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Chronic ethanol consumption upregulates the cytosolic and plasma membrane sialidase genes, but downregulates lysosomal membrane sialidase gene in rat liver

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Abstract

We have previously shown that chronic ethanol feeding stimulates liver cytosolic sialidase (CS) and plasma membrane sialidase (PMS), whereas it decreases lysosomal membrane sialidase (LMS) activities with concomitant alterations in their relative synthetic rate in rat. To understand the molecular mechanism(s) for these changes, we have evaluated the effect of ethanol administration in male Wistar rats as a function of increasing dietary ethanol concentration after 8 weeks of pair-feeding on (i) the expression of CS, PMS, and LMS genes by real-time quantitative polymerase chain reaction method; (ii) their relative transcription rates by nuclear run-on assay; and (iii) the actual amount of these sialidase proteins in the liver fractions of the respective groups by Western blot method. We have demonstrated that the animals fed with 10.6%, 20.8%, and 36% of total calories as ethanol showed a 20% ($P < .05$), 34% ($P < .01$), and 69% ($P < .01$) increase in CS mRNA level, and 22% ($P < .05$), 26% ($P < .01$), and 47% ($P < .01$) increase in PMS mRNA level, but a decrease in LMS mRNA level by 35% ($P < .05$), 50% ($P < .01$), and 80% ($P < .01$), respectively, as compared to controls. Western blot analyses of CS, PMS, and LMS in the liver subfractions showed that changes in protein levels of CS, LMS, and PMS were consistent with the corresponding changes in the respective mRNA levels. Thus, the upregulation of CS and PMS, but not LMS which is downregulated by chronic ethanol, may account for the appearance of asialoconjugates in alcoholics.

1. Introduction

Sialic acids and their derivatives are ubiquitous at the terminal positions of oligosaccharides of glycoproteins and glycolipids in mammalian cells, and their roles have been implicated in various biological phenomena, such as cell proliferation, differentiation, signal transduction, and cell surface interactions [1-5]. Decreased sialylation is one of the characteristic features of alcohol consumption. Chronic ethanol exposure leads to an increase in sialic acid–deficient glycoconjugates, such as carbohydrate-deficient transferrin [6], sialic acid–deficient gangliosides [7], and free sialic acid [8]. The appearance of carbohydrate-deficient transferrin (CDT) in the plasma of chronic alcohol consumers has been established as a viable marker of chronic alcohol consumption [9,10]. Hepatic sialylations of transferrin, an *N*-glycosylated protein and apolipoprotein E, an *O*-glycosylated protein, are markedly impaired in ethanol-fed rats as compared to the pair-fed controls [11-13].

To define the significance and molecular mechanisms of aberrant sialylation in alcoholics, we have focused attention on sialidases and sialyltransferases [12], two of the key enzymes involved in the metabolism of glycoproteins and glycolipids. Sialyltransferases are glycosyltransferases which catalyze the transfer of sialic acid to the nonreducing terminal positions on the carbohydrate groups of glycoproteins and glycolipids in the Golgi compartment [14]. Our studies have shown that chronic ethanol administration decreases the hepatic activity of β-galactoside-α2,6-sialyltransferase (ST6Gal1) in the rat liver via the downregulation of the ST6Gal1 gene [15], which could be the reason for impaired sialylation.

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Sialidase (EC 3.2.1.18) is a key enzyme in catabolism of glycoproteins and glycolipids. The removal of sialic acid from glycoproteins and glycolipids by sialidase greatly influences glycoconjugates molecules, leading to alteration of various cellular functions [1,3]. Mammalian sialidases have been demonstrated to differ from microbial sialidases in various aspects, especially in the presence of multiple forms, even within a single cell, and in their strict substrate specificity. There are 4 types of mammalian sialidases differing in subcellular location as well as in catalytic properties, including substrate specificity: lysosomal membrane sialidase (LMS) [16], cytosolic sialidase (CS) [17], plasma membrane sialidase (PMS) [16], and intralysosomal sialidase [18]. Lysosomal sialidase (*Neu 1*) has a catabolic role in desialylating glycoproteins and glycolipids in lysosomes [19]. Cytosolic sialidase (*Neu 2*) can hydrolyze a wider range of substrates including glycoproteins and gangliosides [20]. Plasma membrane sialidase (*Neu 3*) localizes on the cell surface [21,22] and, by preferentially desialylating gangliosides, may play a major role in various developmental processes. On the other hand, the intralysosomal sialidase hydrolyzes only oligosaccharide substrates and synthetic substrates [18,24] and thus may not have any physiological role in desiaylation of gangliosides [24]. Our earlier studies have shown that there is an increase in sialidase activity in alcoholic condition in the liver [12] and in all compartments of blood including serum, leukocytes, and erythrocytes [23].

