also suggested that protein turnover rates are increased in the diabetic retina. Previously, it was reported that insulin activates genes for protein synthesis and the ubiquitin-proteasome system in the human skeletal muscle (Rome et al., 2003). Therefore, one can imagine that in the diabetic skeletal muscle these genes are down-regulated, although up- or down-regulation of the genes for the ubiquitin-proteasome system in the STZ-induced diabetic mice is controversial (Yechoor et al., 2002). In general, regulation of genes for protein turnover in the retina is different from that in the skeletal muscle under the diabetic condition, apparently reflecting again the difference of glucose influx and resultant energy availability between the two tissues.

To date, only a limited number of retinal proteins such as rhodopsin (Illing et al., 2002; Obin et al., 1996) and the transducin γ-subunit (Obin et al., 2002; Obin et al., 1996) are known to be ubiquitinated. It was reported (Obin et al., 2002) that ubiquitination and subsequent degradation of the transducin γ-subunit were prevented by phosducin, suggesting a unique mechanism for the protection of the light-dependently dissociated transducin complex. Identification of specific proteins under the control of the ubiquitin-proteasome system with altered turnover rates in the diabetic retina may provide further insights into the molecular pathophysiology of the retinopathy.

In summary, genes for both energy-producing oxidative phosphorylation and for energy-consuming protein turnover are generally up-regulated in the retina under the diabetic condition, which is strikingly different from that in the skeletal muscle. Augmented capacity for intermediary metabolism can be utilized in preventive and therapeutic interventions of diabetic retinopathy.

Acknowledgments

References

Barber, A.J., 2003. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Prog. Neuro-Psychoph. Biol. Psych. 27, 283-290.

Barber, A.J., Antonetti, D.A., Kern, T.S., Reiter, C.E., Soans, R.S., Krady, J.K., Levison, S.W., Gardner, T.W., Bronson, S.K., 2005. The Ins2Akita mouse as a model of early retinal complications in diabetes. Invest. Ophthalmol. Vis. Sci. 46, 2210-2218.

Blackshaw, S., Fraioli, R.E., Furukawa, T., Cepko, C.L., 2001. Comprehensive analysis of photoreceptor gene expression and the identification of candidate retinal disease genes. Cell 107, 579-589.

Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813-820.

Cho, G.J., Ryu, S., Kim, Y.H., Kim, Y.S., Cheon, E.W., Park, J.M., Kim, H.J., Kang, S.S., Choi, W.S., 2002. Upregulation of glucose-dependent insulinotropic polypeptide and its receptor in the retina of streptozotocin-induced diabetic rats. Curr. Eye Res. 25, 381-388.

Farjo, R., Yu, J., Othman, M.I., Yoshida, S., Sheth, S., Glaser, T., Baehr, W., Swaroop, A., 2002. Mouse eye gene microarrays for investigating ocular development and disease. Vision Res. 42, 463-470.

Gardner, T.W., Antonetti, D.A., Barber, A.J., LaNoue, K.F., Levison, S.W., 2002. Diabetic retinopathy: more than meets the eye. Surv. Ophthalmol. 47, Suppl. 2, S253-262.

Gerhardinger, C., Costa, M.B., Coulombe, M.C., Toth, I., Hoehn, T., Grosu, P., 2005. Expression of acute-phase response proteins in retinal Muller cells in diabetes. Invest. Ophthalmol. Vis. Sci. 46, 349-357.

Hammes, H.P., Lin, J., Renner, O., Shani, M., Lundqvist, A., Betsholtz, C., Brownlee,

M., Deutsch, U., 2002. Pericytes and the pathogenesis of diabetic retinopathy. Diabetes 51, 3107-3112.

Illing, M.E., Rajan, R.S., Bence, N.F., Kopito, R.R., 2002. A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. J. Biol. Chem. 277, 34150-34160.

Joussen, A.M., Huang, S., Poulaki, V., Camphausen, K., Beecken, W.D., Kirchhof, B., Adamis, A.P., 2001. In vivo retinal gene expression in early diabetes. Invest. Ophthalmol. Vis. Sci. 42, 3047-3057.

Knoll, K.E., Pietrusz, J.L., Liang, M., 2005. Tissue-specific transcriptome responses in rats with early streptozotocin-induced diabetes. Physiol. Genomics 21, 222-229.

