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Further characterization of the impact of ethanol on βLH: alterations in polyribosome association of βLH mRNA

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We have previously reported a decrease in Luteinizing Hormone (LH) levels in serum after *in vivo* acute ethanol exposure in male rats. Accompanying these changes, a rapid and marked decrease of β-LH mRNA was observed. A similar decrease was not detected in the common α-subunit or β-FSH mRNA. The studies presented here examined the possible mechanisms of decreasing β-LH mRNA by using S1 nuclease protection assay to evaluate the effect of acute ethanol exposure on the levels of β-LH heteronuclear RNA (hnRNA). There was no significant difference detected in the level of β-LH hnRNA after ethanol exposure. Polysome distribution analysis was used to evaluate the association and disassociation of β-LH mRNA with polyribosomes since non-polyribosome associated mRNA may be more vulnerable to degradation by RNAases. The results indicated a decrease in the association of the β-LH mRNA with polysomes following acute ethanol exposure. This decrease in polyribosome association would increase the exposure of the β-LH transcript making it more susceptible to RNases. We conclude that the decrease in steady-state β-LH mRNA levels after ethanol exposure occurs because of increasing degradation of the transcript rendered vulnerable by displacement from polysomes and not through a decreased transcriptional rate.

Keywords: ethanol; LH; ribosomes; mRNA

Introduction

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are pituitary hormones necessary for the initiation and maintenance of reproductive processes (Chin *et al.*, 1983; Gharib *et al.*, 1990). They are glycoprotein hormones synthesized in the gonadotrope cells of the anterior pituitary. Each glycoprotein hormone is a heterodimer consisting of a unique β-subunit and a common α-subunit transcribed by different genes (Godine *et al.*, 1982; Chin *et al.*, 1983; Jameson *et al.*, 1984; Garib *et al.*, 1990). Acute and chronic ethanol exposure have been shown to result in depressed concentrations of circulating levels of gonadotropins (Cicero *et al.*, 1978, 1979; Eskay *et al.*, 1978; Chapin *et al.*, 1980; Redmond, 1980; Cicero, 1982; Boyden & Pamenter, 1983; VanTheil, 1983; Mateos *et al.*, 1987; Chung *et al.*, 1988; Esquifino *et al.*, 1989; Widenius *et al.*, 1989; Salonen & Huhtaniemi, 1990; Emanuele *et al.*, 1991, 1992; Rivier *et al.*, 1992; Salonen *et al.*, 1992). Previous studies in our laboratory showed a decreased expression of steady-state β-LH mRNA after acute ethanol exposure to castrated male rats with a 50% suppression seen at 1.5 h and a 90% decrease at 3 h after exposure (Emanuele *et al.*, 1991). A similar suppression in steady-state β-FSH mRNA and common α-subunit mRNA levels was not seen. The present study examined the possible mechanisms of decreasing β-LH mRNA by exploring the effect of acute ethanol on levels of β-LH heteronuclear RNA (hnRNA) to indirectly determine ethanol's impact on transcription. To evaluate the possibility that acute ethanol exposure affects degradation of β-LH mRNA by altering the association of the β-LH mRNA with polyribosomes, polysome distribution analysis was employed.

