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Changes in UsnRNA biosynthesis during rat liver regeneration

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Abstract

Partial hepatectomy (P.H.) induces a partially synchronized growth response of liver under normal regulation of growth. In this phase changes in cellular morphology, radial distribution pattern of cells and other biological as well as major biochemical changes are well documented [24]. Here, we have shown that the cellular content of UsnRNAs altered during this proliferative phase as well. The level of spliceosomal UsnRNAs (U1, U2, U4–U6) gradually decreased by 30–50% upto 48 hrs of P.H. followed by gradual increase to reach the normal level within one month of P.H. The U3 snRNA level on the other hand, was nearly equal to that in normal liver at 48 hrs of P.H. but in 24 and 72 hrs of P.H. its level was high (4 fold) in contrast to that in other UsnRNAs. Thus, it is clear from our data that the level of all the six UsnRNAs decreased during 48 hrs of P.H. compared to that after first 24 hrs. This has been correlated in the kinetics of UsnRNAs' synthesis (in terms of labelling) in isolated hepatocytes, where the rate of labelling of all the six UsnRNAs increased 20–30% in 24 hrs regenerating hepatocytes (R.H.) followed by sharp decrease by 30–50% within next 24 hrs, compared to that in the normal hepatocytes. But from 72 hrs onwards in R.H. the rate of labelling of all the six UsnRNAs again increased by 30–50% (compared to that in normal hepatocytes) followed by decrease of their labelling-rate to reach the normal level in R.H. within one month of P.H. Thus, it may be concluded that the changes in UsnRNAs' level during the proliferative phase of liver regeneration may be either due to the alteration in the rate of synthesis (in terms of labelling) or along with it differential turn over rate; this phenomenon may have some consequences with the regenerative process of liver. (Mol Cell Biochem **141**: 71–77, 1994).

Key words: regenerating rat liver, UsnRNAs, UsnRNA profiles, kinetics of UsnRNAs' labelling

Introduction

Small nuclear RNAs (SnRNAs) of the U family (U1 to U10) are evolutionarily conserved, metabolically stable, nonpolyadenylated RNA molecules present in the nuclei of eukaryotic cells [1, 2]. The most abundant group include U1 through U6 snRNAs of which the first five contain hypermethylated guanosine cap at the 5′ end

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and is transcribed by RNA polymerase II (pol II) [3, 4]. The U6, on the other hand, has γ monomethyl phosphate at the 5′ end [5] and is transcribed by RNA polymerase III (pol III) [4]. Except U3, the five nucleoplasmic UsnRNAs are involved in splicing of pre-mRNA [6]. But nucleolar UsnRNA i.e. U3, is involved in pre-rRNA processing [7–9]. So, the major biochemical role of these UsnRNAs are to process pre-mRNA and pre-rRNA. Moreover, studies on the metabolism of UsnRNAs in proliferating/differentiating cells indicated major quantitative alteration in UsnRNA level with no new appearence or disappearence of any individual snRNA species [10–14]. Preferential expression of certain forms of U1 snRNA during mouse embryo development [15, 16], differential accumulation of U1 and U4 snRNA through embryogenesis in xenopus [17], over expression of U6 snRNA during xenopus development [18] and tissue specific expression of U4 snRNA in Chicken [19] have also been reported. The 3′ end formation of spliceosomal U6 snRNA depends on specificity of the organism and also on the stage of development [20]. It is also reported that U5 snRNA after transfection, can transform primary cells *in vitro* [21]. Thus, quantitative alteration in UsnRNA biosynthesis during cellular proliferation/differentiation, embryonic development, or transforming activity of some particular UsnRNA indicate that any control of cell growth may have some regulation through altered UsnRNA biogenesis.

To study the UsnRNA metabolism during rapid cellular proliferation under normal regulation of growth, we have taken regenerating rat liver as a tool. Ninety per cent by volume (50% by number) of the adult liver is composed of parenchymal cells (hepatocytes) and 5% by volume (40% by number) is composed of nonparenchymal cells (ductal cells, endothelial cells and Kuppfer cells) [22]. The removal of 70% of the liver by partial hepatectomy (P.H.) induces a partially synchronized growth response which involves a hypertrophy phase (lasting for 12 to 16 hr), a replicative phase in which ~ 84% of the hepatocytes undergo DNA synthesis (peak at 24 hrs) and a rapid-cell-division phase, when nearly 70% of the hepatocytes undergo their first set of cell division within 30 hr of P.H.; by 72 hrs of P.H. about 90% of the hepatocytes divide at least once [23, 24]. On the contrary, the nonparenchymal cells undergo DNA synthesis around 42 hr of P.H. [22]. Thus, in the present work, we want to study the UsnRNA metabolism in the proliferative phase (24 to 72 hrs) of liver regeneration when most of the biochemical changes take place.