Kumagai, A.K., 1999. Glucose transport in brain and retina: implications in the management and complications of diabetes. Diab. Metab. Res. Rev. 15, 261-273.

Maechler, P., Wollheim, C.B., 2001. Mitochondrial function in normal and diabetic beta-cells. Nature 414, 807-812.

Martin, P.M., Roon, P., Van Ells, T.K., Ganapathy, V., Smith, S.B., 2004. Death of retinal neurons in streptozotocin-induced diabetic mice. Invest. Ophthalmol. Vis. Sci. 45, 3330-3336.

Mohr, S., Xi, X., Tang, J., Kern, T.S., 2002. Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients. Diabetes 51, 1172-1179.

Mootha, V.K., Lindgren, C.M., Eriksson, K.F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstrale, M., Laurila, E., Houstis, N., Daly, M.J., Patterson, N., Mesirov, J.P., Golub, T.R., Tamayo, P., Spiegelman, B., Lander, E.S., Hirschhorn, J.N., Altshuler, D., Groop, L.C., 2003. PGC-1alpha-responsive genes involved in oxidative phosphorylation are

coordinately downregulated in human diabetes. Nature Genet. 34, 267-273.

Obin, M., Lee, B.Y., Meinke, G., Bohm, A., Lee, R.H., Gaudet, R., Hopp, J.A., Arshavsky, V.Y., Willardson, B.M., Taylor, A., 2002. Ubiquitylation of the transducin beta-gamma subunit complex: regulation by phosducin. J. Biol. Chem. 277, 44566-44575.

Obin, M.S., Jahngen-Hodge, J., Nowell, T., Taylor, A., 1996. Ubiquitinylation and ubiquitin-dependent proteolysis in vertebrate photoreceptors (rod outer segments): evidence for ubiquitinylation of Gt and rhodopsin. J. Biol. Chem. 271, 14473-14484.

Ohtsuka, S., Iwase, K., Kato, M., Seki, N., Shimizu-Yabe, A., Miyauchi, O., Sakao, E., Kanazawa, M., Yamamoto, S., Kohno, Y., Takiguchi, M., 2004. An mRNA amplification procedure with directional cDNA cloning and strand-specific cRNA synthesis for comprehensive gene expression analysis. Genomics 84, 715-729.

Patti, M.E., Butte, A.J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E.J., Goldfine, A.B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C.R., Mandarino, L.J., 2003. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. Proc. Natl. Acad. Sci. USA 100, 8466-8471.

Quackenbush, J., 2002. Microarray data normalization and transformation. Nature Genet. 32, Suppl. 496-501.

Reiter, C.E., Gardner, T.W., 2003. Functions of insulin and insulin receptor signaling in retina: possible implications for diabetic retinopathy. Prog. Retin. Eye Res. 22, 545-562.

Rome, S., Clement, K., Rabasa-Lhoret, R., Loizon, E., Poitou, C., Barsh, G.S., Riou, J.P., Laville, M., Vidal, H., 2003. Microarray profiling of human skeletal

muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. J. Biol. Chem. 278, 18063-18068.

Sato, M., Ohguro, H., Ohguro, I., Mamiya, K., Takano, Y., Yamazaki, H., Metoki, T., Miyagawa, Y., Ishikawa, F., Nakazawa, M., 2003. Study of pharmacological effects of nilvadipine on RCS rat retinal degeneration by microarray analysis. Biochem. Biophys. Res. Commun. 306, 826-831.

Sharon, D., Blackshaw, S., Cepko, C.L., Dryja, T.P., 2002. Profile of the genes expressed in the human peripheral retina, macula, and retinal pigment epithelium determined through serial analysis of gene expression (SAGE). Proc. Natl. Acad. Sci. USA 99, 315-320.

Shimizu-Matsumoto, A., Adachi, W., Mizuno, K., Inazawa, J., Nishida, K., Kinoshita, S., Matsubara, K., Okubo, K., 1997. An expression profile of genes in human retina and isolation of a complementary DNA for a novel rod photoreceptor protein. Invest. Ophthalmol. Vis. Sci. 38, 2576-2585.