In our ongoing studies, we have shown that chronic ethanol feeding stimulates CS and PMS, whereas it decreases LMS activities with concomitant alterations in their relative synthetic rate [24] in rat liver. In this present work, to understand whether these alterations in CS, PMS, and LMS are due to corresponding upregulation or downregulation of their mRNAs, we have evaluated the effect of ethanol on hepatic expression of these 3 sialidase genes by real-time quantitative polymerase chain reaction (PCR) method and also the relative transcription rate by nuclear run-on assay. Finally, to confirm whether the alterations in these gene expressions reflected in the corresponding level of their gene products, the actual amount of these sialidase proteins in the livers of the respective groups was determined by Western blot method. The present study provides evidence that chronic ethanol–mediated alterations in sialidase gene expression could be one of the reasons for the increase in asialoconjugates in alcoholics.

2. Materials and methods

2.1. Chemicals

HEPES, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), EDTA, dithiothreitol, creatine phosphate, creatine phosphokinase, heparin, proteinase K, sodium dodecyl sulfate (SDS), and glycerol were procured from Sigma Chemicals (St Louis, Mo). Adenosine triphosphate, cytidine triphosphate, guanidine triphosphate, ribonuclease inhibitor, and DNase I were purchased from Invitrogen Corporation (Carlsbad, Calif), whereas the yeast tRNA was obtained from Ambion (Austin, Tex). ^{32}P-UTP was procured from Amersham Biosciences (Piscataway, NJ). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male Wistar rats initially weighing 125 to 135 g were procured from Charles River Laboratories (Wilmington, Mass). The rats were placed in plastic cages. The rats were housed in a thermostatically controlled room (28°C) under 12-hour dark/light cycle within a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). All experimental protocols carried out on the animals were approved by the Institutional Animal Care and Use Committee of the George Washington University and that of Veterans Affairs Medical Center (Washington, DC), and followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. After 1 week of acclimatization, the rats were divided into groups and fed the respective dietary regimen as described below.

2.3. Chronic ethanol feeding

Rats were fed a nutritionally adequate ethanol-containing liquid diet for 8 weeks, essentially formulated according to the modified method of Lieber and DeCarli [25]. It contained fat (40% of total calories), protein (20%), ethanol (10.8%-36% of total calories), and the rest from carbohydrates. Ethanol feeding was maintained at 10.8%, 21.6%, or 36% of total dietary calories in the various groups (6 rats per group) for 8 weeks. The control rats were fed the same liquid diet, except that ethanol was replaced by dextrin-maltose and strictly pair-fed the same amount of liquid diet as taken by ethanol-fed rats receiving 36% of total dietary calories from ethanol. To confirm continuing growth of the rats, the body weights of the animals were measured every week. At the end of the feeding period, each rat was killed by aortic exsanguination under pentobarbital anesthesia (50 mg/kg IP). The liver was immediately removed and weighed. A portion of the liver (100 mg) was taken and used for isolation of nuclei. The remaining liver was used immediately and/or quickly frozen in liquid N_2 and stored at 80°C until used for the isolation of RNA.

2.4. RNA isolation

The total RNA was isolated from rat liver tissues from all groups of animals using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) following the manufacturer's instructions. Adequate measures were undertaken to ensure high quality of RNA extracted in our samples. Briefly, 0.1 g of liver tissue was homogenized in 1 mL of Tri-Reagent.

