

## Chapter 7

# CYP2E1, Oxidative Stress, Post-translational Modifications and Lipid Metabolism

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**Abstract** Chronic alcohol-mediated down-regulation of hepatic ST6Gal1 gene leads to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective VLDL assembly and intracellular lipid and lipoprotein transport, which in turn is responsible for alcoholic hepatosteatosis and ALD. The mechanism of ethanol action involves the depletion of a unique RNA binding protein that specifically interacts with its 3'-UTR region of ST6Gal1 mRNA resulting in its destabilization and consequent appearance of asialoconjugates as alcohol biomarkers. With respect to ETOH effects on Cardio-Vascular Diseases, we conclude that CYP2E1 and ETOH mediated oxidative stress significantly down regulates not only the hepatic PON1 gene expression, but also serum PON1 and HCTLase activities

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accompanied by depletion of hepatic GSH, the endogenous antioxidant. These results strongly implicate the susceptibility of PON1 to increased ROS production. In contrast, betaine seems to be both hepatoprotective and atheroprotective by reducing hepatosteatosis and restoring not only liver GSH that quenches free radicals, but also the antiatherogenic PON1 gene expression and activity.

**Keywords** CYP2E1 • Alcohol • PON1 • ROS • Liver

## 7.1 Introduction

Gal $\beta$ 1, 4GlcNAc  $\alpha$ 2,6-sialyltransferase (ST6Gal1) mediates the addition of  $\alpha$ 2,6-linked sialic acid to glycoproteins in the Golgi compartment. Down-regulation of its gene and consequent impaired activity of ST6Gal1 seems to be the major cause for the appearance of asialoconjugates in the blood of long-term alcoholics. Therefore, mechanism(s) involved in the regulation of ST6Gal1 gene is important and clinically relevant. ST6Gal1 is strongly expressed in liver and can be either up- or down-regulated by a number of factors. In our rat alcohol-feeding model, rat ST6Gal1 expression was reduced to as much as 59% by long-term alcohol treatment compared with the pair-fed control group in a dose dependent manner (Rao and Lakshman 1999). It will be of major importance and clinical significance if the regulation of ST6Gal1 gene can be mimicked in a human liver cell model, especially if it can be achieved within a few days of ethanol exposure in a human liver culture system rather than *in vivo* that may take prolonged period of ethanol exposure.

To define the significance and molecular mechanisms of aberrant sialylation in alcoholics, we focused our attention on sialidases and sialyltransferases (Ghosh et al. 1993), two of the key enzymes involved in the metabolism of glycoproteins and glycolipids. Our studies have shown that long-term ethanol administration decreases the hepatic activity of  $\beta$ -galactoside- $\alpha$ 2,6-sialyltransferase (ST6Gal1) in the rat liver via the down-regulation of the ST6Gal1 gene (Rao and Lakshman 1997). In human beings, the vast majority of ethanol is oxidized to acetaldehyde by the hepatocytes of the liver (Crabb et al. 1987). However, whether or not ethanol metabolism by the liver is a prerequisite for its action in down-regulating ST6Gal1 gene has not been established.

On the other hand, CYP2E1-mediated oxidation of ethanol produces a state of oxidative stress by generating reactive oxygen species (ROS) within the cells that is responsible for the progression of alcoholic liver disease or cell damage (Hartley and Peterson 1997; Chen et al. 2001; Nagy 2004). Therefore, it is possible that the mechanism of action of ethanol in regulating ST6Gal1 gene expression may be mediated via ROS. One of the key metabolites generated because of oxidative stress is the  $\alpha$ , $\beta$ -unsaturated aldehyde, 4-hydroxy-2-nonenal (HNE), which may be more harmful than ROS because it has a longer half-life and can easily diffuse into cellular membranes (Dai et al. 1993). In the present report, we have taken advantage of

cultured human wild-type HepG2 liver cells that do not metabolize ethanol because they lack the key ethanol-metabolizing enzymes, namely, cytochrome P450 2E1 (CYP2E1: ethanol-inducible) and alcohol dehydrogenase (ADH). However, when these wild-type liver cells are stably transfected with either CYP2E1 gene or high alcohol dehydrogenase (HAD) gene, they are efficient in metabolizing ethanol and truly reflect ethanol metabolism by human liver *in situ*. Thus, the three HepG2 cell types, the wild-type cells, CYP2E1 cells, and HAD cells are ideal models to clearly define the true action of ethanol in regulating ST6Gal1 gene. It will be shown that ethanol causes the down-regulation of ST6Gal1 gene only in the ethanol-metabolizing liver cells, but not in the wild type. In contrast, acetaldehyde, the immediate product of ethanol oxidation caused the down-regulation of ST6Gal1 gene even in the wild type. Furthermore, we will also show that HNE strongly down-regulates ST6Gal1 gene in CYP2E1 cells. Thus, it is unequivocally demonstrated that ethanol oxidation leading to generation of acetaldehyde and/or ROS is obligatory for its regulatory action on ST6Gal1 gene in human liver.

The complete cDNA for rat ST6Gal1 is 4.2-kb, with a 2.7-kb 3'-untranslated region (UTR) region. Sequence analysis by computer software revealed several conserved sequences among different species within ST6Gal1 3'-UTR. Therefore, it is reasonable that this extremely long 3'-UTR may play important roles in the posttranscriptional regulation of rat ST6Gal1. The 3'-UTR of many eukaryotic mRNAs has been implicated in a variety of cellular processes, such as mRNA stability, processing, polyadenylation, localization, and translational regulation. In each case, functional activity appears to be mediated, in part, by a specific interaction of RNA-binding proteins, which target cellular RNAs to form RNA-protein complexes (Jain et al. 1995; Zaidi and Malter 1995; Wang et al. 1996; Joseph et al. 1998; Ostareck-Lederer et al. 1998; Sakai et al. 1999; Gilmore et al. 2001; Kufel et al. 2003). More and more reports have suggested that mRNA stability is frequently determined by RNA-protein interactions, and those interactions frequently occur within the 3'-UTR. Thus, RNA-protein interactions are crucial for maintaining proper RNA metabolism. We have undertaken to analyze the RNA-protein interactions within the 3'-UTR of ST6Gal1 to characterize the binding protein with which this RNA sequence interacts and to investigate whether chronic ethanol feeding affects the status of this binding protein and consequently that of the ST6Gal1 mRNA transcripts. It will be unequivocally demonstrated in the present investigation that a 41-kDa binding protein specifically binds to the 3'-UTR of ST6Gal1 mRNA and stabilizes the mRNA. Significantly, chronic alcohol feeding decreases this liver cytosol binding protein, leading to impaired binding and destabilization of ST6Gal1 mRNA.

On the other hand, paraoxonase 1(PON1) is a multifunctional antioxidant enzyme tightly associated with high density lipoprotein (HDL) that exhibits not only the capacity to prevent LDL oxidation and destroy oxidized low density lipoproteins (LDL) (Aviram et al. 1998; Shih et al. 1998; Mackness et al. 2003; Navab et al. 1997), but also can detoxify the homocysteine metabolite, homocysteine thiolactone (HCTL), which can pathologically cause protein damage by homocysteinylolation of the lysine residues, thereby leading to atherosclerosis (Jakubowski 2000, 2007;

Aviram and Rosenblat 2004). The importance of PON1 with respect to protection against Cardio-Vascular Diseases (CVD) is supported by recent studies in Type II diabetics showing a strong correlation between decreased HCTLase activity with the severity of CVD (Lakshman et al. 2006; Jakubowski 2007). It has been previously shown that heavy alcohol down-regulates PON1 expression and activity (Sierksma et al. 2002; Rao et al. 2003). It has also been demonstrated that ROS can inhibit PON1 activity (Rao et al. 2003; Horke et al. 2007). Dietary  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFA) could also adversely affect PON status presumably by generating more ROS in spite of their promising hypolipidemic effect. Therefore, any dietary supplement that can protect the depletion of intracellular glutathione (GSH) would be effective in preserving PON1 status.

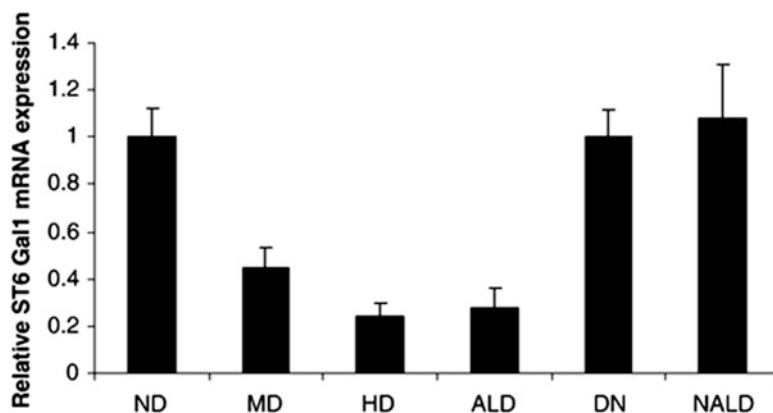
Betaine (trimethyl glycine) is a substrate for the liver enzyme betaine homocysteine methyltransferase (BHMT) which transfers methyl group to convert homocysteine to methionine (Finkelstein et al. 1972). Betaine plays an important role in reducing fatty liver (Barak et al. 1997), depletes homocysteine levels in liver (Kharbanda et al. 2005), and has been reported to restore the decreased liver GSH level in lipopolysaccharide-treated rats (Balkan et al. 2005). Therefore, it is reasonable that dietary betaine may prevent the deleterious effects of heavy alcohol and  $\omega$ -3 PUFA on PON1 status by altering hepatic GSH. We have undertaken this study (i) to explore the effects of feeding high and low  $\omega$ -3 PUFA diets, especially in the presence of chronic heavy alcohol in the diet on PON1 expression and activity and (ii) to test whether betaine can protect these alterations caused by chronic alcohol and  $\omega$ -3 PUFA by restoring hepatic GSH.

## 7.2 Ethical Guideline and Alcoholic Specimen Criteria

This research was approved by the institutional review board and the research and development committee of this medical center as well as those of the participating medical center from Australia. An informed consent was obtained from each patient before taking samples. Postmortem human liver specimens (12 samples in each group; all sample identities were kept anonymous) were purchased from Tissue Transformation Technologies (Edison, NJ) according to the following criteria:

1. Non-alcohol-drinking group (ND): less than 1 alcoholic beverage per day (below 14 g ethanol per day) in the past 10 years before death.
2. Moderate alcohol drinkers (MD): 1 to 3 alcoholic beverage(s) per day (14–42 g ethanol per day) in the past 10 years before death.
3. Heavy alcohol drinkers (HD): more than 6 alcoholic beverages per day ( $\geq$ 84 g ethanol per day).