Turko, I.V., Murad, F., 2003. Quantitative protein profiling in heart mitochondria from diabetic rats. J. Biol. Chem. 278, 35844-35849.

Tusher, V.G., Tibshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA 98, 5116-5121.

Wallace, D.C., 1999. Mitochondrial diseases in man and mouse. Science 283, 1482-1488.

Yechoor, V.K., Patti, M.E., Saccone, R., Kahn, C.R., 2002. Coordinated patterns of gene expression for substrate and energy metabolism in skeletal muscle of diabetic mice. Proc. Natl. Acad. Sci. USA 99, 10587-10592.

Yechoor, V.K., Patti, M.E., Ueki, K., Laustsen, P.G., Saccone, R., Rauniyar, R., Kahn, C.R., 2004. Distinct pathways of insulin-regulated versus diabetes-regulated gene expression: an in vivo analysis in MIRKO mice. Proc.

Natl. Acad. Sci. USA 101, 16525-16530.

Yoshikawa, T., Nagasugi, Y., Azuma, T., Kato, M., Sugano, S., Hashimoto, K., Masuho, Y., Muramatsu, M., Seki, N., 2000. Isolation of novel mouse genes differentially expressed in brain using cDNA microarray. Biochem. Biophys. Res. Commun. 275, 532-537.

(Figure legends)

Fig. 1.   Schematic representation of the system for construction and analysis of the cDNA microarray. See text for detailed explanations.

Fig. 2.   Classification of retinal cDNAs on the microarray. A total of 2,880 cDNA clones derived from the mouse retina were classified based on the 5'-terminal sequence. The percentages of classified cDNAs are represented as a pie chart. Actual cDNA numbers are shown in parentheses.

Fig. 3.   Northern blot analysis with amplified cRNAs for changes in mRNA levels in the diabetic retina. (Left column) Northern blot analysis of retinas from three pairs of control (C) and diabetic (D) mice. Amplified sense-strand cRNAs (0.25 μg per lane) derived from the retinas of three pairs of control and diabetic mice at 3 months after STZ injection were subjected to Northern analysis for COX1, COX3, ATPase subunits 6 and 8, ribosomal protein S6, and ubiquitin mRNAs. The level of rhodopsin mRNA was also determined as a standard showing no significant change. Brackets on the left of chemiluminograms indicate ranges subjected to densitometric quantification. Background correction was done by subtracting the densitometric value of an area with no visible transcripts within the same lane. Below chemiluminograms, the quantified results for each pair are presented as values relative to the control. Ethidium bromide staining of a representative gel is shown at the bottom. Bars represent positions of 28S and 18S rRNAs. (Right column) Graphical summary of the densitometric quantification. Results are presented as mean + S.E. for the three pairs of retinas. Significant differences were determined by the test of population mean: *, $P < 0.03$; **, $P < 0.00005$.

Fig. 4.   Changes in expression levels of genes for oxidative phosphorylation and glucose metabolism. Fold changes in expression levels for mitochondrial DNA-encoded (A) and nuclear DNA-encoded genes for oxidative phosphorylation (B), and genes for glycolysis and the citric acid cycle (C) in the retina of diabetic mice at 1 and 3 months after STZ injection are shown relative to the levels of control mice (mean + S.E.). Significant differences were determined by the test of population means: *, $P < 0.03$; **, $P < 0.002$; ***, $P < 0.00005$.