Samples were left for 5 minutes at room temperature followed by addition of 0.2 ml of bromochloropropane (Molecular Research Center) and shaken vigorously for a few seconds and again left at room temperature for 15 minutes. After centrifugation (12 000 × *g* for 20 minutes) at 4°C, the upper aqueous phase was carefully pipetted out into a sterile tube. The RNA was precipitated by addition of 0.5 ml of isopropanol and incubated at room temperature for 5 to 10 minutes. RNA was pelleted by centrifuging again at 12 000 × *g* at 4°C for 15 minutes. The precipitated RNA was washed in 70% ethanol, briefly air-dried, and then solubilized in Formazol (Molecular Research Center). Total RNA concentrations were measured by absorbance reading at 260 nm using Spectromax 190 (Molecular Devices, Sunnyvale, Calif). The purity of total RNA samples was examined by determining the A_{260}/A_{280} ratio. The ratio was found to be ~2.0 for all the samples. All the samples were further analyzed using agarose gel electrophoresis following standard protocols [26,27]. Isolated RNA was used immediately or stored at −80°C until use.

2.5. Real-time quantitative PCR

Five micrograms of total RNA extracted from each liver was used in 20 μL reverse transcription reaction containing 10 pmol/L Oligo (dT), 10 μmol/L dNTPs, and 1 U of reverse transcriptase. Quantitative real-time PCR was done on an iCycler (Bio-Rad Laboratories, Hercules, Calif) using SYBR green detection system. The amplification of CS, PMS, LMS, and *β*-actin cDNA was run in separate tubes. Using the Competimer techniques (Ambion) for *β*-actin, the amplification efficiencies for CS, PMS, LMS, and *β*-actin were optimized to be approximately equal. Typical PCR reaction mixture included 2 μL of cDNA templates from RT, 10 pmol/L of each primer, SYBR-Green Supermix (2×) (Bio-Rad Laboratories) in a reaction volume of 50 μL. The PCR reaction mixture was subjected to the following cycles: 1× (2 minutes at 94°C) followed by 35× (94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 3 minutes). Gene-specific primers for the 3 sialidases (Operon Biotechnologies Germantown, Md) were designed by using the Omiga 2.0 software (Genetic Computer Group, Madison, Wis) according to the published National Center for Biotechnology Information accession numbers. The primer pairs used were as follows:

(i) For CS (accession number: NM_017130): forward primer: 5′-CCGTCCAGGACCTCACAGAG-3′, and reverse primer: 5′ -TCACTGAGCACCATGTACTG-3′,
(ii) For PMS (accession number: NM_054010): forward primer: 5′-CCTACTTGATATCATGCTGG-3′, and reverse primer: 5′-TTAGTTGCTACTAGGGCTGG-3′, and
(iii) For LMS (accession number: NM_031522): forward primer: 5′-TTGGAGTAAGGATGACG-GCC-3′, and reverse primer: 5′-TCAAAGCGTGC-CGTAGACGC-3′.

The primer pair for *β*-actin was purchased from Ambion. Polymerase chain reaction products were directly monitored by measuring the increase of fluorescence due to the binding of SYBR green to double-stranded DNA. Relative mRNA expression was determined by the cycle threshold (ΔC_t) method [28]. The C_t is the cycle at which the PCR product crosses the detection threshold, usually at mid-log stage of PCR amplification. Ratios of target gene and *β*-actin (relative gene expression numbers) were calculated by subtracting the C_t of *β*-actin from the C_t of the target gene and raising 2 to the power of this difference. Target gene mRNA expressions are thus expressed relative to *β*-actin expression.

2.6. Nuclear run-on assay

The nuclei from control rats and rats fed with 36% of dietary calories from ethanol were prepared as previously described [29]. Transcription was performed as described [29] in a final volume of 200 μL containing 50 mmol/L HEPES, pH 7.5, 50 mmol/L NaCl, 2.2 mmol/L $MgCl_2$, 0.05 mmol/L EDTA, 5 mmol/L dithiothreitol, 1.0 mmol/L adenosine triphosphate, 1.0 mmol/L cytidine triphosphate, 1.0 mmol/L guanidine triphosphate, 100 μCi [α-^{32}P]UTP, 20 U of ribonuclease inhibitor, 2 mmol/L creatine phosphate, 2 μg of creatine phosphokinase, 20 μg of heparin, 25% glycerol, and nuclei equivalent to 200 μg of DNA from each liver. After incubating for 30 minutes at 30°C, the reaction mixture was treated with 50 U of DNase I for 10 minutes, followed by digestion with 150 μg/ml proteinase K and 0.5% SDS for 30 minutes at 37°C. After the incubation, 10 μg of yeast tRNA was added as a carrier and the labeled nuclear RNA was extracted by Tri-reagent as described above. The final pellet was dissolved in 30 μL of 10 mmol/L Tris/HCl, pH 8.0, 1 mmol/L EDTA. cDNA templates of CS, PMS, and LMS for use in nuclear run-on assay were amplified by PCR using specific primers mentioned above. Glyceraldehyde-3-dehydrogenase (GAPDH) cDNA template for use in nuclear run-on assay was amplified using the following primers: forward primer: 5′-AAACCCATCACCATCTTCCA-3′, and reverse primer: 5′-CCACAGTCTTCTGAGTGGCA-3′. Each specific amplified cDNA was purified from gel, denatured, and immobilized on nitrocellulose filter paper strips as described by Sambrook et al [27]. Hybridization of the labeled RNA to immobilized specific sialidases and GAPDH cDNA was performed according to the standard procedures [26,27]. In densitometric analysis, CS, PMS, and LMS transcription rate are represented after normalizing it to the GAPDH transcription rate.