Four explant liver samples with advanced alcoholic liver disease (ALD) (end-stage cirrhosis without viral hepatitis or other defined liver disease) and 5 normal subjects (partial donation; controls without liver disease) were also used to study the expression

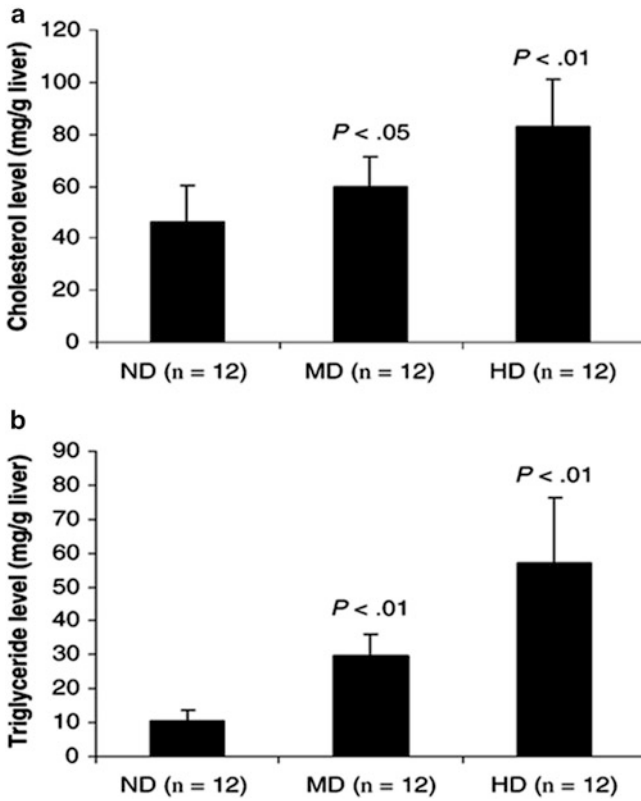


**Fig. 7.1 Real-time RT-PCR analyses of ST6Gal1 mRNA in human liver specimens in the study groups.** Total RNA from the ND group (n=12), the MD group (n=12), the HD group (n=12), the end-stage ALD group (n=4), the normal liver donors (n=5), and the NALD group (n=12) was reverse transcribed and used in the real-time PCR. The RNA levels were normalized to the level of  $\beta$ -actin. Each sample analysis was performed in triplicate independently; and each bar graph represents the mean  $\pm$  SEM of 12 samples in each group except for the ALD and DN groups, in which *bar graph* represents the mean  $\pm$  SEM of 4 and 5 samples, respectively. DN indicates normal liver donors

of ST6Gal1. Twelve liver biopsy samples taken from patients with chronic liver disease of nonalcoholic etiology were used as the nonalcoholic liver disease (NALD) group.

### **7.2.1 Ethanol Per Se, Not Other Liver Pathological Conditions, Mediates the Down-Regulation of ST6Gal1 Gene**

Our previous study (Gong et al. 2007) showed that ST6Gal1 mRNA was significantly down-regulated in liver samples from long-term alcoholics without liver disease. To rule out that other liver pathological conditions may also affect the expression of ST6Gal1, we repeated our real-time quantitative RT-PCR analyses on liver samples from nondrinkers, moderate drinkers, and heavy drinkers together with liver explant samples from alcoholic liver disease subjects as well as liver samples from hepatitis subjects without alcohol exposure (NALD group). The results are shown in Fig. 7.1. Thus, compared with the nonalcohol group, ST6Gal1 mRNA level was decreased on average by 50% ( $p < 0.01$ ) and by 70% ( $p < 0.01$ ) in moderate- and heavy-alcohol-drinking groups, respectively, just as we reported previously (Gong et al. 2007). Similar results were also found in liver explants from advanced ALD patients compared with normal subjects (ND). The ST6Gal1 mRNA level was decreased by as much as 65% ( $p < 0.01$ ) in end-stage alcoholic liver disease subjects, whereas ST6Gal1 mRNA level was not changed or slightly increased in the NALD, compared

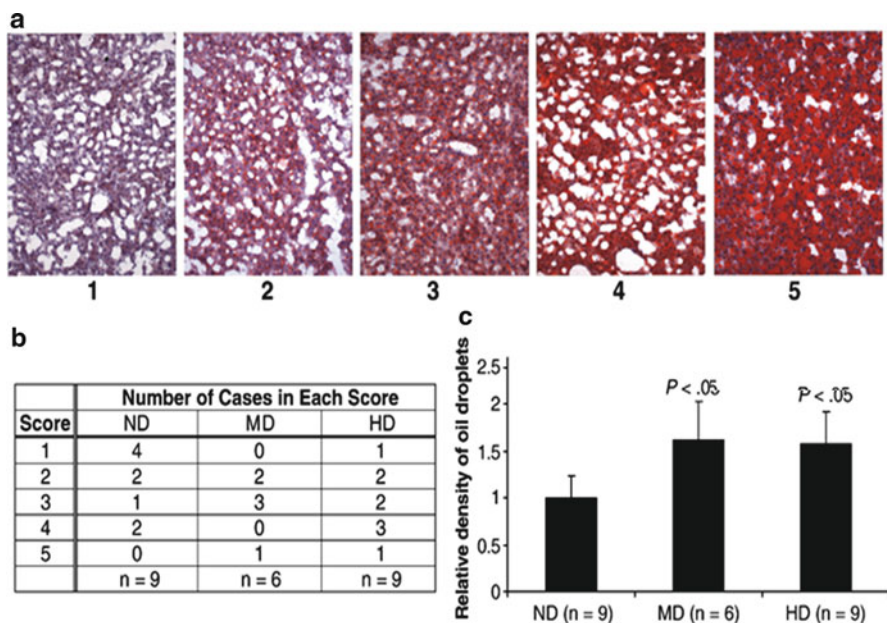


**Fig. 7.2 Liver cholesterol and triglyceride levels in the study groups.** Aliquots of each liver lipid extract were analyzed for (a) cholesterol and (b) triglycerides as described in the methods section. Each sample analysis was performed in duplicate and repeated at least three times on different dates. The concentration for cholesterol and triglyceride is expressed as milligram per gram of liver. Bar graph represents the mean  $\pm$  SEM of 12 specimens in each group

with normal controls. These results indicate that ethanol per se may be the major cause for the down-regulation of ST6Gal1 gene in alcoholics.

### **7.2.2 Liver Lipid Deposition Directly Correlates with the Amount of Alcohol Consumed**

Liver total cholesterol level was increased by more than 30% ( $p < 0.05$ ) and 75% ( $p < 0.01$ ) in the MD and HD groups, respectively, compared with the ND group (Fig. 7.2a). Triglyceride was increased by more than 100% ( $p < 0.01$ ) in the MD group and by more than 300% ( $p < 0.01$ ) in the HD group compared with the ND group (Fig. 7.2b).



**Fig. 7.3 Histopathology of human liver specimens and lipid deposits in the study groups.** Cryosections of tissues were prepared as described in the materials and methods section. Sections were stained with oil red O and counterstained with hematoxylin. All  $ca \times 120$ ; bar =  $ca 100 \mu m$ . (a) Representative photomicrographs from the standard set used to calibrate scoring of lipid accumulation. Numbers *below* each *image frame* represent the score for that frame, with 1 indicating the least and 5 indicating the most lipid accumulation. (b) Number of samples from each group that fits into each score according to the standard in Fig. 7.3a as determined subjectively by the *scoring panel*. (c) Quantification of oil droplets in oil red O–stained sections as determined by the Image-Pro Plus version 6.1 method. Bar graph represents the mean  $\pm$  SEM of nine specimens from the ND group, six specimens from the MD group, and nine specimens from the HD group, with the ND group set at 1 for convenience

### 7.2.3 Alcohol Consumption Correlates Positively with Hepatic Steatosis

To investigate how alcohol consumption affects liver pathologically, we performed histopathology of liver tissues from the ND ( $n=9$ ), MD ( $n=6$ ), and HD ( $n=9$ ) groups. In hematoxylin and eosin–stained sections, discrete, blank vacuoles were uncommon in the livers from the ND and MD groups and prominent in the livers of the HD group (data not shown). In oil red O–stained liver sections, bright red–stained lipid droplets were scored as described in the methods section. One calibrator image for every score is shown in Fig. 7.3a. The number of cases put into each score as determined by the blinded scoring panel in the ND, MD, and HD groups is listed in Fig. 7.3b. As the amount of alcohol consumption increased, there was a clear shifting from lower score scale to higher score scale. The mean density of lipid accumulations determined with the Image-Pro Plus v6.1 software on the digital photomicrographs of the various experimental groups is shown in Fig. 7.3c. There

was greater than 70% more lipid deposition in the drinking groups (MD+HD combined) compared with the ND group.

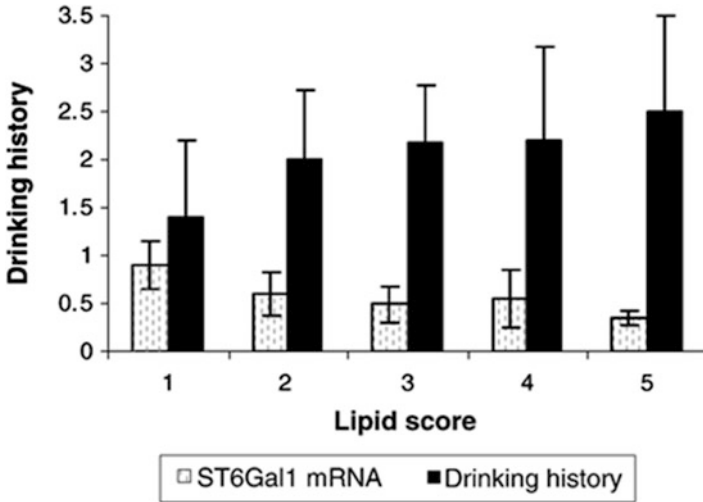
### ***7.2.4 Strong Multivariate Relationships Exist Between Alcohol-Drinking History, ST6Gal1 Gene Expression, and Liver Lipid Deposits***

There was a strong relationship between alcohol-drinking history, ST6Gal1 mRNA expression, and liver lipid score ( $F=8.68$ ,  $p<0.001$ ). Alcohol-drinking history and ST6Gal1 mRNA expression together accounted for 56.6% of the lipid score variance, and both predictors made a significant correlation to prediction accuracy (for ST6Gal1,  $p<0.001$ ; for drinking history,  $p<0.01$ ) in a multivariate model. When both alcohol history and lipid deposit score were coded as ordinal, the Pearson correlation was 0.35 ( $p=0.09$ ). However, by examining the 95% confidence intervals around the mean liver lipid deposit scores for each of the 3 levels of drinking history, we found that subjects with a history of heavy or moderate drinking had significantly more lipid deposits (mean = 3.11, confidence interval = 2.26–3.96 and 2.11–3.89 for heavy and moderate drinkers, respectively) than subjects with no drinking (mean = 2.11, confidence interval = 1.26–2.96,  $p<0.05$ ). The univariate relationship between ST6Gal1 mRNA expression and drinking history was very strong and significant ( $F=337.09$ ,  $p<0.0001$ ,  $R^2=0.97$ ). Mean expression levels by drinking history are shown in Fig. 7.4.

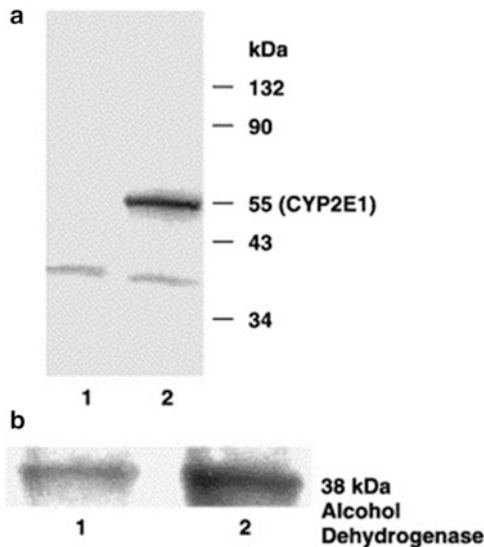
### ***7.2.5 Expression of CYP2E1 and ADH in the Respective HepG2 Cell Lines***

To confirm that CYP2E1 and ADH were expressed in the stably transfected CYP2E1 and HAD cells, respectively, but not in the wild type, Western blot analysis was carried out with the extracts of the respective cells and the wild-type cells using anti-human CYP2E1 and anti-human ADH. As shown in Fig. 7.5a, a 55-kDa band corresponding to CYP2E1 was identifiable only in the extracts of CYP2E1 cells but not in wild-type cells. Furthermore, CYP2E1 activity, as measured by p-nitrophenol oxidation rate, was found to be 53.2 pmol/min/mg of protein in the CYP2E1 cells, thus agreeing with the results of Yang and Cederbaum (1997). CYP2E1 activity was undetectable in the wild-type cells. Similarly, it can be seen in Fig. 7.5b that a strong 39-kDa band corresponding to human ADH subunit was identifiable in the extracts of HAD cells but only a faint one in the wild type. Furthermore, ADH activity, as measured by ethanol oxidation was found to be 216.2 nmol/h/mg of protein similar to the results reported by Clemens et al. (1995). Negligible ADH activity was detectable in the wild-type cell extracts.