Fig. 5.   Changes in expression levels of genes for protein turnover. Fold changes in expression levels of genes for protein synthesis (A) and the ubiquitin-proteasome system (B) in the retina of diabetic mice at 1 and 3 months after STZ injection are shown relative to the levels of control mice (mean + S.E.). Significant differences were determined by the test of population means: *, $P < 0.03$; **, $P < 0.002$; ***, $P < 0.00005$.
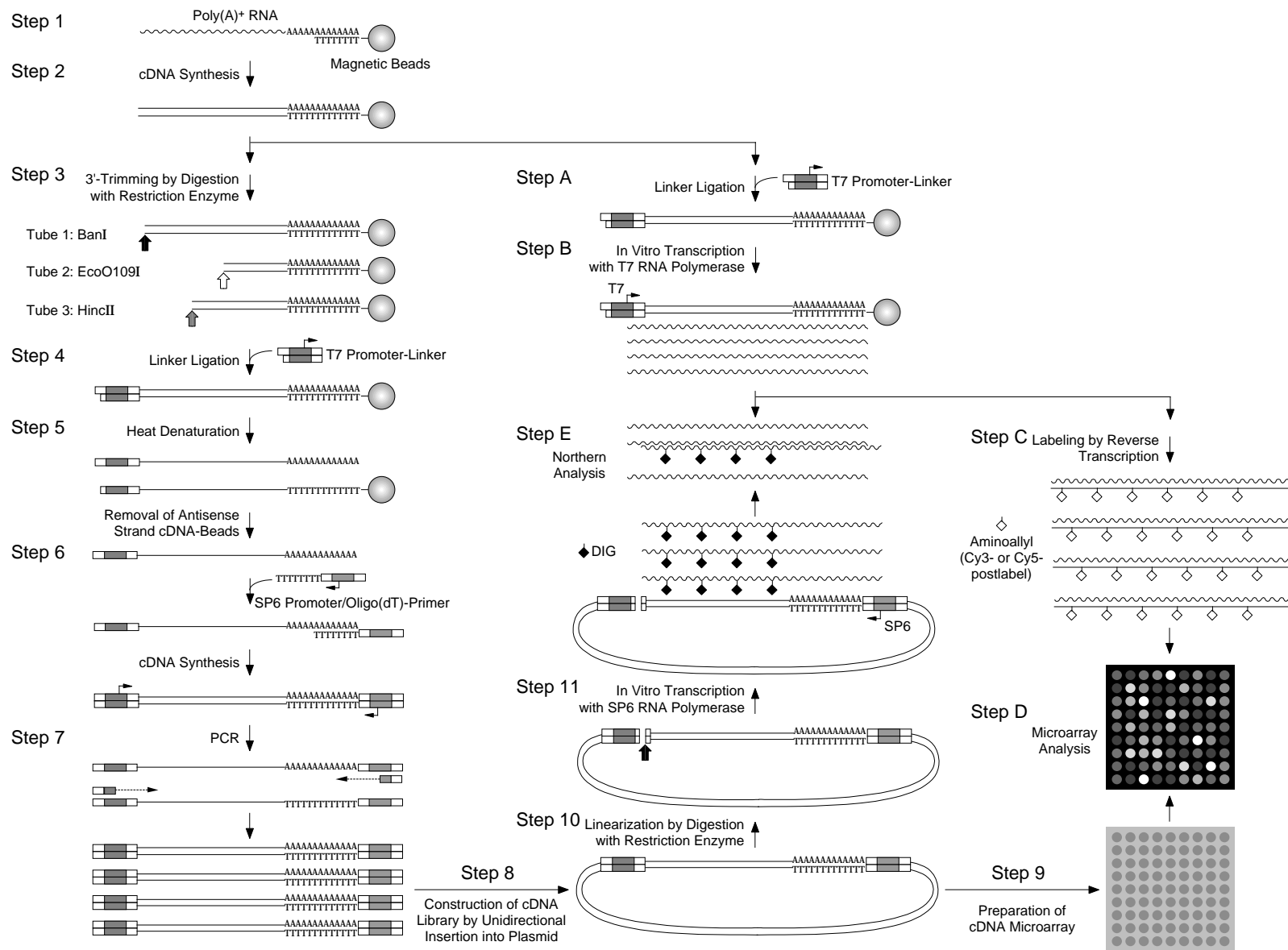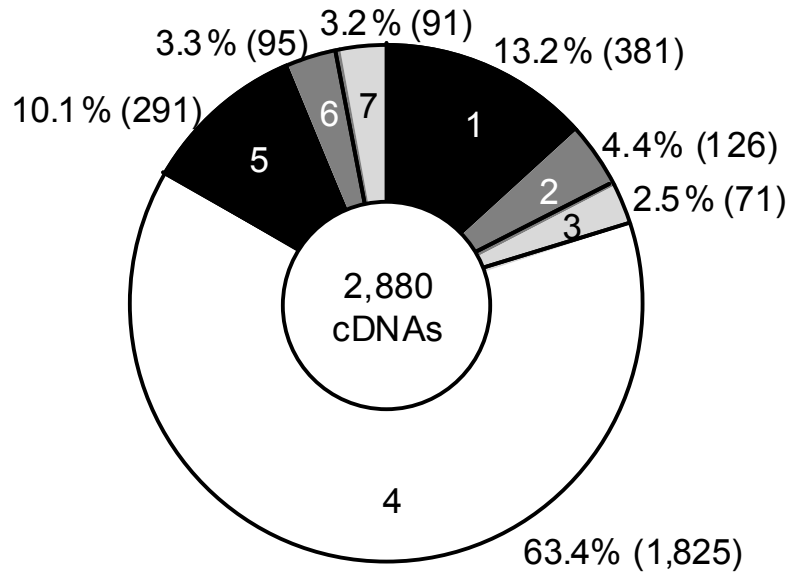
Fig. 1