2.7. Western blot analysis

The liver lysosomal, plasma membrane, and cytosolic subcellular fractions were prepared as described previously [30,31]. Each specific liver subcellular fraction was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting to polyvinylidene

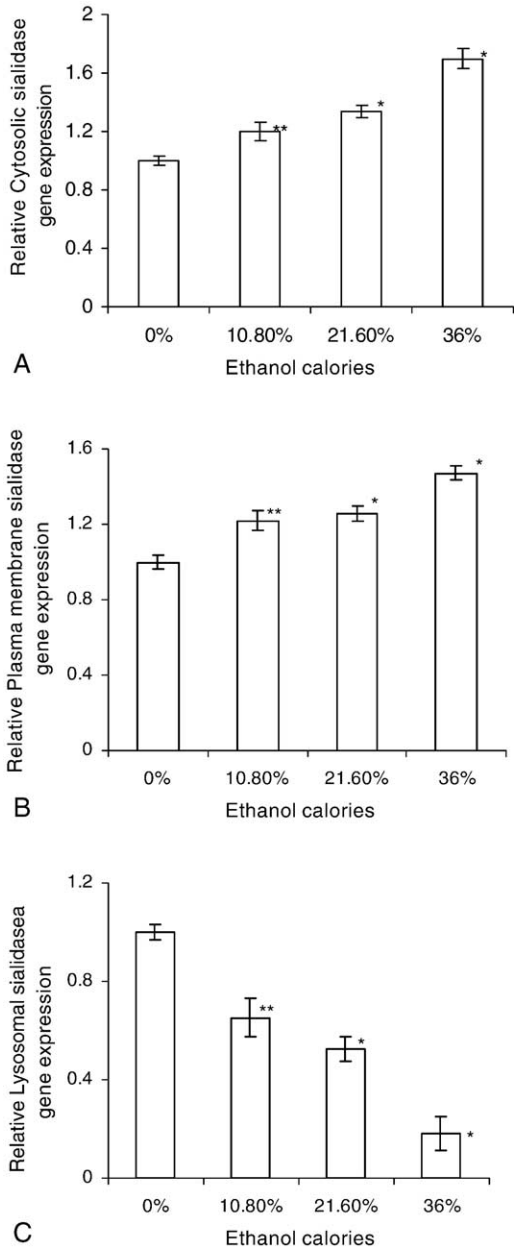

Fig. 1. A-C, Effect of ethanol feeding on expression of CS (A), PMS (B), and LMS (C) mRNA levels in the rat liver. Total RNA was isolated from the livers of rats maintained on 0%, 10.8%, 21.6%, or 36% dietary ethanol calories for 8 weeks as described under Materials and methods. Real-time PCR analysis was used to assess mRNA expression in the liver homogenates among the group. Results are expressed as mean ± SE for 6 animals per group. The *P* values of statistical significance in the ethanol groups compared to the corresponding control groups were at **P* < .01 and ***P* < .05 levels.