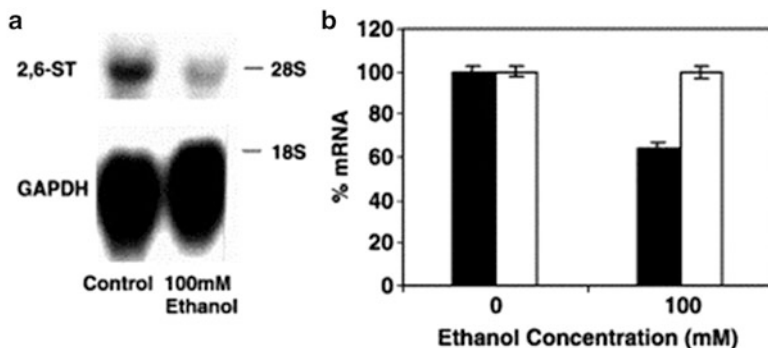




**Fig. 7.4 Multivariate analyses of ST6Gal1 gene expression, drinking history, and liver lipid deposit in the study groups.** The relative ST6Gal1 mRNA expression from all samples in the ND (n=12), MD (n=12), and HD (n=12) groups was plotted together with drinking history against liver lipid score. Drinking history was coded as heavy=3, moderate=2, and none=1 to obtain a mean. *Error bars* represent the 95% confidence interval around the mean



**Fig. 7.5 Western blot analysis of CYP2E1 and HAD cells.** Twenty micrograms equivalent of total protein extracts from CYP2E1 and HAD cells were subjected to Western blot analyses using the polyclonal CYP2E1 and ADH antibodies, respectively. (a) *Lane 1* indicates wild-type cell extract; *lane 2*, CYP2E1 cell extract. (b) *Lane 1* indicates wild-type cell extract; *lane 2*, HAD cell extract



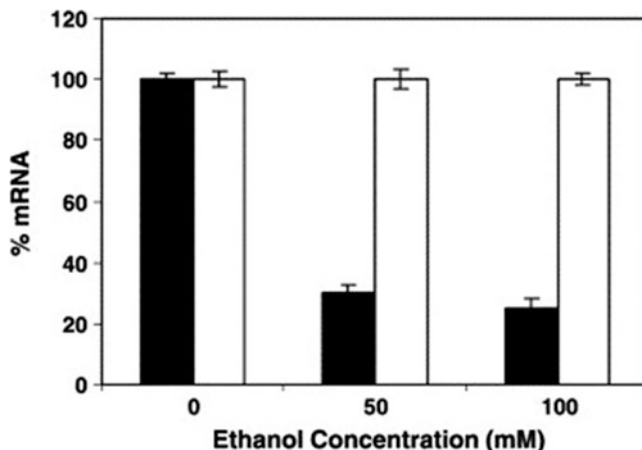
**Fig. 7.6** Effect of ethanol on ST6Gal1 mRNA level in CYP2E1 cells. CYP2E1 cells were incubated without or with 100 mmol/L ethanol for 72 h and the total RNA was extracted and subjected to Northern blot analysis. (a) A representative Northern blot analysis of ST6Gal1 mRNA from total RNA extracts of control and 100 mmol/L ethanol-treated cell extracts. *Lane 1*, RNA from CYP2E1 cells without ethanol exposure. *Lane 2*, RNA from the same cell line after exposure to 100 mmol/L of ethanol. (b) Relative levels of ST6Gal1 mRNA. Each value is the mean  $\pm$  SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

### 7.2.6 Effect of Ethanol on ST6Gal1 mRNA Expression in CYP2E1 Cells

The time course effect of 100 mmol/L ethanol on ST6Gal1 mRNA level in CYP2E1 cells showed 5% (NS) decrease at 24 h, 8% (NS) at 48 h, and 39% at 72 h. Therefore, the concentration effect of ethanol was carried out at the 72-h time point. A representative Northern blot analysis of ST6Gal1 mRNA from the total RNA extracts of control and ethanol-treated cells is shown in Fig. 7.6a. Figure 7.6b shows the effect of ethanol concentration on the relative levels of ST6Gal1 mRNA in CYP2E1 cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 46% ( $p < 0.05$ ) at 100 mmol/L ethanol compared with untreated CYP2E1 cells. The housekeeping gene GAPDH expression was not affected by ethanol treatment under identical conditions.

### 7.2.7 Effect of Ethanol on ST6Gal1 mRNA Expression in HAD Liver Cells

Because ethanol effect on ST6Gal1 mRNA was not evident before the 72-h point (data not shown), the concentration effect of ethanol was carried out at the 72-h time point. Figure 7.7 shows the effect of ethanol concentration on the relative levels of

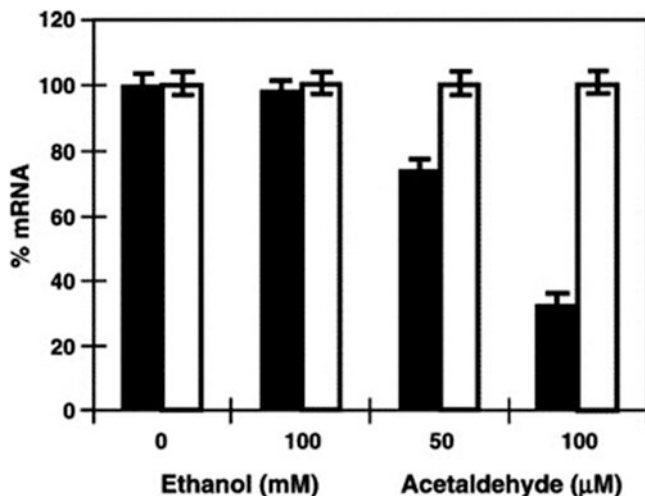


**Fig. 7.7** Effect of ethanol exposure time on ST6Gal1 mRNA level in HAD cells. HAD cells were incubated without or with 50 and 100 mmol/L ethanol for 72 h and the total RNA was extracted and subjected to Northern blot analysis. *Lane 1* is RNA from untreated HAD cells, *lane 2* is RNA from HAD treated with 50 mmol/L ethanol for 72 h, and *lane 3* is RNA from same cell line treated with 100 mmol/L ethanol for 72 h. Expression of human ST6Gal1 dramatically down-regulated in treated cells. Each value is the mean  $\pm$  SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

ST6Gal1 mRNA in HAD cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 70% ( $p < 0.05$ ) at 50 mmol/L ethanol and by 76% ( $p < 0.05$ ) at 100 mmol/L ethanol as compared with untreated HAD cells. The housekeeping gene GAPDH expression was not affected by ethanol treatment under identical conditions.

### 7.2.8 Effect of Ethanol and Acetaldehyde on ST6Gal1 mRNA Expression in Wild-Type HepG2 Liver Cells

The concentration effects of ethanol and acetaldehyde were carried out at the 72-h time point. Figure 7.8 shows that the effect of ethanol concentration on the relative levels of ST6Gal1 mRNA in wild-type cells decreased negligibly after 72 h of incubation with 100 mmol/L ethanol. In contrast, the relative level of ST6Gal 1 mRNA was decreased by 35% ( $p < 0.05$ ) at 50  $\mu$ mol/L acetaldehyde and by 69% ( $p < 0.05$ ) at 100  $\mu$ mol/L acetaldehyde compared with untreated wild-type cells. The housekeeping gene GAPDH expression was not affected by ethanol or acetaldehyde exposure under identical conditions.



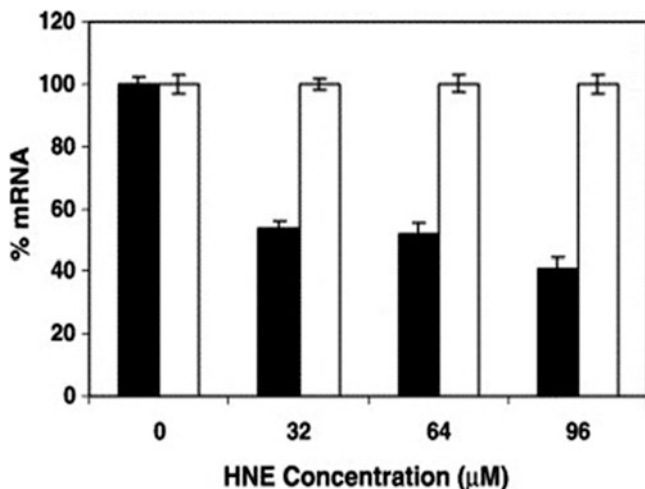
**Fig. 7.8** Effect of ethanol and acetaldehyde on ST6Gal1 mRNA level in wild-type cells. Wild-type cells were incubated without or with 100 mmol/L ethanol or 50 and 100 µmol/L acetaldehyde for 72 h and the total RNA was extracted and subjected to Northern blot analysis. Each value is the mean  $\pm$  SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

### 7.2.9 Effect of HNE on ST6Gal1 mRNA Expression in CYP2E1 Cells

The concentration effect of HNE was carried out at the 72-h time point. Figure 7.9 shows the effect of HNE concentration on the relative levels of ST6Gal1 mRNA in CYP2E1 cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 46% ( $p < 0.02$ ) at 32 µmol/L HNE, by 49% ( $p < 0.02$ ) at 64 µmol/L HNE, and by 61% ( $p < 0.02$ ) at 96 µmol/L HNE compared with untreated CYP2E1 cells. The housekeeping gene GAPDH expression was not affected by HNE treatment under identical conditions.