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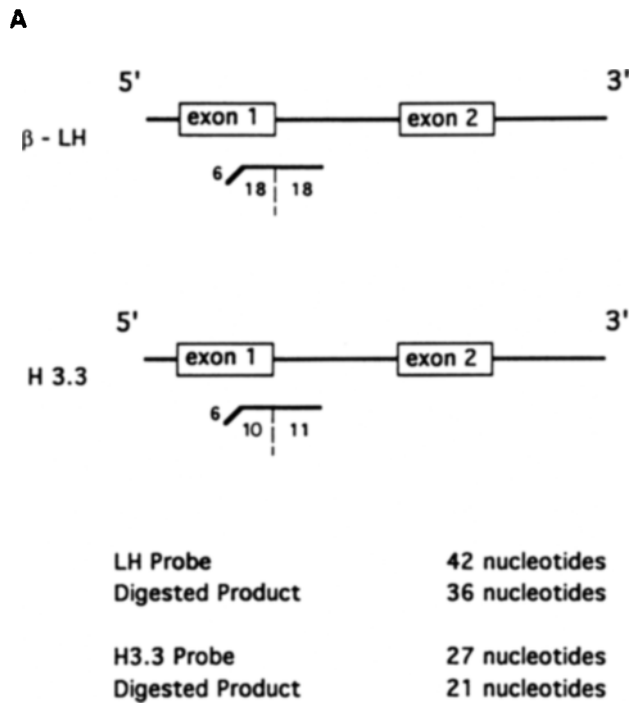
Results

S1 nuclease protection assays were performed to quantitate the levels of hnRNA for β-LH in order to determine if the transcription of the β-LH hnRNA was affected by acute ethanol exposure. Animals treated 3.0 h previously with ethanol were chosen since this was the time point where a 10-fold decrease in steady-state mRNA levels was previously seen (Emanuele *et al.*, 1991). An oligonucleotide for β-LH was designed to be complementary to 18 nucleotides of β-LH exon 1, 18 nucleotides of β-LH intron, leaving six nucleotides on the 3' end that were not complementary to exon 1 (Figure 1A). The oligonucleotides were designed to distinguish between undigested probe and the RNA:DNA hybrids created from hybridization and S1 nuclease treatment of pituitary RNA and probe. The product representing LH hnRNA, the RNA:DNA hybrids formed, were 36 nucleotides long and distinct from the 42 nucleotide undigested probe when electrophoresed on a 15%/8 M Urea polyacrylamide gel. A second oligonucleotide was designed to be complementary to H3.3. It also had six non-complementary nucleotides added to its 3' end. The undigested probe was 27 nucleotides long, while the product representing the RNA:DNA hybrids were 21 nucleotides long (Figure 1A). H3.3 was used in the reaction as a loading control for amount of RNA added to the reaction.

It was necessary to pool three pituitaries in order to detect β-LH hnRNA. Pituitaries from control and ethanol-treated animals 3 h post-injection were used for these studies. The serum ethanol levels were 58 mM for ethanol-treated and undetectable for control animals. There was no statistically significant difference detected by ANOVA in the level of β-LH hnRNA when comparing control and ethanol samples (Figure 1B). Amount of RNA per reaction was corrected for with H3.3 levels detected on the gel.

Polysome distribution analysis is a useful assay to assess the relationship of a particular mRNA species and polysomes versus monosomes (Kleene *et al.*, 1984; Murphy *et al.*, 1992). Association of mRNA with polysome implies the 'translatability' of mRNA. If a message is shifted from the polysomes to monosomes, the assumption exists that less message is available for translation. Also, the mRNA may be more accessable to attack by endogenous RNAses. To conduct the polysome distribution analysis, cytoplasmic extracts of anterior pituitary glands from control and ethanol-treated animals were fractionated through 10 to 40% (wt/vol) sucrose gradients. As in the S1 protection assays, animals treated 3.0 h previously with ethanol were chosen. The gradient was divided into 10 fractions, RNA was extracted from each gradient fraction and subjected to Northern blot analysis.