1: Mitochondrial DNA-encoded genes
2: Phototransduction
3: Translation
4: Other known genes
5: Known ESTs
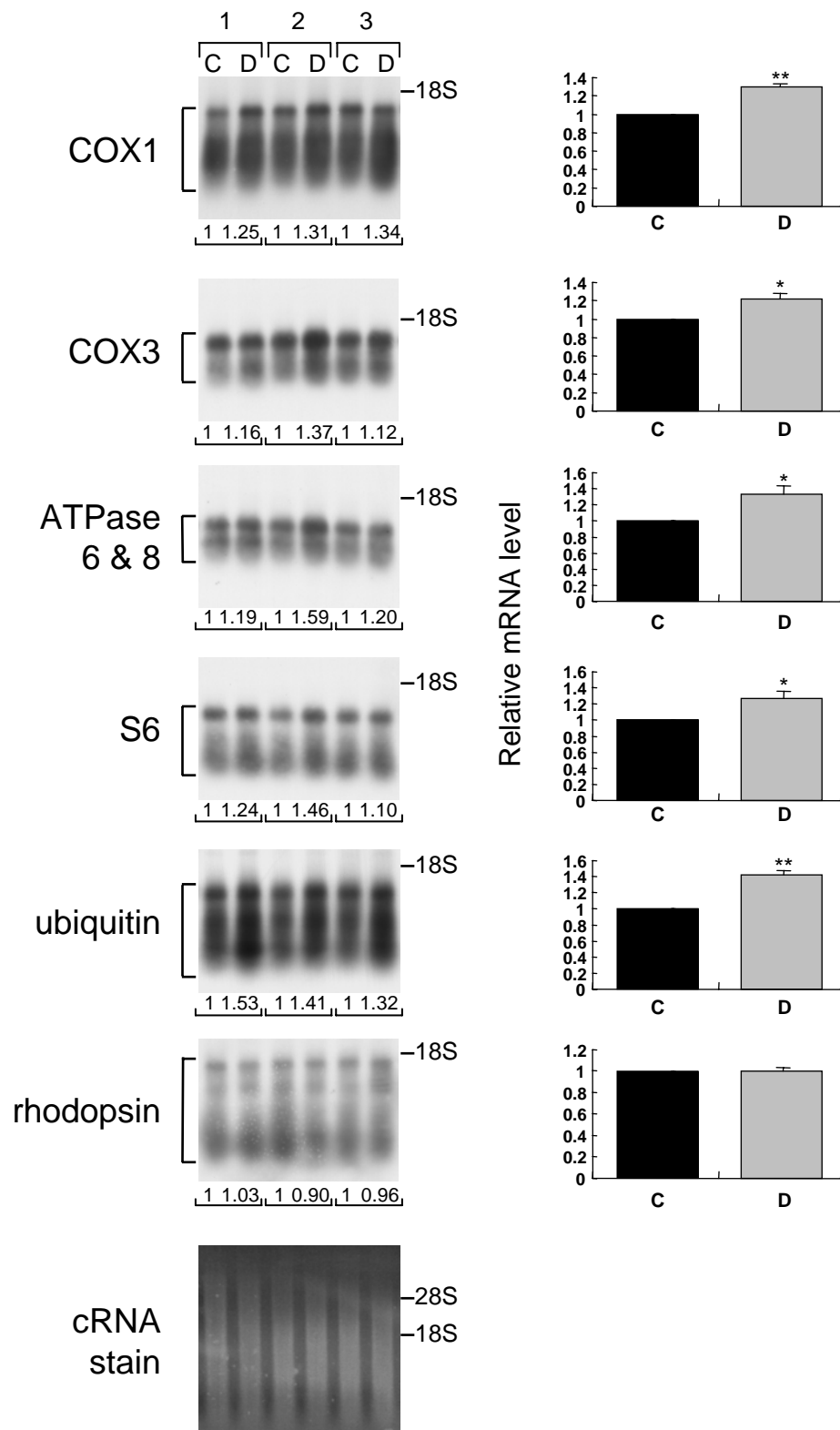