Materials and methods

Bovine Serum Albumin (BSA) fraction V and collagenase type IV, were from Sigma Chemical Company, USA. HAM's F-12 medium was from GIBCO-BRL, USA. ^{3}H-Orotic acid (10000 mCi/mmole) was from BARC, Trombay, India. Other chemicals were of analytical grade.

Animals, partial hepatectomy and perfusion of liver

Male albino rat weighing 180–200 gms were routinely used for partial hepatectomy. Seventy percent hepatectomy was achieved by removing the left lobe and the median lobe of liver according to the method of Higginis and Anderson [25]. Restorative growth of liver took place by a rapid-cell-division phase which continued from 24 to 72 hrs of partial hepatectomy [24]. Rats were sacrificed at 24 hrs interval upto 96 hr and also after one month of P.H. Sham-operated normal rat livers were used as control. Livers were perfused through the portal vein by perfusion buffer (0.08 M NaH_2PO_4/Na_2HPO_4, pH 7.4) containing heparin (10 units/ml) to get rid of blood contamination.

Subcellular fractionation, RNA extraction and UsnRNA fractionation

Nuclei and cytoplasmic supernatant were prepared from perfused rat liver as described in [26] with slight modification. Perfused liver was passed through the metal net, single cells were washed with PBS (Phosphate Buffered Saline) at 500 rpm for 5 min at room temperature. The cells were suspended in 10 vols. (10 volumes/gm) of homogenisation buffer (2.5% Citric acid, 0.2% Triton-X-100), kept in ice for 10 min and homogenised with 10–15 strokes in Dounce homogeniser fitted with B pestle. The suspension was then centrifuged at 1,500 g for 10 min at 4° C. The supernatant fraction (SF) was stored in ice. The pellet containing the crude nuclei was washed once with the homogenisation buffer. The pellet was suspended in suspension buffer (2.5% Citric acid) and passed through a cushion containing 2.5% Citric acid, 15% glycerol at 1,500 g for 15 min at 4° C. Pellet containing the purified nuclei was washed once with the suspension buffer and kept in ice. The 'SF' was centrifuged at 15,000 g for 15 min at 4° C. The pellet (SF-P)

and the supernatant fraction (SF-1) were saved and kept in ice. RNAs were extracted from the purified nuclei, whole cells and also from 'SF-P' by using guanidine isothiocynate, according to the method of Chomczynski and Sacchi [27] and from 'SF-1' by Phenol: Chloroform extraction.

For electrophoretic analysis, RNAs were dissolved in 80% deionised formamide, heated to 85° C for 3 min, chilled immediately in ice and electrophoresed on 12% polyacrylamide – 7 M Urea gel as described in [28]. The gel was ethidium bromide stained and photographed. Then the gel was silver stained [29], dried and scanned densitometrically at 410 nm using a densitometric scanner (SHIMADZU MODEL-CS-9000). We have observed that most of the cytoplasmic RNAs were present in the SF-P fraction compared to the SF-1 (data not shown).

Identification of UsnRNA bands on the scanning profile were accomplished by comparing their mobilites with that of known cytoplasmic marker RNAs namely 4S, 4.5S, 5S and 5.8S, as well as by direct comparison to snRNAs isolated from whole nuclei [30, 31]. Quantitation by densitometry after silver staining was done under the condition that has given a linear response with the RNA concentration [29]. Relative abundance of each UsnRNA was calculated by normalizing the areas of the peaks with respect to that of corresponding 5S rRNA [28, 32–34].