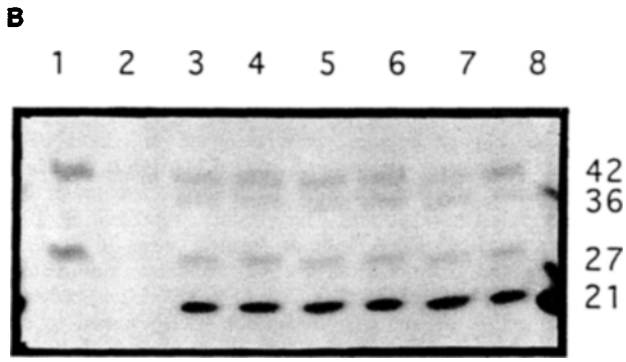


Figure 1 S1 nuclease protection assay of LH heteronuclear RNA (hnRNA). (**A**) Diagram of the exon-intron spanning oligonucleotides used in the S1 nuclease protection assay and sizes of expected products. (**B**) Autoradiograph of the S1 nuclease protection assay of LH heteronuclear RNA. The products at 42 and 27 represent undigested LH and H3.3 probes, respectively. The H3.3 RNA:DNA product ran at 21 and the βLH hnRNA RNA:DNA product ran at 36. Lane 1 is untreated probe, lane 2 is undigested probe alone (no RNA was added to the reaction). Lanes 3, 5 and 7 are reactions with control sample, and lanes 4, 6 and 8 are reactions with ethanol-treated samples (3 h post-injection)

In order to compare the fractions of control and ethanol treated samples, the absorbance at 254 nm was taken of each fraction (before precipitation for RNA isolation). Figure 2 shows that the fractions being compared were equivalent between control and ethanol treated animals. Results from Northern blots probed with β-LH cDNA show a shift from the heavy polysome fractions (9 and 10) to the lighter polysome fractions (6–8) for ethanol treated animals (Figure 3B) compared to control animals (Figure 3A). Data from the densitometric scan of these blots is shown in Figure 4A. In control animals, the heavy polysome fractions 9 and 10 contained 86% of total hybridized β-LH mRNA. Conversely, in ethanol treated rats, these fractions only contained 43% of the total mRNA. Most of the shift was to fraction 8 which increased three-fold from 13% in control to 43% of total in the ethanol treated animals. This localization of β-LH

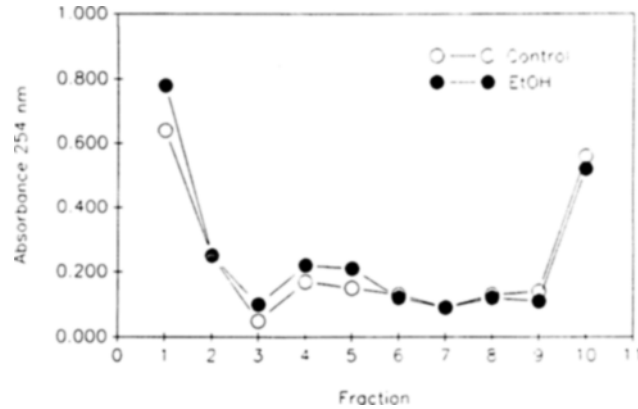


Figure 2 Analysis of fractions from the polysome distribution of RNA from control and ethanol treated animals 3.0 h after treatment. Optical density values on sucrose gradients of intact polysomes (HKM Buffer) for control and ethanol samples (3.0 h post treatment). Fraction 10 represents the bottom of the gradient and contains polysomes

mRNA to polysome fractions was due to specific ribosomal association since transcripts were released from polysomes in the presence of EDTA (Figure 3C and D).

The Northern blots for the intact polysomes and dissociated polysomes were stripped and reprobed with α-subunit cDNA and β-FSH cDNA. The densitometric scan of the blots are presented in Figure 4B and C. There was no shift in association of the α-subunit mRNA with polysomes when comparing the ethanol-treated and control animals. Similar results were seen when the blots were probed with β-FSH (Figure 4C).

To determine the time course of the shift in β-LH mRNA association with polysomes, polysome distribution analysis of β-LH mRNA was assessed 24 h after ethanol injection (Figure 5A and B). The results showed no change in the association of β-LH mRNA with polysomes between control and ethanol treated samples with the heavy polysome fractions (9 and 10) containing 64% and 72% of the total mRNA respectively. The polysome distribution analyses were repeated for both the 3.0 h and 24 h timepoints showing similar results to those presented above and corroborating the previous findings. Thus, this data was reproducible.