difluoride membrane and finally analyzed by Western blot technique using the corresponding specific sialidase antiserum (generous gift of Prof Taeko Miyagi, Miyagi Prefectural Cancer Center, Miyagi, Japan) [16,32]. Protein samples corresponding to 14 μg were solubilized in 2× Laemmli buffer in the presence of 2-mercaptoethanol. The samples were boiled and cooled on ice before being used for Western blot analysis. The samples were separated on a 4% to 20% Tris-HCl Criterion Precast gel (Bio-Rad Laboratories) and subsequently transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories) by electroblotting. Nonspecific binding sites on the membranes were blocked overnight at 4°C with Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 5% dried skimmed milk. The membranes were then incubated overnight at 4°C with respective antisialidase serum (1:5000) diluted in TTBS–5% dried skimmed milk. After washing with TTBS, the membrane was further incubated for 1 hour at room temperature with the secondary antibody of rabbit anti-IgG peroxidase–conjugated polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif; 1:1000 dilution in TTBS–5% dried skimmed milk). After washing the membrane with TTBS and then Tris-buffered saline, the respective sialidase protein bands were visualized by the enhanced chemiluminescence detection technique using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life and Analytical Sciences, Boston, Mass) as described by the manufacturer. The membrane was stripped using the Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, Ill), and GAPDH protein was detected with rabbit anti-GAPDH polyclonal antibody (1:1000; Santa Cruz Biotechnology) and rabbit anti-IgG peroxidase–conjugated polyclonal antibody (1:1000 dilution in TTBS–5% dried skimmed milk). After development, the membrane was scanned and analyzed using the FluorChem 8800 (Alpha Innotech, Leandro, Calif). In densitometric analysis, CS, PMS, and LMS protein levels are represented as a ratio relative to that of the GAPDH level.

2.8. Protein assays

The protein concentrations of the various fractions were determined according to the Bradford method [33] using bovine serum albumin as a standard.

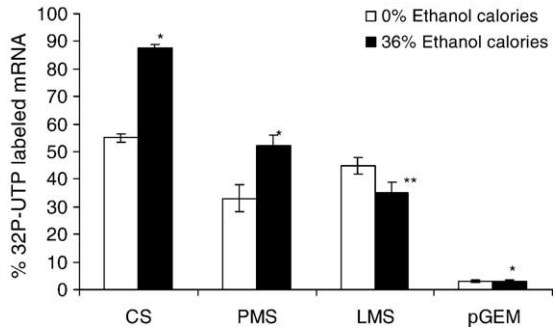

Fig. 2. Effect of chronic ethanol feeding on the nuclear transcription rate of CS, PMS, and LMS mRNA. Densitometric analyses of transcription rate of CS, PMS, and LMS are represented after being normalized with transcription rate of GAPDH and are plotted as % [^{32}P]UTP-labeled mRNA. The *P* values of statistical significance in the ethanol groups compared to the corresponding control groups are at **P* < .01 and ***P* < .05 levels.