In vitro *labelling of UsnRNAs in isolated hepatocytes*

Viable hepatocytes were isolated from sham-operated normal liver and partially hepatectomized rat liver (as described earlier) according to the method of Öbrink [35]. The abdomen of the rat under light ether-anaesthesia was opened and 0.25 ml of heparin (5000 IU/ml) was injected through the iliolumber vein. The Ca^{2+} free perfusion buffer (0.14 M NaCl, 0.0067 M KCl, 0.01 M HEPES, pH 7.4) was passed through the portal vein at a flow rate of 20 ml/min for a period of 20 min. The collagenase buffer (0.0066 M NaCl, 0.0067 M KCl, 0.0047 M $CaCl_2$. 0.1 M HEPES pH 7.6 containing 15 mg/ml of BSA and 0.5 mg/ml collagenase) was then passed through the portal vein as before with recirculation for 10 min. Cells were liberated by gently shaking the perfused liver in cell suspension buffer (0.14 M NaCl, 0.0067 M KCl, 0.01 M HEPES, pH 7.4 containing 7.5 mg/ml of BSA). The cell suspension was centrifuged at 500 rpm for 2 min at room temperature, followed by washing the cell pellet twice with the same buffer. Viable cells, in suspension, was found to be 75 to 85% as determined by Trypan blue exclusion method.

Isolated hepatocytes, suspended at a concentration of 10^6 cells/ml in HAM's F-12 medium buffered with 25 mM HEPES-0.014 M $NaHCO_3$, pH 7.4 containing 15% (V/V) FCS and ^{3}H-Orotic acid (10 Ci/10^6 cells/ml), were incubated at 37° C with constant shaking over a period of 3.5 hr. The cell suspension (5 ml at each time point) was collected at 1 h interval upto 3 hr and also after 3.5 hr of incubation. Cells were centrifuged at 5000 rpm for 2 min at room temperature and washed twice with PBS. Whole cellular RNAs were extracted from these cell pellets and fractionated on polyacrylamide gel by electrophoresis as described before. The gel was ethidium bromide stained and photographed. Fluorography of the gels were done on KODAK X-OMAT AR-5 film through 1 M Sodium Salicylate impregnation according to the method of Chamberlin [36]. Identification of UsnRNAs' bands on the fluorogram was done as described in silver stained gel. Bands in the X-ray film and in the negative of ethidium bromide stained gel were densitometrically scanned as before. Quantitation by densitometry was done following the procedure of Choudhury *et al.* [28] to give a linear response both with X-ray film and the negative of ethidium bromide stained gel. To study the kinetics of labelling of each of the six UsnRNAs during liver regeneration, area of each labelled UsnRNA band was normalised to that of the total unlabelled area of the corresponding 5S rRNA [28, 32–34] and plotted against different period of incubation.

Results

Alteration in the profile of UsnRNA during liver regeneration

We have studied alterations of UsnRNA profiles during the proliferative phase of liver regeneration i.e. from 24 to 96 hrs of P.H. It was apparent from ethidium bromide stained gel (gel not shown) that there was some alteration in UsnRNA content at least at 48 hrs of P.H. The gel was then silver stained for densitometric scanning. It was revealed from Fig. 1 that there was gradual decrease in abundance of the five spliceosomal UsnRNAs (U1, U2, U4–U6) upto 48 hrs of P.H. At this phase (i.e. after 48 hrs of P.H.) the abundance of U1 and U2 snRNAs decreased nearly 30%, while in case of U4–U6 snRNAs the decrease in abundance was about 50%. Then their abun-