Discussion

Previous studies have shown that acute ethanol exposure has an effect on pituitary endocrine function. Following an acute ethanol exposure, there is decreased serum LH (Cicero *et al.*, 1978, 1979; Eskay *et al.*, 1978; Chapin *et al.*, 1980; Redmond, 1980; Cicero, 1982; Boyden & Pamenter, 1983; VanTheil, 1983; Mateos *et al.*, 1987; Chung *et al.*, 1988; Esquifino *et al.*, 1989; Widenius *et al.*, 1989; Salonen & Huhtaniemi, 1990; Emanuele *et al.*, 1991, 1992; Rivier *et al.*, 1992; Salonen *et al.*, 1992) and decreased steady-state β-LH mRNA levels (Emanuele *et al.*, 1991). The rapid and significant fall in β-LH mRNA suggests a decrease in β-LH mRNA synthesis and/or a decrease in β-LH mRNA stability.

To address the possibility of acute ethanol exposure decreasing β-LH mRNA synthesis, nuclear run-off assays were performed. However, the levels of β-LH mRNA were too low to detect and a comparison of control and ethanol-treated β-LH mRNA transcriptional rates could not be made (data not shown). In order to analyse the effects of ethanol on β-LH mRNA transcription, S1 nuclease protection assays were performed using a probe complementary to an intron-exon junction of β-LH mRNA. This probe was hybridized to newly transcribed mRNA which has not yet been processed,

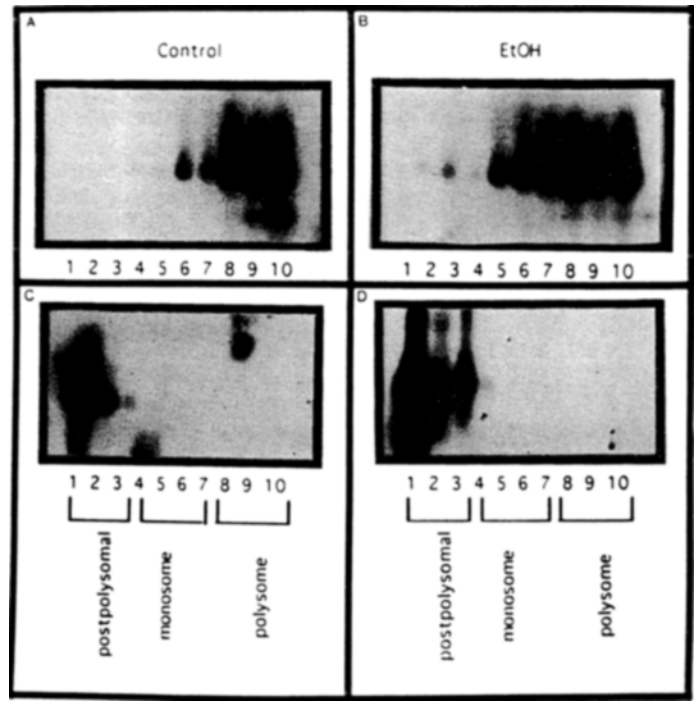


Figure 3 Polysome distribution of β-LH mRNA from control and ethanol treated rats 3.0 h after treatment. Sucrose gradients of RNA from cytoplasmic extracts of pituitairies from control and ethanol treated animals (3.0 h post treatment). Polysomes are intact in HKM buffer (**A**, **B**) and dissociated in HKE buffer (**C**, **D**). A much larger percentage of the β-LH message was noted in fractions 6–8 in the ethanol exposed pituitaries, corresponding to the monosomes where less efficient translation occurs