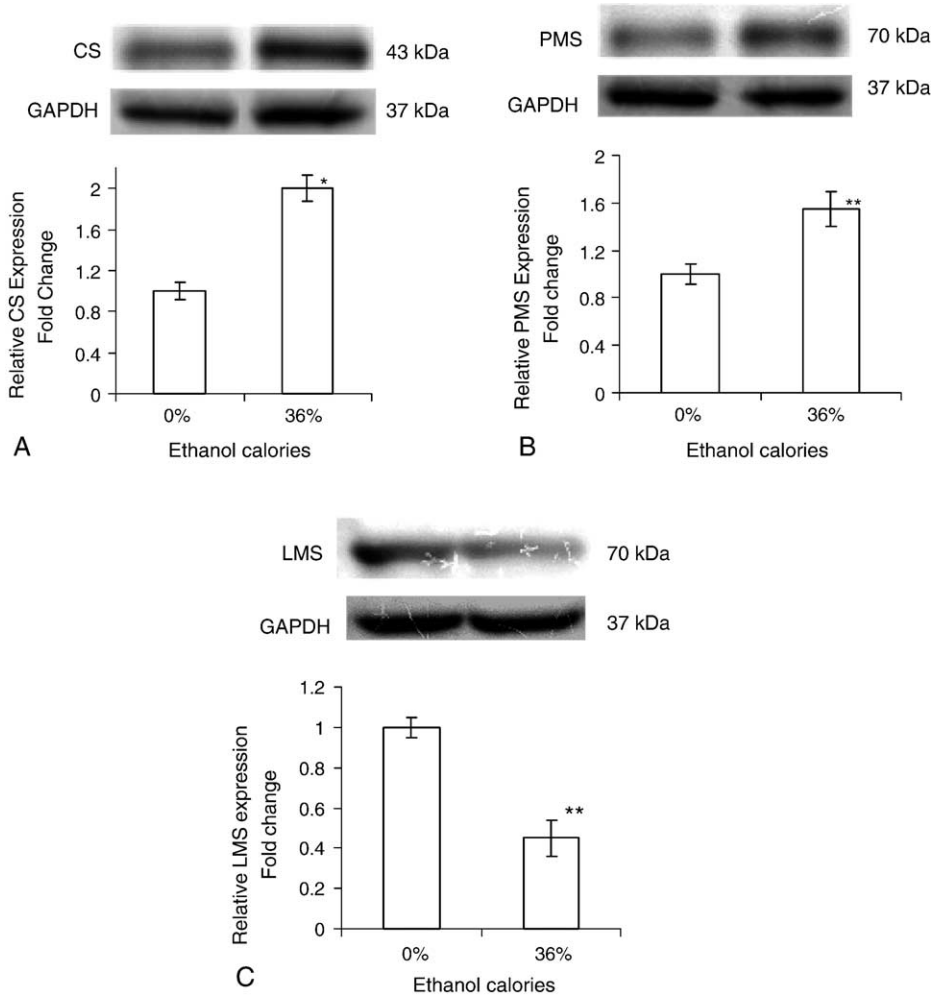

Fig. 3. A-C, Effect of chronic ethanol feeding on of CS, PMS, and LMS proteins by Western blot analysis. The various liver subcellular fractions (cytosol, plasma membrane, and lysosomal membrane) were prepared from rats on 0% (CN) or 36% dietary ethanol calories for 8 weeks. An aliquot of each fraction was subjected to Western blot analysis with the corresponding specific antisialidase antibody as described in Materials and methods. Western blot and densitometric analyses of CS (A), PMS (B), and LMS protein (C) were performed. The P values of statistical significance in the ethanol groups compared to the corresponding control groups are at $^{*}P < .01$ and $^{**}P < .05$ levels.

2.9. Statistical analysis

The data are expressed as mean ± SE. All groups within each data set were compared by 1-way analysis of variance test followed by Tukey test.

3. Results

3.1. Effect of alcohol on rat liver CS, PMS, and LMS mRNA

Fig. 1A to C shows that there was a dose-dependent increase in the mRNA level of CS and PMS with increase in dietary ethanol. In contrast, the mRNA level of LMS decreased with the increase in ethanol concentration. Animals fed with 10.6%, 20.8%, and 36% of total calories as ethanol showed a 20% ($P < .05$), 34% ($P < .01$), and 69% ($P < .01$) increase in CS (Fig. 1A), and 22% ($P < .05$), 26% ($P < .01$), and 47% ($P < .01$) increase in PMS (Fig. 1B) mRNA level, but a decrease in mRNA level of LMS (Fig. 1C) by 35% ($P < .05$), 50% ($P < .01$), and 80% ($P < .01$), respectively, as compared to controls.

3.2. Transcription rate of CS, PMS, and LMS mRNA

Fig. 2 shows that feeding rats with 36% of their dietary calories as ethanol resulted in 59% ($P < .01$) and 55% ($P < .01$) increase in the rate of transcription of CS and PMS, respectively, but the rate of transcription for LMS decreased by 22% ($P < .05$) as compared to the control group. There was no hybridization of the nascent mRNA to a nonspecific sequence of DNA (pGEM-11f plasmid DNA).

3.3. Effect of ethanol on protein levels of rat liver CS, PMS, and LMS

The Western blot analysis of different subcellular fractions (plasma membrane, lysosomal, and cytosol) of the liver with the respective specific polyclonal sialidase antibodies showed a single specific polypeptide band at

70 kd for PMS and LMS, and a 43-kd band for CS. The molecular size of the protein bands corresponded well to the previously reported apparent molecular mass of the various sialidases [24]. The densitometric analysis of Western blots (Fig. 3A-C) showed a 2-fold ($P < .01$) and 1.5-fold ($P < .05$) increase in the relative level of CS (Fig. 3A) and PMS (Fig. 3B) proteins, respectively, but a 46% ($P < .05$) decrease in that of LMS (Fig. 3C) protein in the rats fed 36% of their dietary calories as ethanol as compared to control rats.