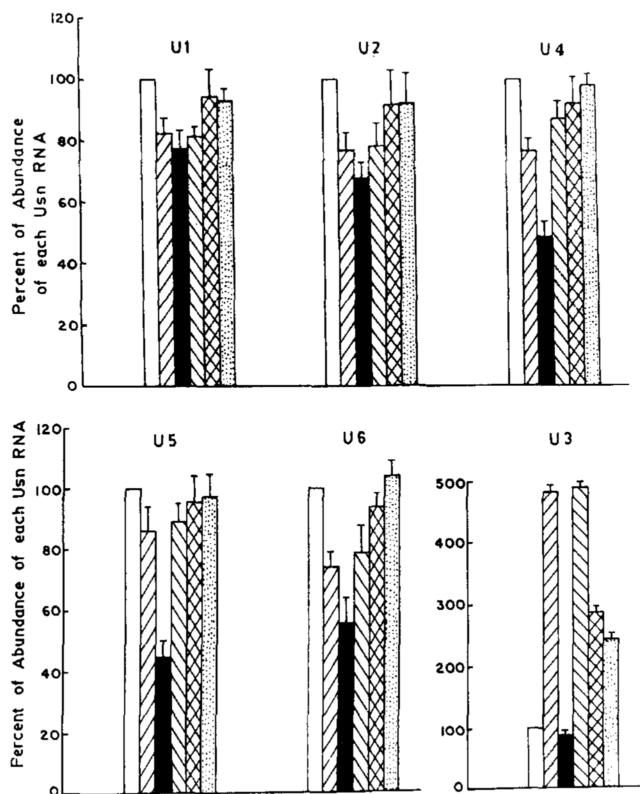

Fig. 1. Profile of each UsnRNAs in sham-operated normal liver and livers of 24 hr, 48 hr, 72 hr, 96 hr and one month of P.H. Silver stained gel was scanned densitometrically. Relative abundance of each UsnRNAs was obtained by normalizing the area of each UsnRNA peak in the silver stained gel with that of corresponding 5S rRNA. Percent of abundance was calculated by taking the relative abundance of each UsnRNA in Sham-operated normal as control i.e. percent of abundance of each UsnRNA is the relative abundance of each UsnRNA in P.H. liver sample, divided by the relative abundance of each UsnRNA in normal liver sample, multiplied by 100. □ normal; ▨ 24 hrs of P.H.; ■ 48 hrs. of P.H.; ▧ 72 hrs of P.H.; ▩ 96 hrs. of P.H.; ▤ one month of P.H.;

dance gradually increased and reached normal level within one month of P.H. On the contrary, the abundance of U3 snRNA was nearly same as in normal after 48 hrs of P.H. Its abundance sharply increased about 4 fold compared to that in normal after 24 hrs and 72 hrs of P.H. Thereafter, the level of U3 gradually decreased until one month of P.H. Thus, during proliferative phase of liver regeneration, the abundance of UsnRNAs altered considerably.

Kinetics of UsnRNAs' labelling during liver regeneration

Rate of UsnRNAs' labelling during proliferative phase of liver regeneration i.e 24 to 96 hrs of P.H. was studied *in vitro* in isolated hepatocytes, as described in materials and methods. During *in vitro* culture of isolated hepatocytes (from regenerating rat liver), it has been observed that UsnRNAs could be significantly labelled with radioactive orotic acid only after 1 hr of incubation at 37° C (data not shown). The rate of labelling of all the six UsnRNAs reached plateau after 3 hrs of incubation at 37° C (Fig. 2). At the first replicative phase of liver regeneration i.e after 24 hrs of P.H., the rate of labelling of all the six UsnRNAs increased 20–30% compared to that in normal hepatocytes. But after 48 hrs of P.H. the levels of labelled UsnRNAs sharply decreased to approximately 30 to 50% with respect to that in normal hepatocytes (Fig. 2). However, during last phase of proliferation of liver regeneration, i.e 72 hrs after P.H. the levels of all the six labelled UsnRNAs considerably increased (nearly 30–50%), followed by gradual decrease in their rates of labelling to restore normal rate within one month of P.H. Therefore, in the proliferative phase of liver regeneration the biosynthetic rate (in terms of labelling) of all the six UsnRNAs are not same. The upregulation of their rate of synthesis (in terms of labelling) after 24 and 72 hrs of P.H. along with downregulation after 48 hrs of P.H. might have some consequences with the regenerative process of liver growth.

Discussion

The major metabolic alterations during liver regeneration after P.H. have been reviewed in great details [23, 24]. The total RNA concentrations both in the nucleus and in the cytoplasm (including cytoplasmic poly A^+ mRNA) reached to a maximum level within 24 hrs of P.H., but their level decreased sharply within 48 hrs of P.H. Whereas the nuclear poly A^+ mRNA level remained unaltered during this period [37, 38]. Though, there was no remarkable change in liver specific mRNA transcription rate or its concentration within first 24 hrs of P.H. a definite increase in concentration of actin mRNA level and higher transcription rate for tubulin mRNA within 24 hrs of P.H. have been reported [39]. That, the rate of pre-rRNA synthesis and ribosomal protein synthesis did not alter during the proliferative phase of liver regeneration after P.H. have been well documented; on the contrary, the ribosome biosynthesis increased considerably during this phase of liver regeneration [39–41]. However, the activation of pre-rRNA synthesis in regenerating rat liver due to increased RNA Polymerase I activity has also been reported [42]. But it has been observed from Fig. 1 that the profile of UsnRNAs changed considerably during the proliferative phase of