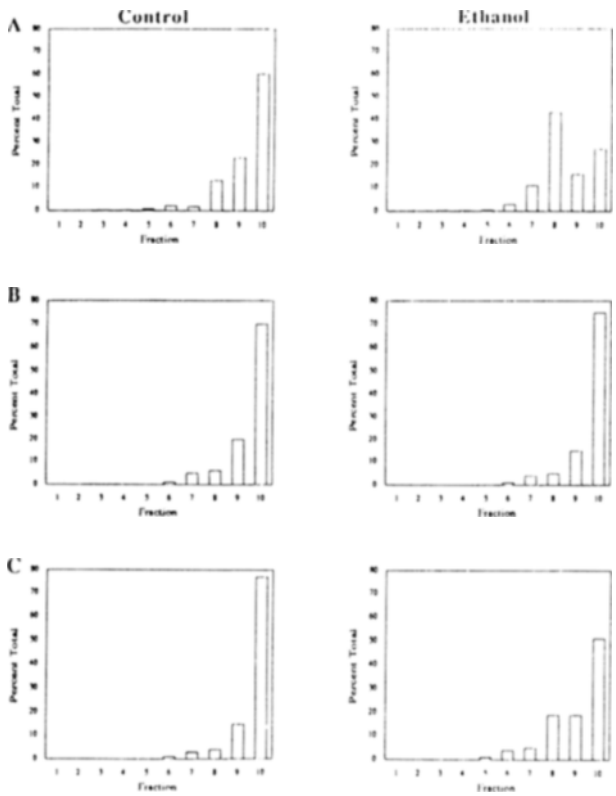


Figure 4 Polysome distribution of gonadotropin subunit mRNA from control and ethanol treated rats 3.0 h after treatment. Northern blots of intact polysomes were stripped and reprobed with cDNA for the common α subunit and β-FSH subunit. The autoradiographs were scanned and values for each fraction are percent total signal of all the fractions. (**A**) β-LH, (**B**) common α-subunit, (**C**) β-FSH. The ethanol-induced shift to the monosome fractions (6–8) in the β-LH mRNA is not demonstrated in the common α-subunit or β-FSH, where the majority of the message is in the polysome fractions (9 and 10)

hnRNA. This assay assumes equal splicing rates for control and ethanol-treated samples and has been successfully used in other similar studies (Chen *et al.*, 1987). Since such processing is very rapid, measurement of this hnRNA provides a good indirect index of the rate of transcription.

A comparison of the amount of newly transcribed RNA for control and ethanol-treated samples using S1 nuclease protection disclosed no significant difference. The samples used in this study were from animals injected with ethanol 3 h previously. This is the same time point when β-LH mRNA was decreased 8–10-fold (Emanuele *et al.*, 1991). This result does not completely exclude the possibility of β-LH mRNA transcriptional rate being altered by acute ethanol exposure. However, if the transcription of β-LH mRNA decreased, one would expect a decrease in the amount of hnRNA for β-LH in the ethanol-treated samples. This lack of suppression suggests that the sharp fall in steady-state level of β-LH mRNA induced by ethanol was due to degradation of the mature β-LH mRNA transcript.

The half-life of β-LH message is approximately 24 h (Carroll *et al.*, 1991) and a decrease in β-LH mRNA is observed 3.0 h after ethanol exposure. Therefore, the decreased steady-state level of β-LH mRNA must be due, at least in part, to an increase in mRNA degradation. Since the studies presented use an *in vivo* model, direct analysis of the half-life of β-LH mRNA in control and ethanol-treated samples is prohibitive. In order to evaluate the half-life of β-LH mRNA, transcriptional inhibitors, such as actinomycin D, are given to the animals and the amount of β-LH mRNA determined at various time points after treatment with the inhibitor. In *in vivo* studies, the above experiment is extremely difficult since it is a challenge to determine whether the effects on the mRNA of interest is a result of the ethanol exposure or the result of the inhibitor itself on the animal. As a result of these difficulties, an evaluation of the half-life of β-LH mRNA after ethanol exposure could not be determined. Nonetheless, given that there must be increased β-LH message degradation, we sought to determine by what mechanism this could occur. We hypothesized that one such