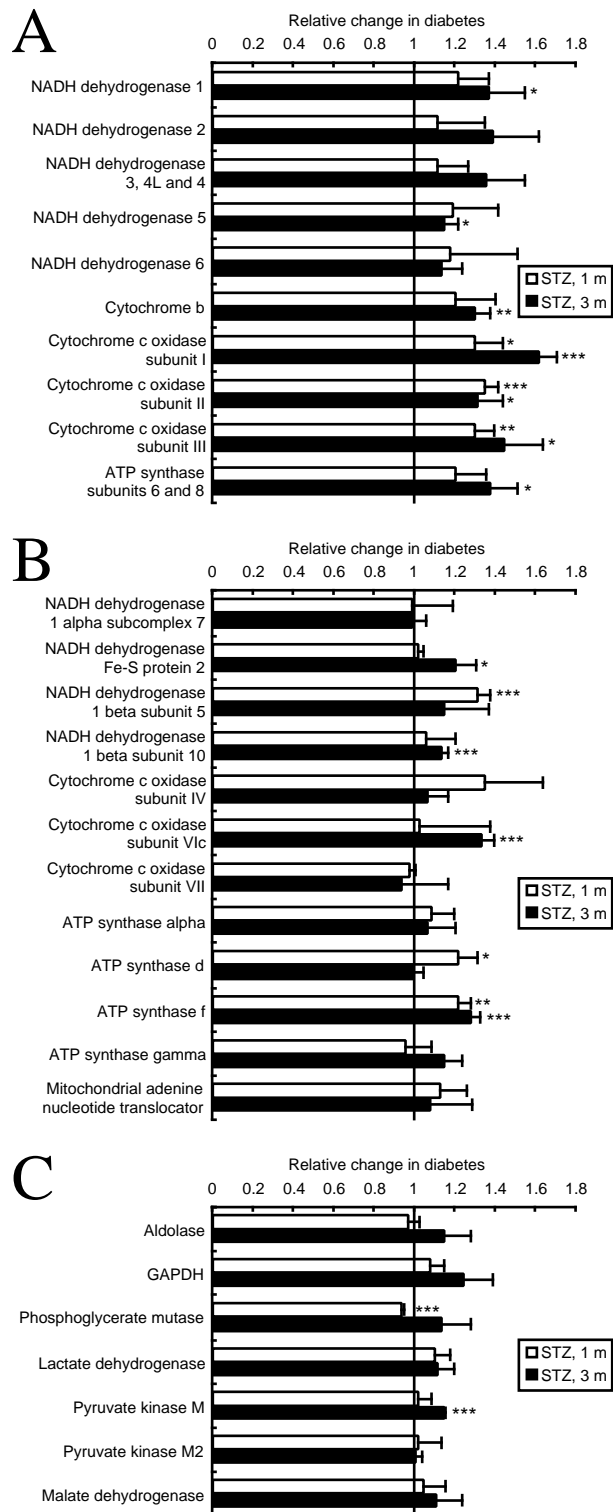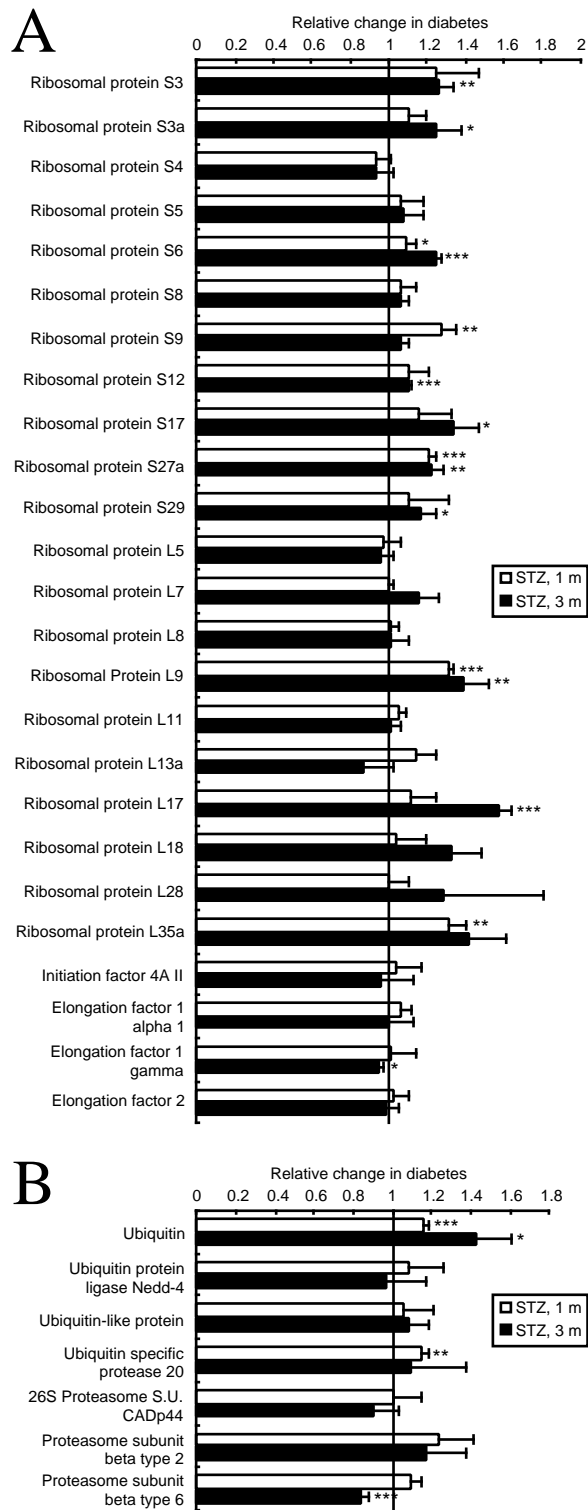6: Novel ESTs
7: Bad and short sequences