### 7.2.10 Partial Identification of the Liver Cytosol Protein That Forms Complex with the 3'-UTR of Rat ST6Gal1 mRNA

The interaction of the cytosol fraction from control rat liver with  $^{32}\text{P}$ -labeled ST6Gal1 mRNA probe of 2029-bp length covering nucleotides immediately downstream from the stop codon was analyzed by electrophoretic gel mobility shift assays (EMSA) on 8% native polyacrylamide gels. As can be seen in Fig. 7.10a, incubation of the  $^{32}\text{P}$ -labeled ST6Gal1 mRNA probe with as little as 1 µg of cytosol

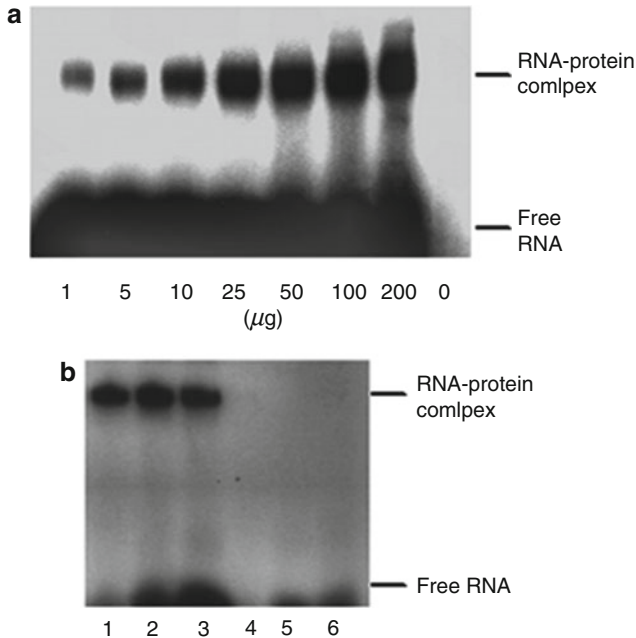


**Fig. 7.9** Effect of HNE on ST6Gal1 mRNA level in CYP2E1 cells. CYP2E1 cells were incubated without or with indicated concentrations of HNE for 72 h and the total RNA was extracted and subjected to Northern blot analysis. Each value is the mean  $\pm$  SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

protein retarded the migration of the probe, leading to the appearance of a RNA-protein complex radioactive band (Fig. 7.10a, lane 1). There was an increase in the intensity of this band with increasing amount of the cytosol protein up to 50  $\mu\text{g}$ , beyond which no further increase occurred (Fig. 7.10a, lanes 1–7). Prior incubation of the cytosol fraction with 100-fold molar excess of unlabeled cold RNA probe completely quenched the binding of  $^{32}\text{P}$ -labeled RNA probe as evidenced by the loss of the intensity of the RNA-protein complex band (Fig. 7.10b, lane 4). Likewise, RNA-protein complex formation was abolished by treating the cytosol fraction with proteinase K or preheating at 56°C for 10 min (Fig. 7.10b, lanes 5 and 6).

### 7.2.11 Importance of Secondary Structure of RNA in RNA-Protein Complex Formation

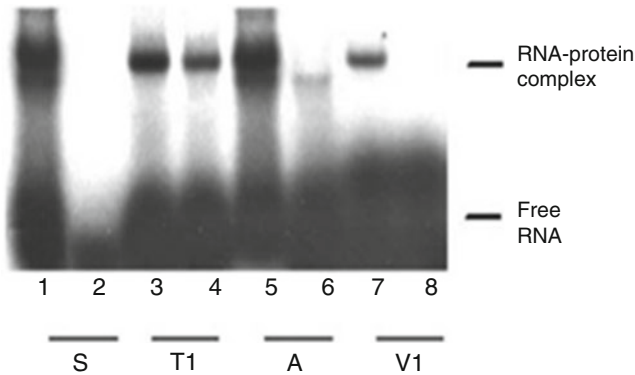
Figure 7.10b (lanes 1–3) shows the interaction of the cytosol protein with the RNA probe that was first heat-denatured at 90 °C for 10 min and either cooled down rapidly on ice to maintain the denatured structure (Fig. 7.10b, lane 2) or cooled down gradually to room temperature to allow the stable RNA secondary structure to form (Fig. 7.10b, lane 3), compared with normal binding reaction with unheated RNA probe (Fig. 7.10b, lane 1). It is obvious from the figure that the same strong signal for the RNA-protein complex band could be observed with both denatured and renatured RNA probes.



**Fig. 7.10** Characterization of a cytosol protein that bind to ST6Gal1 mRNA. (a) Effect of the amount of cytosol protein from rat liver on 3'-UTR of ST6Gal1 mRNA-protein complex formation. Indicated amounts of cytosol fraction were incubated with  $^{32}\text{P}$ -labeled RNA probe (2.0nM final concentration) in the standard binding assay. (b) Effect of RNA secondary structure, competitive binding, and pretreatment with proteinase K or heat on RNA-protein complex formation. The standard binding assay was carried out with the cytosol fraction and the  $^{32}\text{P}$ -labeled RNA that was unheated (lane 1), preheated to  $90^\circ\text{C}$  for 10 min, and then rapidly cooled to  $4^\circ\text{C}$  (lane 2) or gradually cooled to room temperature (lane 3), competitive binding with 100-fold excess of cold mRNA probe (lane 4), cytosol fraction treated with proteinase K (lane 5), or prior heating (lane 6)

### 7.2.12 *The RNA-Protein Interaction Protects 3'-UTR from RNase Digestion*

To investigate whether the formation of protein-ST6Gal1 complex plays any functional role in ST6Gal1 mRNA stability, RNase digestion assays were performed with the longest RNA probe (probe 1) either before or after the binding reactions. As can be seen from Fig. 7.11, the specific protein binding to the 3'-UTR of ST6Gal1 completely protected it from digestion by RNases S, V1, and A and partially protected it from RNase T1. It is worthy to note that RNase S is nonspecific for digesting RNA; RNase V1 cleaves only base-paired nucleotide, whereas RNase A cleaves 3'-U and C residues. On the other hand, RNase T1 cleaves only at the 3'-G residue; therefore, it is possible that some RNA sequence may remain intact after RNase T1 digestion.



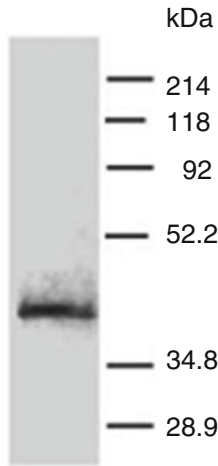
**Fig. 7.11** Effects of various RNases on the binding of 3'-UTR of ST6Gal1 mRNA to the cytosol protein. The standard binding assay was carried out with same amount of  $^{32}\text{P}$ -labeled RNA probe either before or after treatment with various RNases prior to incubation with the cytosol binding protein. Lanes 1 and 2, before and after treatment with RNase S; lanes 3 and 4, before and after treatment with RNase T1; lanes 5 and 6, before and after treatment with RNase A; lanes 7 and 8, before and after treatment with RNase V1. In all of the assays in Figs. 7.16 and 7.17, the formation of RNA-protein complex was determined by EMSA

### 7.2.13 *Partial Characterization of Protein Interacting with 3'-UTR of ST6Gal1 mRNA by UV Cross-Linking*

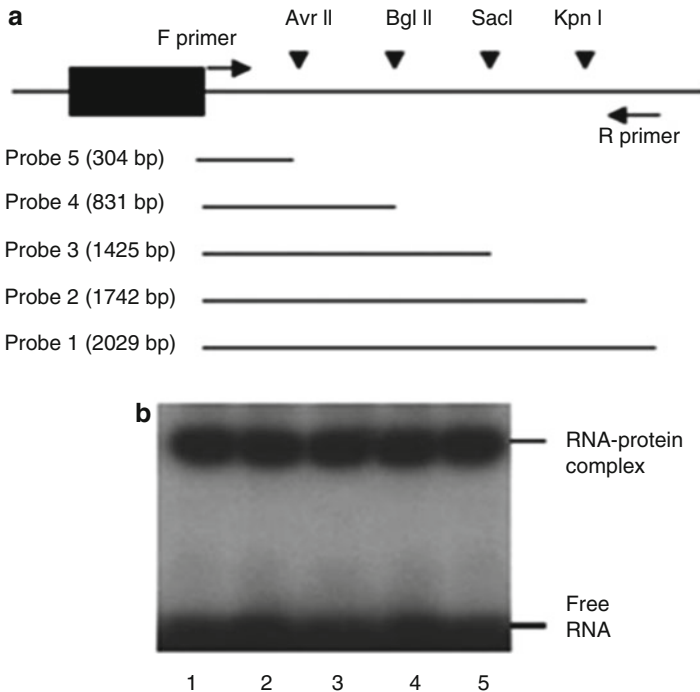
As a direct method to determine the molecular mass of this protein under denatured condition, the products of the binding reaction with the  $^{32}\text{P}$ -labeled ST6Gal1 RNA probe were UV cross-linked, and the noncross-linked RNA was removed by RNase T1 digestion. The resulting  $^{32}\text{P}$ -labeled RNA-protein complex was separated by SDS-PAGE along with a set of molecular mass standards on a separate lane. As shown in Fig. 7.12, a single band corresponding to 41 kDa was seen.

### 7.2.14 *Thirteen-Base Pair Motif of ST6Gal1 mRNA Is Sufficient for the Specific Protein to Bind*

To identify the shortest possible nucleotide sequence of 3'-UTR of ST6Gal1 with which the binding protein interacts, the 2029-bp of the RT-PCR template was digested with restriction enzymes, and the sequentially truncated templates were used for RNA probe synthesis and EMSA (Fig. 7.13a). As shown in Fig. 7.13b, all of the probes listed in Fig. 7.13a interacted with the protein, indicating that probe 5, which is only 304-bp immediate downstream from the stop codon, was clearly sufficient to facilitate the binding interaction. However, it was difficult to visualize the bands by EMSA, even with prolonged exposure with RNA probes that were shorter than probe 5, due to the limited incorporation of [ $^{32}\text{P}$ ]UTP. Therefore, after narrowing the binding region

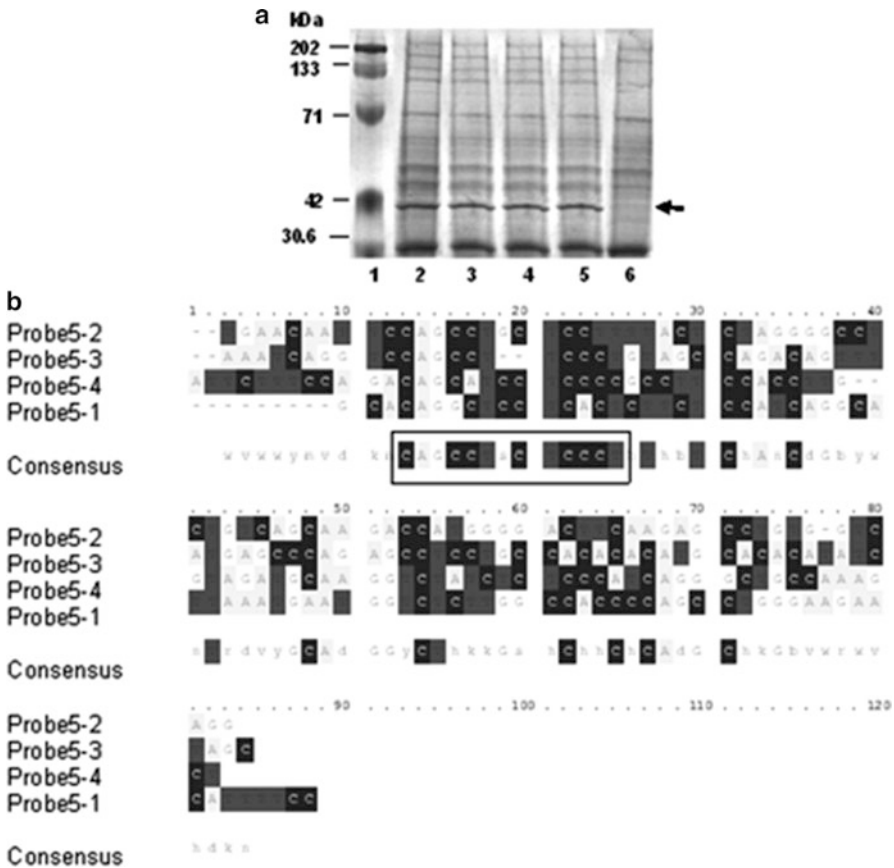


**Fig. 7.12 Characterization of the binding protein by UV cross-linking.** The standard binding reaction was carried out between  $^{32}\text{P}$ -labeled probe and cytosol protein. The complex was UV cross-linked as described under Materials and Methods, and the protein samples were analyzed by SDS-PAGE gel. The positions of pre-stained Broad Range molecular mass markers (Bio-Rad) are shown in *middle*