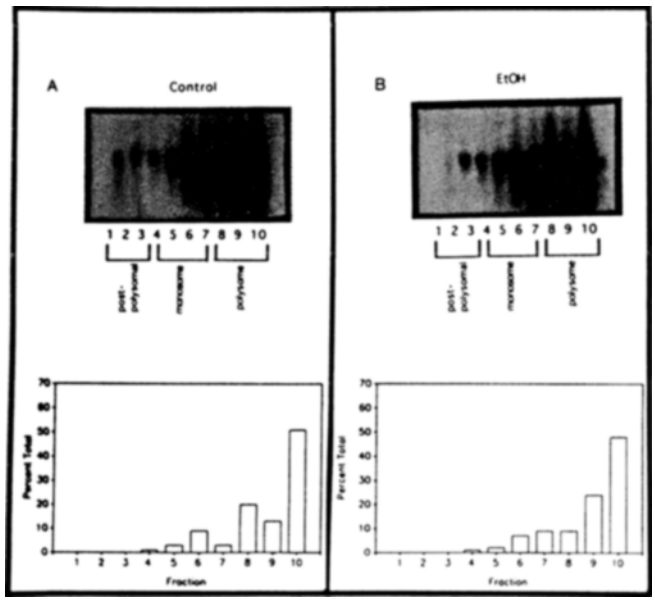


Figure 5 Polysome distribution of β-LH mRNA of control and ethanol treated rats 24 h after treatment. Northern blots probed with cDNA for β-LH (**A**, **B**). Autoradiographs were scanned and the values represented as percent total signal

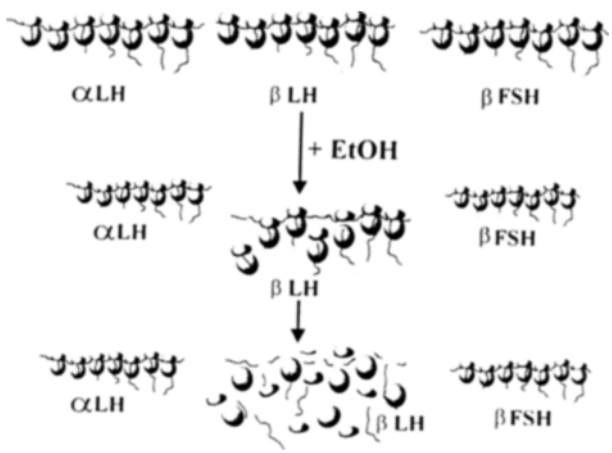


Figure 6 Model of ethanol's effect on β-LH association with polysomes. The data presented here and in our previous publication (Emanuele *et al.*, 1991) leads us to conclude that ethanol is affecting the β-LH message through degradation of the mRNA rather than through transcriptional inhibition. The shift of the β-LH mRNA from polysomes to monosomes could either be the result of, or related to, the degradation of the β-LH mRNA after ethanol exposure. The mechanism of this effect is still unknown, but it could be a result of some signaling mechanism and/or increased RNase activity or RNase access after ribosome rearrangement. However, the effect is specific for β-LH, in that the α-common subunit and β-FSH are not affected in this manner

mechanism might be a shift of β-LH mRNA off polyribosomes where they are relatively protected from degradation.