Fig. 2

Fig. 3

Fig. 4

Fig. 5

4. Table

Table 1

Genes significantly* up-regulated 1 month after STZ injection in the mouse retina

No.	Accession	Gene name	Fold change
1	J01420	Cytochrome c oxidase subunit I (mitochondrial genome)	1.21
2	X13605	Replacement variant histone H3.3	1.19
3	X51703	Ubiquitin	1.16
4	J01420	Cytochrome c oxidase subunit III (mitochondrial genome)	1.13
5	J01420	ATP synthase subunits 6 and 8 (mitochondrial genome)	1.08

*Delta=1.25, FDR 23.1%.

4. Table

Table 2

Genes significantly* up-regulated 3 months after STZ injection in the mouse retina

No.	Accession	Gene name	Fold change
1	AF032872	Potassium channel regulatory protein KChAP	1.60
2	AB049357	Cytochrome c oxidase subunit I (mitochondrial genome)	1.57
3	L08075	Phosducin	1.53
4	AF315374	ATP synthase lipid-binding protein P3 precursor (Atp5g3)	1.36
5	J01420	ATP synthase subunits 6 and 8 (mitochondrial genome)	1.35
6	U47430	Mitochondrial DNA type 1 control region and flanking tRNA genes (mitochondrial genome)	1.35
7	AF201948	PIL protein	1.32
8	J01420	Cytochrome c oxidase subunit III (mitochondrial genome)	1.31
9	M20152	Cytochrome c oxidase subunit VIc	1.31
10	X51703	Ubiquitin	1.31
11		Novel EST	1.27
12	BC002044	Ribosomal protein S17	1.27
13	S70447	F_1F_0-ATP synthase complex Fo membrane domain f subunit	1.26
14	X76772	Ribosomal protein S3	1.23
15	AF073992	Heterogenous nuclear ribonucleoprotein U (hnRNP U)	1.21

*Delta=1.25, FDR 9.3%.