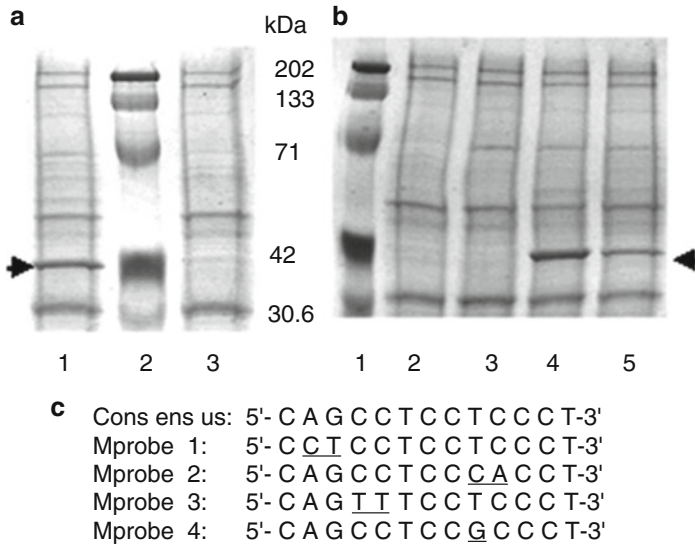
**Fig. 7.13 Schematic chart for mapping of the protein-ST6Gal1 interaction.** (a) The 2029-bp RT-PCR fragments were digested with KpnI, SacI, BglII, and AvrII. The corresponding fragments with the T7 RNA polymerase promoter sequence were gel purified and used for generating RNA probes. *Black bar*, coding region for ST6Gal1. (b) All five RNA probes synthesized from the templates in (a) were used in the binding reaction, and the RNA-protein complexes were visualized by EMSA. *Lanes 1–5* indicate the complexes for RNA probes 1–5, respectively





**Fig. 7.14 Identification of the ST6Gal1 3'-UTR mRNA sequences that the cytosol protein(s) interact with:** (a) Four biotin-labeled 80-bp RNA probes (5-1, 5-2, 5-3, and 5-4) that cover the 304-bp region (probe 5 in Fig. 7.19a) were incubated with liver cytosol protein and then added to SA-PMP beads. The bound proteins were eluted and run on SDS-PAGE gel and were stained. *Lane 1*, Kaleidoscope protein molecular mass marker (Bio-Rad). *Lanes 2–5*, proteins eluted from SA-PMP beads with the above biotin-labeled probes 5-1, 5-2, 5-3, and 5-4. *Lane 6* is a negative binding control. *Arrowhead* points to a 41-kDa specific protein band. (b) Sequence alignment analysis for the cDNA region that is complementary to the four 80-bp RNA probes covering the entire 304-bp region. A 13-bp consensus sequence is repeated four times within the 304-bp immediately downstream of the stop codon. The consensus sequence is *boxed*

to 304-bp downstream from the stop codon (probe 5), 5'-biotin-labeled RNA probes were made within this region and were used to further map the binding region. Therefore, four 5'-end biotin labeled 80-bp RNA probes covering the entire length of the 304-bp RNA probe 5 were synthesized and tested in the standard binding reaction. Surprisingly, in contrast to other nonspecific faint bands that were observed when the cytosol fraction was incubated with the magnetic beads alone (Fig. 7.14a, lane 6), a strong band corresponding to 41 kDa was observed with equal binding intensity exclusively for each of these 80-bp probes when they were incubated with the cytosol fraction and the magnetic beads (Fig. 7.14a, lanes 2–5). Further analysis revealed that

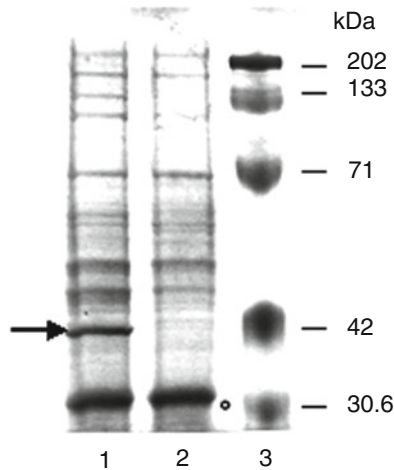


**Fig. 7.15 Identification of the minimal sequence (element) that is essential for the ST6Gal1 RNA-protein interaction.** (a) RNA probe corresponding to the 13-bp consensus sequence (lane 1) and nonconsensus sequence (lane 3) were used in the binding reaction. Kaleidoscope protein molecular mass marker (Bio-Rad) (lane 2). (b) Mutagenesis analysis. RNA probes corresponding to the 13-bp consensus sequence were mutated as indicated in (c). Binding reactions were performed as described under Materials and Methods. Lane 1, kaleidoscope marker; lane 2, Mprobe 1; lane 3, Mprobe 2; lane 4, Mprobe 3; lane 5, Mprobe 4. (c) Alignment of the consensus sequence and the mutated sequences. The mutated nucleotides are *underlined*

all of the four 80-bp probes shared the 13-bp consensus sequence as shown in Fig. 7.14b. Further mapping of the 80-bp probe showed that only the RNA probe corresponding to the 13-bp consensus sequence showed binding (Fig. 7.15a, lane 1) but not to the probe corresponding to the remaining nonconsensus sequence (Fig. 7.15a, lane 3). Significantly, when the nucleotides AG and TC were mutated to CT and CA, respectively, in the 13-bp consensus sequence, the corresponding RNA probes completely lost their binding ability (Fig. 7.15b, lanes 1 and 2). However, when only the nucleotide T was mutated to nucleotide G (Fig. 7.15b, lane 5), the corresponding RNA probe still showed partial binding ability. The mutation of other nucleotides, like CC to TT, did not affect the binding (Fig. 7.15b, lane 4).

### 7.2.15 The Specificity of the Cytosolic Binding Protein that Interacts with ST6Gal1 mRNA

ST8Sia1 is another member of sialyltransferase family, which links the sialic acid residues to another sialic acid residue of brain glycoconjugates through an  $\alpha$ 2,8-bond. In an attempt to test the specificity of this binding protein to ST6Gal1 mRNA, it was reasonable to test whether this binding protein interacted with ST8Sia1 mRNA



**Fig. 7.16 Specificity of the protein that binds to 3'-UTR of ST6Gal1.** Biotin-labeled RNA probe synthesized from ST6Gal1 mRNA (*lane 1*) and ST8Sia1-mRNA (*lane 2*) 3'-UTRs were used separately in the standard binding assay as described in Fig. 7.5 legend. *Lane 3*, kaleidoscope protein marker (Bio-Rad). *Arrowhead* points to a 41-kDa specific protein band that is present only in *lane 1* but not in *lane 2*

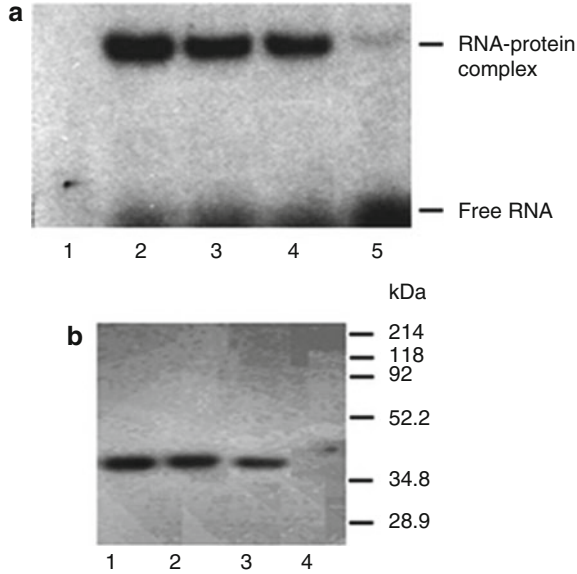
probe. Biotin-labeled ST8Sia1 RNA probe was used along with the labeled ST6Gal1 probe to study the specificity of their binding to the liver cytosolic fraction. As shown in Fig. 7.16, a band corresponding to 41 kDa was found only with ST6Gal1 probe (*lane 1*) but not with ST8Sia1 probe (*lane 2*) under identical conditions.

### 7.2.16 *Effects of Chronic Ethanol Exposure In Vivo on the Status of the Binding Protein*

As shown in Fig. 7.17a, there was a dietary ethanol concentration- dependent decrease in the binding intensity of the RNA-protein complex band that virtually disappeared when the cytosol fraction from the livers of rats fed with 36% of their dietary calories because ethanol was tested in the standard binding reaction. The intensity of the UV cross-linked  $^{32}\text{P}$ -labeled RNA protein complex band also progressively decreased with increasing concentration of dietary ethanol (Fig. 7.17b).

### 7.2.17 *Influence of Chronic Ethanol and Betaine on Animal Body and Liver Weights*

The initial and final body weights, final liver weights and the liver weights as per cent of body weights of various experimental groups are given in Table 7.1. It can be seen that both LFO and HFO Control groups gained significant body weights. On the other



**Fig. 7.17 Effects of various concentrations of dietary ethanol feeding on the expression of binding protein.** The formation of <sup>32</sup>P-labeled RNA probe and cytosol protein complex was determined by EMSA (a) and by UV cross-linking (b). (a) RNA-protein complex with the cytosol fraction from: *lane 1*, negative control without the cytosol fraction in the reaction; *lane 2*, control liver; *lane 3*, 10.8% ethanol group; *lane 4*, 21.6% ethanol group; and *lane 5*, 36% ethanol group. (b) UV cross-linked RNA-protein complex with the cytosol fraction from: *lane 1*, control liver; *lane 2*, 10.8% ethanol group; *lane 3*, 21.6% ethanol group; and *lane 4*, 36% ethanol group

hand, the corresponding LFOE and HFOE Alcohol groups did not gain as much body weight compared to their controls. In contrast, the gains in weights of the LFOEB and HFOEB groups were markedly decreased compared to their corresponding LFOE and HFOE groups, respectively. Moreover, the hepatosomatic index increased by 23% ( $p < 0.05$ ) in both LFOE and HFOE Alcohol groups. The corresponding increases in hepatosomatic index values for the LFOEB and HFOEB groups were 23% ( $p < 0.05$ ) and 32% ( $p < 0.05$ ), respectively. For the sake of the ease of interpretations with regard to the influence of chronic ethanol and high  $\omega$ -3 PUFA, all results described below are compared against the low  $\omega$ -3 PUFA control group.