Messenger RNA association with polyribosomes was analysed using polysomal distribution analysis of β-LH mRNA. Polysomal distribution analysis enables one to detect actively translated messages by determining the amount of mRNA associated with the heavy polysome fractions in comparison to lighter polysome and monosome fractions (Kleene *et al.*, 1984; Murphy *et al.*, 1992). In this assay, sucrose gradient centrifugation was utilized to separate polysomes from monosomes. Control and ethanol samples were analysed and their polysome profiles were compared. A reduced percentage of total cytoplasmic β-LH mRNA was found to be associated with heavy polysomes from pituitaries examined 3 h after ethanol injection compared to saline controls. In saline treated rats, 86% of total hybridized β-LH mRNA was associated with efficiently translating heavy polysomes (fractions 9 and 10). In contrast, 3 h after ethanol injection, this was reduced to 43% of total. This phenomenon was transient, since the difference in subcellular association of β-LH mRNA had disappeared by 24 h after ethanol administration. This ethanol induced shifting of mRNA off the most efficiently translating ribosome subfraction was specific for β-LH mRNA since the α-subunit and β-FSH mRNA profile patterns were unaffected by ethanol. The decreased binding of the β-LH transcript with the heavier polysome fractions provides a partial explanation for the apparent increase in β-LH mRNA degradation. The dissociation of the message from the polysomes would expose the β-LH transcripts making them more susceptible to nucleases, and therefore to increased degradation (Figure 6). This correlation of mRNA stability with polysome-association has been noted by others (Brawerman, 1989; Jäck *et al.*, 1989; Bandyopadhyah *et al.*, 1990). Further in accord with this mechanism is our previous work showing no change in the steady state levels of common α- or β-FSH mRNA after ethanol exposure (Emanuele *et al.*, 1991) and in studies reported here, there was no shifting of common α- or β-FSH mRNA off heavy polysomes.

In summary, our data indicates that the decrease in the steady-state mRNA levels of β-LH after ethanol exposure previously reported by us (Emanuele *et al.*, 1991) occurs because of increasing degradation of the transcript and not through a decreased transcriptional rate. Studies showing that ethanol causes a transient shift of cytoplasmic A-LH mRNA off heavy polysomes provide a partial explanation for this increased degradation, in that they indicated an increased vulnerability of non-heavy ribosome associated mRNA to degradation.

Materials and methods

Animals

All animals used were adult male Sprague-Dawley rats (aged 60–90 days, weighing 250–300 g) obtained from Harlan, Indianapolis, IN. In order to enhance α-subunit, β-LH and α-FSH mRNA and serum LH and FSH levels, the rats were surgically castrated, using light pentobarbital anesthesia (50 mg/kg). After a 2 week recovery, animals were either given a single intraperitoneal (i.p.) injection of ethanol in a dose of 3 g/kg or saline and sacrificed by decapitation at 3.0 h or 24 h after the i.p. injection. The reason that these time points were chosen was that we have previously shown that serum LH and β-LH mRNA are reduced compared to control 1.5 and 3 h after ethanol treatment, but return to control levels by 24 h (Emanuele *et al.*, 1991). The most dramatic fall is at 3 h. The brain was removed from the cranial cavity, and the pituitary stalk severed. The anterior pituitary was isolated and frozen in liquid nitrogen.

Blood ethanol determination

Samples were taken from trunk blood at time of decapitation. Blood ethanol levels were assessed using an enzymatic assay from Sigma Corporation (St Louis, MO).

RNA isolation and Northern blotting

Total RNA was isolated by homogenizing the anterior pituitaries in a guanidinium-thiocynate solution (GIT) and using a modification of the Chomczynski and Sacchi method (Emanuele *et al.*, 1991). Total RNA was fractionated through agarose (1.4%)-formaldehyde gels and transferred to Nytran

(Schleicher and Schuell). Blots were hybridized under stringent conditions with ^{32}P-labeled probes. The β-LH, β-FSH and the α-LH cDNA probes used were obtained from Dr William Chin (Boston, MA). After hybridization, the membranes were washed three times at high stringency (0.2 × SSC, 0.5% sodium dodecyl sulfate (SDS) at 65°C) to remove excess labeled probe. The filters were blotted dry and exposed to film at −70°C (Kodak Hyperfilm MP). Autoradiographs were analysed with a scanning densitometer and readings expressed as arbitrary densitometer units (ADU).