4. Discussion

Glycolsylation of proteins in the liver is one of the important functions that have many other ramifications, including secretory activities and also immunochemical interactions of liver proteins. The steady-state levels of these glycoproteins and glycolipids are determined by the relative actions of the glycosyltransferases and sialidases that are responsible for their synthesis and degradation, respectively. Our ongoing studies [24] have clearly shown that ethanol feeding at 36% dietary calories increased the hepatic activity of CS and PMS, but decreased LMS activity as compared to control animals. The relative synthetic rates of CS and PMS increased in a dose-dependent manner in rats fed with 10.8%, 21.6%, and 36% of their calories as ethanol as compared to controls, whereas the relative synthetic rate of LMS was inhibited in these groups [24].

In this study, we have demonstrated that, as compared to controls, liver mRNA levels of CS and PMS increased, whereas LMS mRNA decreased with increase in dietary ethanol calories in a concentration-dependent manner (Figs. 1 and 2). These changes are consistent with the corresponding activities of the respective sialidases and their relative synthetic rates as published by us earlier [24].

The changes in mRNA levels can be the consequence of alterations in transcription rates or of modulation of posttranscriptional events. Examination of the rate of transcription of CS, PMS, and LMS mRNA in control and ethanol-fed rats showed that ethanol increased the transcription rates of CS and PMS but decreased that of LMS (Fig. 2C). The relative changes in transcription rates of CS, PMS, and LMS were commensurate with their relative changes in mRNA levels. Significantly, the relative levels of the respective sialidases also exhibited corresponding changes (Fig. 3A-C).

Regulation of the expression of eukaryotic genes can occur at various steps during the formation of mature mRNA, including transcription, RNA processing in the nucleus, degradation of primary transcripts or processing intermediates, mRNA transport from the nucleus, and degradation of cytoplasmic mRNA. The regulation of the expression of a particular gene might involve more than one of these steps. Control at the level of transcription has been demonstrated for a variety of genes, including developmental genes [34,35], tissue-specific genes [36,37], hormonally controlled genes [38], and heat shock genes [39]. There is increasing evidence for posttranscriptional regulation [40-43]; for example, α_{2u}-globulin gene expression in rat liver by glucocorticoids has been shown to occur, at least in part, during the nucleocytoplasmic transport of mRNA [44]. Regulation by influencing the stability of mRNA has been shown in hepatocytes. For example, αCP2 increases collagen mRNA stability by binding to its 3′-UTR and thereby increases type 1 collagen protein levels [45]. Our results suggest that different sialidases can differ in their regulatory response in various ways under the influence of ethanol. For the proteins CS and PMS, an increase in transcription rate seems to be the major form of regulation. However, because the transcription rate of LMS decreased by only 22% (Fig. 2C), whereas its protein level decreased markedly by 46% (Fig. 3C), it seems reasonable to conclude that posttranscriptional control may also be involved in its regulation.

Based on these findings, it seems that LMS gene may not be physiologically important in chronic alcohol–mediated generation of various asialoconjugates compared to PMS and/or CS genes that we have shown to be upregulated by chronic alcohol exposure. Evidences in favor of the PMS playing a major role in desialylation are compelling. For example, in human neuroblastoma cells, 2 ganglioside-degrading sialidase activities were exhibited: one was PMS, whose specific activity increased drastically during cell proliferation, and an LMS activity that showed no such activity increase during cell growth [46,47], indicating that PMS plays the major role in various developmental processes. A similar analogy has been provided suggesting the significance of PMS in the catabolism of sialoconjugates [48]. Furthermore, a positive correlation between the PMS activity and the turnover rate of radiolabeled sialic acid compounds on the cell surface was observed with Rous sarcoma virus–transformed chick embryonic fibroblasts [49].

It is known that most sialic acids are associated with plasma membrane in eukaryotic cells [50], and thus, it is most likely that the increased presence of asialoconjugates [51,52] and free sialic acid observed after alcohol exposure [53] is promoted by the increased PMS activity. We do not yet know the functional significance of alcohol-induced upregulation of these sialidases, but this is potentially very relevant in understanding alcohol abuse and alcoholism as well as the pathological conditions observed in alcoholics.

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