### 7.2.18 *Influence of Chronic Ethanol and Betaine Liver PON1 mRNA Expression in Rats Fed Low and High $\omega$ -3 PUFA Diets*

As shown in Fig. 7.18, liver PON1 mRNA expression was decreased by 23% ( $p < 0.01$ ) in the high  $\omega$ -3 PUFA-fed group compared to the low  $\omega$ -3 PUFA group. Chronic ethanol feeding decreased liver PON1 mRNA expression by 25% ( $p < 0.01$ )

**Table 7.1** Effects of ETOH and Betaine in low and high fish oil diets on body weight, liver weight, and liver to body weight ratio

Dietary groups (n=4)	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Final liver weight (g)	Hepatosomatic index
LFO	130.1±3.9	244.5±5.9	114.4±6.2	7.8±0.4	3.2±0.16
LFOE	160.0±4.8	233.4±4.8 <sup>a</sup>	73.4±4.8 <sup>a</sup>	7.5±0.3	3.2±0.20
LFOEB	157.2±3.2	185.2±5.2 <sup>b</sup>	28.2±2.2 <sup>b</sup>	7.2±0.4	3.9±0.24 <sup>a</sup>
HFO	159.4±2.9	268.2±7.3	108.8±4.3	8.4±0.4	3.1±0.18
HFOE	178.3±4.2	228.3±6.7 <sup>c</sup>	50.3±4.6 <sup>c</sup>	8.8±0.6 <sup>a</sup>	3.8±0.21 <sup>c</sup>
HFOEB	177.6±3.0	201.9±3.5 <sup>d</sup>	24.3±3.0 <sup>d</sup>	8.1±0.4	4.1±0.26 <sup>c</sup>

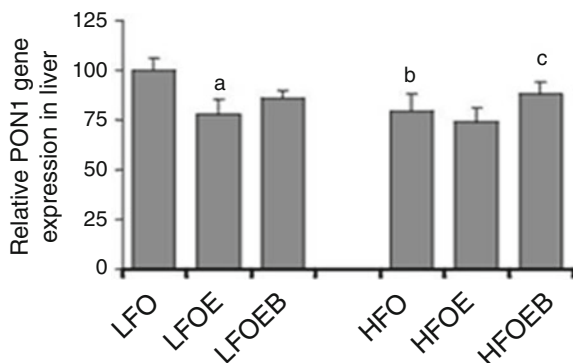
Values are means ± SEM. Means in a column with different superscripts differ significantly ( $p < 0.05$ ) as calculated by Tukey's test

<sup>a</sup> Compared to LFO group

<sup>b</sup> Compared to the corresponding LFOE or HFOE groups

<sup>c</sup> Compared to HFO group

<sup>d</sup> Compared to HFO or HFOE. Hepatosomatic Index = (Liver weight × 100) / body weight

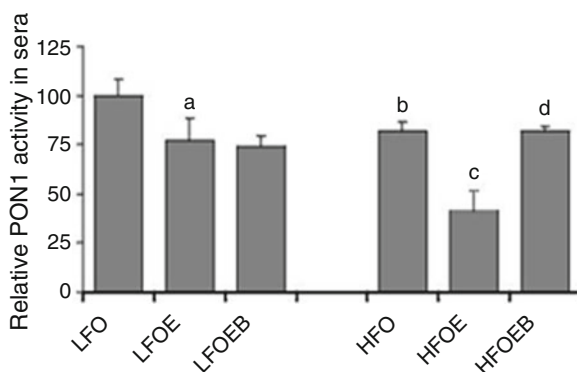


**Fig. 7.18 Influence of chronic ethanol and betaine on PON1 mRNA expression in livers of rats fed low and high  $\omega$ -3 PUFA diets.** The animals in the indicated groups ( $n=4$ ) were pair-fed their respective Lieber-DeCarli control or alcohol containing liquid diets for 8 weeks after which the animals were killed and each liver was analyzed for PON1 mRNA. The relative expression of PON1 mRNA in the HFO groups is expressed as percent of the corresponding values in the LFO groups. The data are means  $\pm$  SEM. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.01$  compared to LFO group; *b*:  $p < 0.01$  compared to LFO group; *c*:  $p < 0.01$  compared to HFOE group

in low  $\omega$ -3 PUFA group, and 30% ( $P < 0.01$ ) in high  $\omega$ -3 PUFA group compared to the low  $\omega$ -3 PUFA control group. Betaine significantly restored liver PON1 mRNA expression to 90–95% ( $p < 0.01$ ) in high  $\omega$ -3 PUFA alcohol groups compared to low  $\omega$ -3 PUFA control group.

### ***7.2.19 Influence of Chronic Ethanol and Betaine on Serum PON1 and HCTLase Activities in Rats Fed Low and High $\omega$ -3 PUFA Diets***

As shown in Figs. 7.19 and 7.20, high  $\omega$ -3 PUFA significantly decreased serum PON1 activity by 20% ( $p < 0.05$ ) and homocysteine thiolactone (HCTL)ase activity by 28% ( $p < 0.05$ ). Correspondingly, serum PON1 activity decreased by 23% ( $p < 0.05$ ) and 58% ( $p < 0.01$ ) while serum HCTLase activity decreased by 25% ( $p < 0.05$ ) and 59% ( $p < 0.01$ ) in the low and high  $\omega$ -3 PUFA ETOH groups, respectively. Significantly, betaine stimulated serum PON1 activity by 200% ( $p < 0.01$ ) in the high  $\omega$ -3 PUFA alcohol group to the level of low  $\omega$ -3 PUFA group while betaine had marginal effect in low  $\omega$ -3 PUFA alcohol group. Betaine caused similar changes in serum HCTLase activities in the high  $\omega$ -3 PUFA alcohol group.



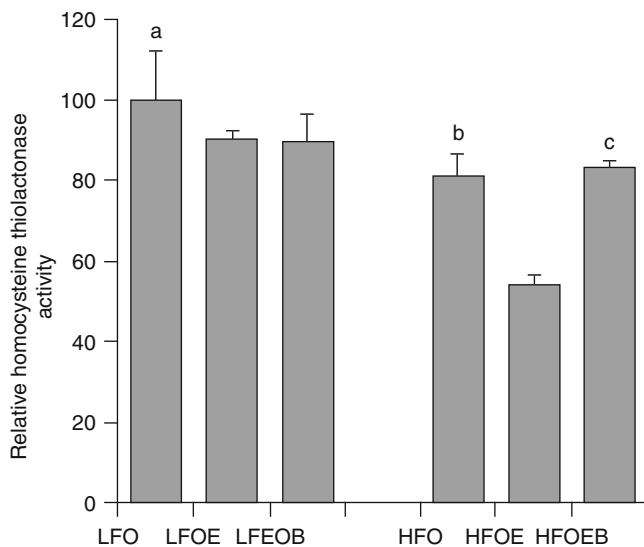
**Fig. 7.19 Influence of chronic ethanol and betaine on serum PON1 activity in rats fed low and high  $\omega$ -3 PUFA diets.** The animals in the indicated groups ( $n=4$ ) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of the betaine for 8 weeks after which the animals were killed and serum sample was analyzed for PON1 activity. The relative PON1 activity in the various experimental groups is expressed as percent of the corresponding values in the LFOE groups. The data are means  $\pm$  SEM. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.01$  compared to LFO group; *b*:  $p < 0.05$  compared to LFO group; *c*:  $p < 0.01$  compared HFO group; *d*:  $p < 0.01$  compared to HFOE group

### 7.2.20 Influence of Chronic Ethanol, $\omega$ -3 PUFA and Betaine on Liver GSH Levels in Rats Fed Low and High $\omega$ -3 PUFA Diets

As shown in Fig. 7.21, GSH in the liver was very similar in both groups of rats fed low or high  $\omega$ -3 PUFA diet. However, chronic alcohol significantly decreased liver GSH levels by 27% ( $p < 0.05$ ) in low  $\omega$ -3 PUFA group and by 38% ( $p < 0.01$ ) in high  $\omega$ -3 PUFA group. Betaine restored liver GSH levels in both low and high  $\omega$ -3 PUFA alcohol groups nearly to the level in low  $\omega$ -3 PUFA control group ( $p < 0.05$ ).

### 7.2.21 Influence of Chronic Ethanol, $\omega$ -3 PUFA and Betaine on Serum ALT

As shown in Fig. 7.22, serum ALT increased moderately by 28% ( $p < 0.01$ ) in LFOE group compared to the LFO group that was significantly restored to the level of LFO group by betaine supplementation. In contrast, serum ALT increased dramatically by 60% ( $p < 0.01$ ) in HFOE compared to the HFO group. Betaine feeding significantly blocked the increase in serum ALT level in HFOEB group than in the HFO group ( $p < 0.05$ ).



**Fig. 7.20 Influence of chronic ethanol and betaine on serum homocysteine thiolactonase activity in rats fed low and high  $\omega$ -3 PUFA diets.** The animals in the indicated groups ( $n=4$ ) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of the betaine for 8 weeks after which the animals were killed and serum sample was analyzed for homocysteine thiolactonase activity as described in the Methods section. Homocysteine thiolactonase activity expressed as percentage in sera. The data are means  $\pm$  SEM. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.01$  compared to HFO group; *b*:  $p < 0.01$  compared to HFOE group; *c*:  $p < 0.01$  compared to HFOE

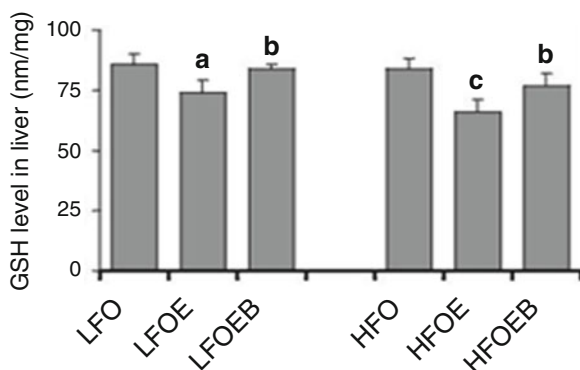
### 7.2.22 Influence of Chronic Ethanol, $\omega$ -3 PUFA and Betaine on Liver Lipid Score

As seen in Fig. 7.23, oil red O stained liver sections of low  $\omega$ -3 PUFA alcohol fed rats showed no significant changes of fat deposition compared to low  $\omega$ -3 PUFA. Whereas betaine in low  $\omega$ -3 PUFA alcohol diet significantly reduced fat deposition compared to low  $\omega$ -3 PUFA alcohol and low  $\omega$ -3 PUFA groups ( $p < 0.05$ ); steatosis increased significantly in rats fed high  $\omega$ -3 PUFA diet versus low  $\omega$ -3 PUFA ( $p < 0.05$ ). Ethanol significantly increased liver steatosis in rats fed high  $\omega$ -3 PUFA compared to all other groups ( $p < 0.01$ ). Betaine significantly prevented liver steatosis that occurred in both low and high  $\omega$ -PUFA alcohol groups as well as high  $\omega$ -PUFA group ( $p < 0.01$ ).