Polysome distribution analysis

Five pituitaries from each treatment group were homogenized in 1 ml of HKM buffer [20 mM HEPES, pH 7.6, 100 mM KCl, 20 mM MgCl2, 0.5% Triton X100, 3 mM 2-mercatoethanol, 300 U/ml RNAsin (Promega, Madison, WI)] and centrifuged at 12 000 *g* for 10 min at 4°C to pellet nuclei. The supernatants were overlaid onto a linear sucrose gradient 10–40% (wt/wt) made in HKM buffer with a 60% (wt/wt) sucrose cushion (Kleene *et al.*, 1984; Murphy *et al.*, 1992). Gradients were centrifuged using a Beckman SW41 rotor for 105 min at 41 000 r.p.m. (288 000 *g*). After centrifugation, gradients were unloaded manually into fractions (1–10) and the absorbance at 254 nm was determined. SDS was then added to a final concentration of 0.5% to each of the fractions. Each fraction was extracted with phenol:chloroform (1:1) twice. The fractions were ethanol precipitated with 2 vol 100% ethanol and 10 mg tRNA. The pellets were resuspended in TE (10 mM Tris-HCl and 1 mM EDTA) with 0.1% SDS. Northern blot analysis was then carried out on the fractions. As a control, equivalent supernatants were prepared and centrifuged in sucrose gradients in buffers in which the $MgCl_2$ was replaced by EDTA (10 mM).

S1 nuclease protection assay

Oligonucletides used in the assay (NBI; Plymouth, MN) were resuspended in deionized distilled water at a concentration of 1 pmol/ml. The 5′ end of the oligo was labeled according to Maniatis *et al.* (1989). Purification of the labeled oligonucleotide was performed by polyacrylamide gel electrophoresis followed by elution of the labeled oligonucleotide from the gel. The β-LH intron-exon probe oligonucleotide sequence was: 5′-TGGGCCCTACCATCTTACCTGGAGCCTCTCC-ATTCTGTTCCT-3′. The control oligonucleotide sequence, histone 3.3, used in the experiments was: 5′-GGCCTCACT-TGCCTCCTGCAAGTAGAA-3′. Each oligonucleotide contained six non-complementary nucleotides on the 3′ end.

Total RNA was isolated from three pooled pituitaries for each treatment group and resuspended in DEPC water (see RNA Isolation). A reaction mixture was made containing 80% formamide, 1 × S1 hybridization buffer (4 M NaCl, 0.4 M PIPES, 0.2 M EDTA), 5 mg tRNA, 1 μl of labeled probe and RNA to a total volume of 100 ml. The mixture was heated at 70°C for 15 min and immediately placed at 48°C for 12–16 h. Following this incubation, 300 μl of S1 digestion buffer (66 mM NaOAC, 0.3 M NaCl, 4.0 mM $ZnSO_4$) and 100 U of S1 nuclease was added and incubated at 37°C for 60 min. The digestion was stopped by addition of 200 μl of phenol and 200 μl of chloroform: isoamyl alcohol (49:1). The mixture was centrifuged (12 000 *g*) at 4°C for 10 min. The aqueous layer (top) was removed and 1 ml of 100% EtOH was added to precipitate the protected fragments. The tube was placed in a dry ice and methanol bath for 15 min and then centrifuged at 12 000 *g* for 15 min at 4°C. After the ethanol was aspirated, the pellet was dried and resuspended in deionized distilled water. Loading dye (90% formamide, 0.5% TBE, 0.1% Bromophenol Blue, 0.1% Xylene cyanol) was added and samples were heated to 90°C for 10 min. The samples were electrophoresed through a 15% polyacrylamide gel in 1 × TBE buffer at 150 V until the bromophenol blue dye had reached the end of the gel. The gel was then exposed to X-ray film.

Statistics

Data for the S1 nuclease protection assays were analysed by two-way ANOVA with Bonferroni adjustments.

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