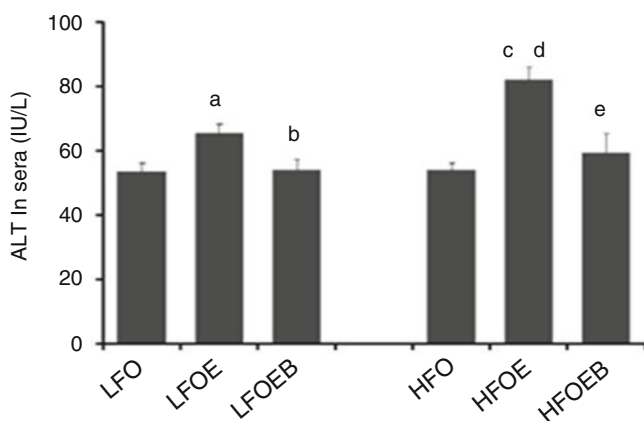
## 7.3 Significance of the Results

As part of our continuous efforts to better understand how ethanol affects human liver pathologically, we have previously reported that long-term ethanol causes down-regulation of ST6Gal1 gene (Gong et al. 2007). In the current expanded study,

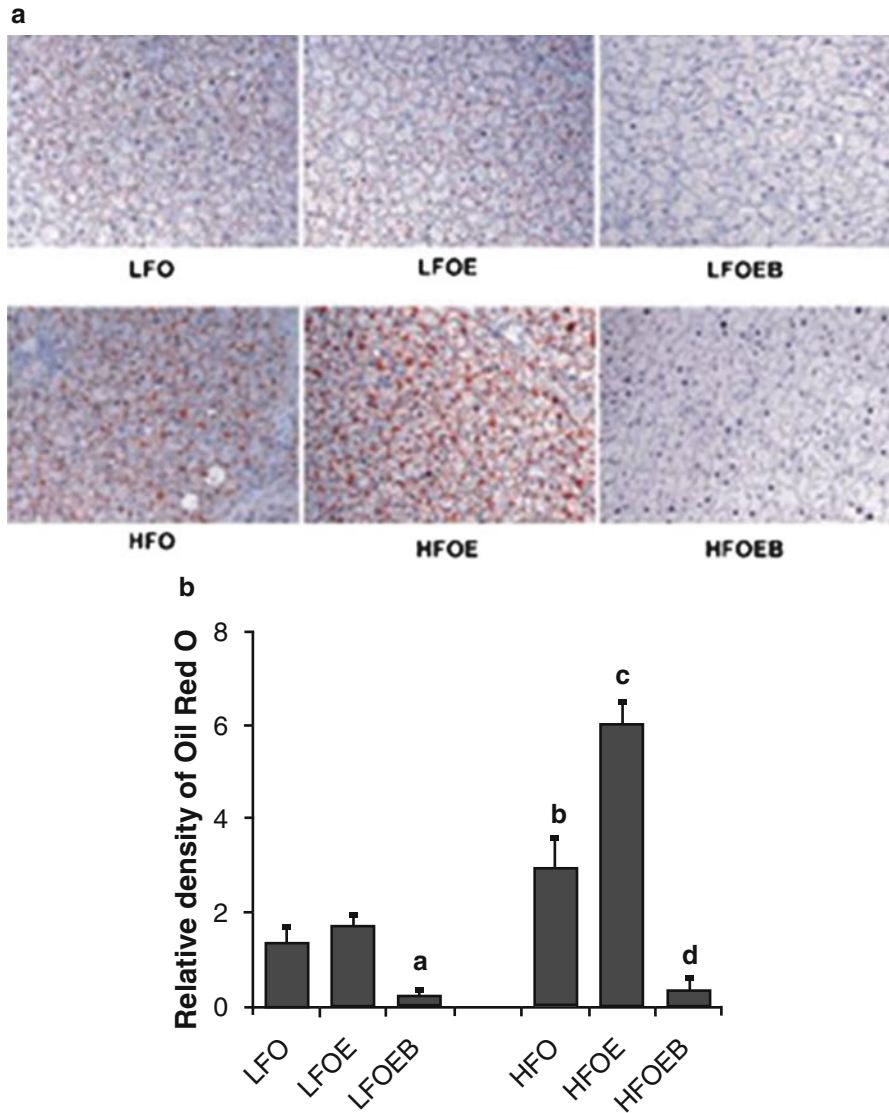




**Fig. 7.21 Influence of chronic ethanol, w-3 PUFA, and betaine on liver GSH level in rats fed low and high w-3 PUFA diets.** The animals in the indicated groups (n=4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each liver was analyzed for reduced GSH level in liver quantified as described in the Methods section. The data are means±SEM. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.05$  compared to LFO group; *b*:  $p < 0.05$  compared to the corresponding LFOE or HFOE groups; *c*:  $p < 0.01$  compared to HFO group



**Fig. 7.22 Influence of chronic ethanol, w-3 PUFA, and betaine on serum ALT in rats fed low and high w-3 PUFA diets.** The animals in the indicated groups (n=4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each serum sample was analyzed for ALT level. The relative ALT activity in the various experimental groups is expressed as international units per liter. The data are means±SEM. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.01$  compared to LFO group; *b*:  $p < 0.05$  compared to the corresponding LFOE group; *c*:  $p < 0.01$  compared to HFO or LFO groups; *d*:  $p < 0.01$  compared to LFOE group; *e*:  $p < 0.01$  compared to HFOE group



**Fig. 7.23 Influence of chronic ethanol, w-3 PUFA, and betaine on liver steatosis in rats fed low and high w-3 PUFA diets.** The animals in the indicated groups were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each liver section was stained with oil red O, counter stained with hematoxylin, and the extent of lipid deposition in the liver was quantified. **(a)** Representative liver histochemistry of each group. **(b)** A bar graph shows the relative lipid score analyses in various groups. The data are means  $\pm$  SEM from three animals. Statistical significance of variance was calculated using Tukey's test; *a*:  $p < 0.05$  compared to LFOE group; *b*:  $p < 0.05$  compared to the corresponding LFO groups; *c*:  $p < 0.01$  compared to HFO or LFO groups; *d*:  $p < 0.01$  compared to HFOE or HFO or LFOE

we have not only confirmed our previous findings, but also have provided a direct evidence that the down-regulation of ST6Gal1 is most likely due to long-term ethanol exposure, but not due to pathologic conditions (Fig. 7.1).

Liver sections stained with oil red O from the various experimental groups clearly show that even moderate drinking of alcohol causes fat deposits in the liver that exacerbate after heavy drinking compared with the non-drinking group (Figs. 7.2 and 7.3). It must be pointed out that the samples belonging to metabolic and genetic obesity were excluded from the study analyses. Both histometric methods demonstrated much more lipid accumulation in the livers of the drinking groups than in the nondrinking groups and thus histologically (Fig. 7.3) support our biochemical findings (Fig. 7.2). These results, together with molecular biology evidence (Fig. 7.1), imply that the steatosis found in the drinking groups compared with the nondrinking groups may be more specific to ethanol consumption. Our results are consistent with other reports that ST6Gal1 gene expression is unaltered or up-regulated in nonalcoholic or neoplastic liver diseases (Petretti et al. 2000; Wang et al. 2003; Dall'Olio et al. 2004).

When drinking history, ST6Gal1 mRNA level, and liver lipid deposit are all considered in the multivariate statistical analysis, there is a strong correlation among drinking history, ST6Gal1 gene expression, and liver lipid deposit (Fig. 7.4).

We have also used human hepatocyte HepG2 cell lines as a model to study the effects of ethanol on the regulation of ST6Gal1 gene expression because ethanol metabolism takes place primarily in the liver. That the expression of ST6Gal1 mRNA was affected by ethanol only in ethanol-metabolizing cells but not in the wild type (Figs. 7.6, 7.7 and 7.8) clearly demonstrates the importance of ethanol metabolism for eliciting this regulatory response. The two major hepatic ethanol-metabolizing enzymes are CYP2E1 and alcohol dehydrogenase. Both of these enzymes oxidize ethanol to acetaldehyde, which is more toxic than ethanol itself. The fact that acetaldehyde, the immediate product of ethanol oxidation, is effective in down-regulating ST6Gal1 mRNA confirms that ethanol may mediate this response via acetaldehyde.

On the other hand, CYP2E1-mediated oxidation of ethanol also produces a state of oxidative stress by generating ROS within the cells that can lead to the generation of a key metabolite, the  $\alpha,\beta$ -unsaturated aldehyde, HNE. HNE may be more harmful than ROS because it has a longer half-life and can easily diffuse into cellular membranes (Esterbauer et al. 1991). Under normal conditions, intracellular HNE concentration is less than 1.0  $\mu\text{mol/L}$ , but it can reach a level of as high as 100  $\mu\text{mol/L}$  under oxidative stress (Burchynski et al. 2001). The fact that HNE strongly down-regulates ST6Gal1 mRNA even at 32  $\mu\text{mol/L}$  (Fig. 7.9) strongly supports the concept that oxidative stress caused by ethanol oxidation via CYP2E1 could also play a key role in regulating this gene.

Sialyltransferases are a family of enzymes consisting of more than 18 glycosyltransferases that catalyze the transfer of sialic acid from cytidine monophospho-N-acetylneuraminic acid to the nonreducing terminal positions on the oligosaccharide chains of glycoproteins and glycolipids (Harduin-Lepers et al. 2001). Terminal sialic acids are key determinants of carbohydrate structures involved in a variety

of biologic events, such as viral-host recognition (Gagneux et al. 2003), cell-cell adhesion (Schauer 1985; Pilatte et al. 1993; Lin et al. 2002) and tumor cell invasiveness (Zhu et al. 2001). In particular, alteration of sialic acids, generally found in the nonreducing terminus of most glycoproteins and glycolipids, has been associated with long-term alcohol exposure (Ghosh et al. 2001). A number of reports have provided evidences that long-term alcohol exposure interferes with the metabolism of the complex glycoconjugates of certain circulating as well as membrane-bound species in human and experimental animals (Lakshman et al. 1999; Stibler and Borg 1981), resulting in either the appearance of a number of carbohydrate-deficient glycoconjugates including carbohydrate-deficient transferrin (Stibler and Borg 1981), sialic acid-deficient apolipoprotein J (Ghosh et al. 2001), and  $\alpha$ 1-acid glycoprotein in the plasma of long-term alcohol consumers (Tsutsumi et al. 1994).

Ethanol exposure down-regulates the expression of ST6Gal1 mRNA in two ethanol-metabolizing human liver cell lines, the CYP2E1 cells and HAD cells but not in wild-type HepG2 cells, which do not metabolize ethanol. Thus, this study assumes major importance and clinical relevance because ST6Gal1 gene regulation in a human liver cell model is demonstrated within a few days of ethanol exposure, whereas it's *in vivo* regulation in liver generally takes prolonged period of ethanol exposure. The possible mechanism(s) of action ethanol seems to be mediated via acetaldehyde as well as via ROS. Further work is warranted to shed light as to how acetaldehyde and ROS regulate the expression of ST6Gal1 and what signaling pathways are involved.

ST6Gal1 mediates the transfer of  $\alpha$ 2,6-linked sialic acid to glycoproteins. Down-regulation of the expression of this gene and consequent impaired activity of ST6Gal1 has been associated with the increase of asialoconjugates in the blood of chronic alcoholics and retention of glycoproteins in the liver. In our previous studies (Ghosh and Lakshman 1997; Rao and Lakshman 1997, 1999), we showed that chronic ethanol feeding in rats caused a marked decrease (59% reduction compared with controls) of the ST6Gal1 activity as well as its mRNA level in liver that was due to decreased stability of ST6Gal1 mRNA. In our present work, we have further characterized the mechanism of action of ethanol in the destabilization of ST6Gal1 mRNA. We report here for the first time the identification and partial characterization of a liver cytosol protein that specifically binds to the 3'-UTR of the ST6Gal1 mRNA and plays a role in its stability. Significantly, chronic ethanol feeding decreases the intracellular concentration of this binding protein leading to the destabilization of ST6Gal1 mRNA and its down-regulation by rapid degradation.

The observed binding intensity of RNA-protein complex as a function of protein concentration (Fig. 7.10a) reveals that the binding reaction seems to be saturated at 50  $\mu$ g of cytosol protein when the RNA concentration is kept at 2.0nM. Unfortunately, it is premature to carry out Scatchard analysis using the crude cytosol fraction to determine the dissociation constant for this binding protein. This has to wait until the purification of this protein to homogeneity is achieved. Nonetheless, if the stoichiometry of interaction of the RNA with the binding protein is mole per mole, our data imply that the relative abundance of this binding protein in normal

rat liver cytosol fraction is approximately 0.0035% of total cytosol protein (based on our finding that the molecular mass of the binding protein is 41 kDa). The specificity of this binding is demonstrated by virtual absence of the binding in the presence of 100-fold excess of the unlabeled probe (Fig. 7.10b, lane 4). The polypeptide nature of this binding protein is confirmed by its sensitivity to proteinase K and heat treatment (Fig. 7.10b, lanes 5 and 6). Based on the fact that the binding protein interacts equally well with both native and denatured RNA probes (Fig. 7.10b, lanes 2 and 3), it is clear that the proper secondary structure of RNA is not an absolute prerequisite for this interaction to occur. The fact that the RNA-protein complex was resistant to digestion by various types of RNases, whereas the naked RNA was totally destroyed on treatment by the same RNases, clearly shows that the binding protein specifically protects the RNA from degradation (Fig. 7.11). UV cross-linking experiments revealed a molecular mass of the binding protein to be around 41 kDa (Fig. 7.12). The fact that the 3'-UTR of ST8Sia1, another related sialyltransferase, failed to show any interaction with this binding protein (Fig. 7.16) strongly supports our finding this binding protein is highly specific for interaction with the ST6Gal1 3'-UTR.

Based on the fact that the secondary structure of ST6Gal1 3'-UTR is not critical for this specific protein-ST6Gal1 interaction (Fig. 7.10b, lanes 2 and 3), it is logical that this protein should interact with the ST6Gal1 3'-UTR in a sequence specific manner. Our data shows that the RNA probe that covers the 304 bp immediate downstream from the stop code of ST6Gal1 3'-UTR shows tight binding to this binding protein (Fig. 7.13). Surprisingly, all four RNA probes (5-1, 5-2, 5-3, and 5-4) of 80-bp length spanning the entire 304-bp probe showed equal binding intensity to the binding protein (Fig. 7.14a). Sequence alignment analysis of these four probes revealed that the corresponding cDNA sequences for the four 80-bp RNA probes share 13-bp consensus sequences (Fig. 7.14b). Further analysis confirmed that this consensus sequence is not present in other areas of the ST6Gal1 3'-UTR and coding region. The fact that only the 13-bp consensus sequence but not the remaining nonconsensus sequence on the 80-bp region showed binding activity (Fig. 7.15a) clearly establishes that this 13-bp consensus sequence serves as the specific binding site for this protein. Mutagenesis analysis conclusively proved that the conserved nucleotides AG and TC are critical for the RNA-protein interaction (Fig. 7.15b). Thus, a mutation of even a single nucleotide seems to affect the binding.

Although the identity of the 41-kDa protein is yet to be determined, its specificity and high-affinity interaction with the narrow UTR region of ST6Gal1 mRNA clearly indicates its critical role in regulating the ST6Gal1 mRNA metabolism as evidenced by the influence of chronic ethanol exposure that leads to its destabilization (Ghosh and Lakshman 1997; Rao and Lakshman 1997, 1999). The formation of the RNA-protein complex progressively decreased with increasing dietary ethanol concentration leading to its virtual disappearance in the livers of rats fed with 36% of the total calories as ethanol (Fig. 7.17a, b). A parallel 45% ( $p < 0.05$ ) decrease in plasma SIJ in the 36% ethanol group compared with the control group fully confirms our concept that chronic ethanol feeding destabilizes ST6Gal1 mRNA level by decreasing

the amount of this specific binding protein that interacts with the 3'-UTR of ST6Gal1 mRNA and leads to the generation of asialoconjugates in the blood of alcoholics.

Our present study demonstrates for the first time another unique interaction of a liver cytosolic-specific binding protein with the 3'-UTR region of ST6Gal1 mRNA that protects it from degradation in normal rat liver. We further show that chronic ethanol exposure down-regulates this mRNA by depleting this specific binding protein leading to ethanol-mediated destabilization of ST6Gal1 mRNA. It is significant to point out that the amount of ethanol consumption, when it is fed at 36% of the total dietary calories, amounts to 12–14 g/kg body weight per day, a value that is comparable with ethanol drinking by human heavy alcoholics (>100 g/day that is equivalent to  $\geq 6$  drinks/day). Thus, this specific defect caused by chronic ethanol exposure in alcoholics is the most likely cause for the blood appearance of asialoconjugates such as carbohydrate-deficient transferrin (Stibler et al. 1987), sialic acid-deficient apolipoprotein J (Ghosh et al. 2001), and asialo- $\alpha 1$ -acid glycoprotein (Tsutsumi et al. 1994) that serve as excellent biomarkers for chronic alcohol consumption.

It is significant to point out that our data are consistent with the existing concept that dietary  $\omega 3$ -fatty acids in low amounts (2.8% of the total dietary calories) may reduce liver injury by increasing antioxidants such as GSH peroxidase and catalase (Nanji et al. 1995), whereas in high amounts (13.8% of the total dietary calories)  $\omega 3$ -fatty acids seem to be detrimental as evidenced by marked increase in serum ALT in chronic alcohol-fed high  $\omega 3$ -fatty acids group (Fig. 7.22). This was further confirmed by enormous increase in liver lipid score in the chronic alcohol-fed high  $\omega 3$ -fatty acids unlike the corresponding low  $\omega 3$ -fatty acids group. These results are in agreement with the previous studies using intragastric alcohol feeding rat model (French et al. 1993; Nanji et al. 1995; Tsukamoto et al. 1995). The ability of betaine in chronic alcohol-fed low and high  $\omega 3$ -fatty acids diets significantly ameliorated the increased serum ALT level strongly suggests that betaine prevents liver injury induced by ethanol (Fig. 7.22). This is further confirmed by our finding that betaine prevents liver steatosis as evidenced by significant decrease in liver lipid score in betaine supplemented chronic alcohol-fed low and high  $\omega 3$ -fatty acids groups (Fig. 7.23a, b). Presumably, the action of betaine may be mediated via increased generation of liver S-adenosylmethionine (SAM) level (Barak et al. 1997) that is required to methylate phosphatidylethanolamine to phosphatidylcholine, which may restore the impaired VLDL synthesis and secretion (Purohit et al. 2007).

Consistent with numerous alcohol feeding studies, the present study confirms that alcohol-fed animals do not seem to gain as much weight as the control animals in spite of pair-feeding lead to increased hepatosomatic index. Strikingly, betaine feeding seems to further reduce the gain in body weight that results in greater increase in the Hepatosomatic index (Table 7.1). It must be pointed out that, apart from the liver gross appearance liver histopathology (Fig. 7.23) as well as ALT (Fig. 7.22) data clearly point out that the hepatosteatorosis and liver injury caused by chronic ethanol are markedly alleviated by betaine feeding in both low and high  $\omega 3$ -fatty acid fed animals.

With respect to chronic alcohol and Atherogenesis, our present study clearly shows that feeding high  $\omega$ -3 PUFA diet for 8 weeks in rats significantly down-regulates not only the hepatic PON1 gene expression (Fig. 7.18), but also serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities. Reduced PON1 activity in high  $\omega$ -3 PUFA versus low  $\omega$ -3 PUFA suggests that fish oil has a dose effect and implicates its susceptibility to increased ROS production. These results are consistent with a previous study showing decreased PON1 activity in fish oil-fed rats compared to the rat groups fed separately with triolein and tripalmitin (Kudchodkar et al. 2000). Significantly, chronic ethanol-mediated further decreases in liver PON1 mRNA expression (Fig. 7.18) and serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities in high  $\omega$ -3 PUFA fed animals imply that chronic ethanol-induced cytochrome P450 2E1 (CYP2E1) exacerbates the accelerated generation of ROS in the presence of high  $\omega$ -3 PUFA that may be responsible for these deleterious effects on PON1 status. Chronic heavy ethanol is known to induce CYP2E1 that mediates the generation of ROS during ethanol oxidation (Marí and Cederbaum 2000; Morimoto et al. 1994). The greater decreases in serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities relative to modest corresponding changes in hepatic PON1 mRNA levels (Fig. 7.18) caused by chronic ethanol further confirm the susceptibility of PON1/HCTLase enzymes to oxidative stress prevailing in ethanol and high  $\omega$ 3-fatty acid-fed conditions (Figs. 7.19 and 7.20).

Based on our present study as well as on the current literature in this area, we have summarized in Fig. 7.16, our suggested hypotheses on the possible mechanism/s of action/s of  $\omega$ 3-fatty acids and betaine in ameliorating chronic alcohol-mediated alterations in PON1 and HCTLase activity in relation to ROS and GSH status. Accordingly, in addition to ROS, homocysteine is a well known factor involved in vascular disease (Griendling et al. 2000; McCully 1993). High fish oil and alcohol have been reported to increase plasma homocysteine by 20% (Piolot et al. 2003) and by 62% (Stickel et al. 2000), respectively, in rats suggesting that they may lead to earlier cardiovascular and liver abnormalities by synergistically increasing tissue homocysteine level. Excess of homocysteine, in turn, is likely to increase S-adenosylhomocysteine (SAH) levels, leading to decreased SAM:SAH ratios that trigger the endoplasmic reticulum stress response and associated liver cell apoptosis (Esfandiari et al. 2005, 2007). Betaine can convert homocysteine to methionine by transmethylation and homocysteine to cysteine by transsulfuration, and thereby can reduce the levels of toxic homocysteine in the blood. Our study fully supports this concept since betaine significantly increased both PON1 and HCTLase activities. To our knowledge, this is the first time high  $\omega$ -3 PUFA as well as chronic heavy ethanol have been shown to markedly affect serum HCTLase activity (Fig. 7.20). These findings could explain why heavy alcoholics, especially when they are also on high  $\omega$ -3 PUFA diet are susceptible to cardiovascular diseases.

Further, our findings of a significant decrease in endogenous liver GSH in chronic ethanol-fed high  $\omega$ -3 PUFA group (Fig. 7.21) support the critical role of GSH in the regulation of PON1/HCTLase activities (Figs. 7.20 and 7.21). The ability of betaine to restore the decreased level of liver GSH found in chronic ethanol-fed high  $\omega$ -3

PUFA group with concomitant restoration of serum PON1/HCTLase activities strongly suggests that the protective role of betaine may be mediated via increases in thiol group containing superoxide dismutase and GSH both of which help in quenching free radicals (Balkan et al. 2005). Important mechanism of betaine is to activate cystathionine pathway to synthesize cysteine and glycine that are precursors for the synthesis of GSH. Liver is the major organ to synthesize GSH and export to other organs to quench free radicals generated by excessive alcohol metabolism. Further work is necessary to delineate the molecular mechanism of action of betaine on PON1 regulation.

## 7.4 Conclusions

We conclude that chronic alcohol-mediated down-regulation of hepatic ST6Gal1 gene leads to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective VLDL assembly and intracellular lipid and lipoprotein transport, which in turn is responsible for alcoholic hepatosteatosis and ALD. The mechanism of ethanol action involves the depletion of a unique RNA binding protein that specifically interacts with its 3'-UTR region of ST6Gal1 mRNA resulting in its destabilization and consequent appearance of asialoconjugates as alcohol biomarkers. With respect to ETOH effects on CVD, we conclude that CYP2E1 and ETOH mediated oxidative stress significantly down regulates not only the hepatic PON1 gene expression, but also serum PON1 and HCTLase activities accompanied by depletion of hepatic GSH, the endogenous antioxidant. These results strongly implicate the susceptibility of PON1 to increased ROS production. In contrast, betaine seems to be both hepatoprotective and atheroprotective by reducing hepatosteatosis and restoring not only liver GSH that quenches free radicals, but also the antiatherogenic PON1 gene expression and activity.

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