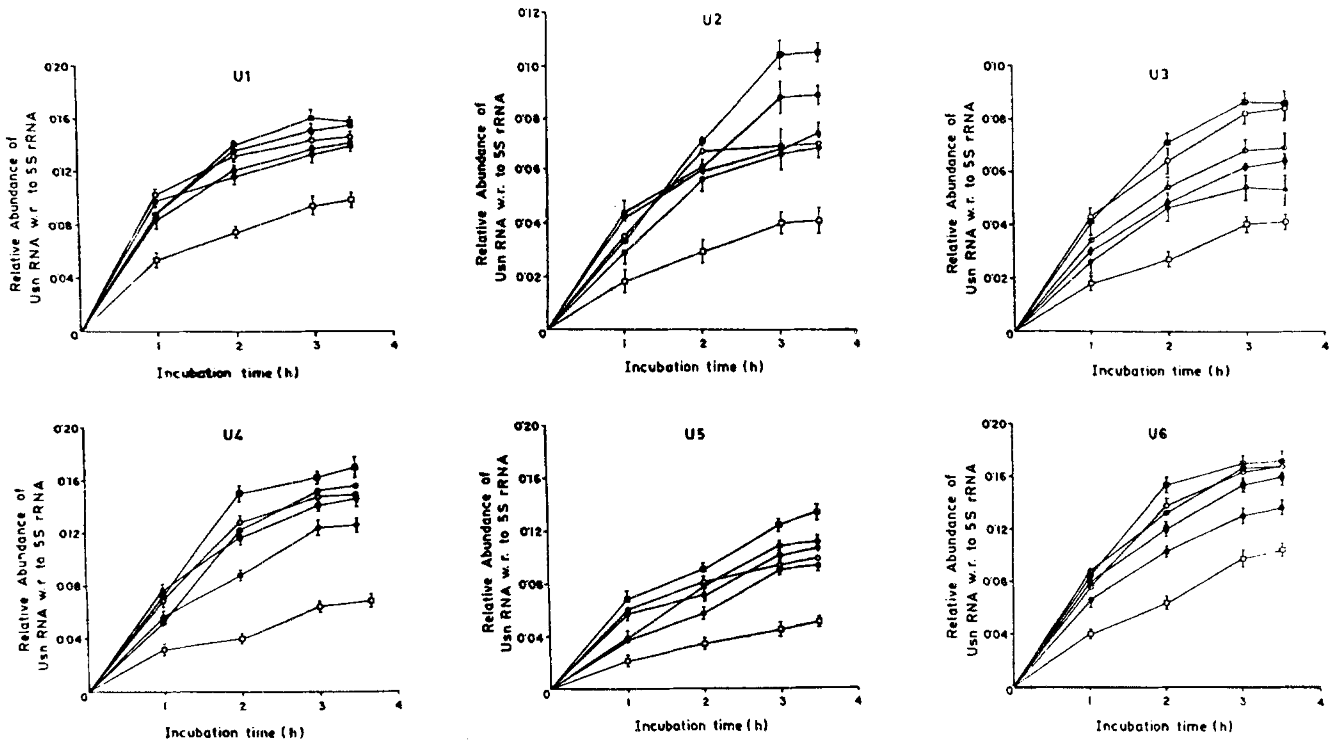

Fig. 2. Kinetics of each UsnRNA's labelling in isolated hepatocytes from sham-operated normal liver and livers of 24 hr, 48 hr, 72 hr, 96 hr and one month of P.H. Relative abundance of each labelled UsnRNA at each time point was obtained by normalizing the area of each labelled UsnRNA band with that of total 5S rRNA in the ethidium bromide stained gel. (Area of each RNA band was calculated by densitometric scanning of either fluorogram or the ethidium bromide stained gel.) ●—● normal; ○—○ 24 hrs of P.H.; □—□ 48 hrs of P.H.; ■—■ 72 hrs of P.H.; ◐—◐ 96 hrs of P.H.; ♦—♦ one month of P.H.

liver regeneration. The levels of five UsnRNAs (except U3) which constitute the active spliceosomal complex gradually decreased (30 to 50%) upto 48 hrs of P.H. followed by sharp increase to reach the normal level within one month of P.H. In contrast, U3 snRNA increased 4 fold around 24 hrs of P.H. followed by a sharp decrease within next 24 hrs (Fig. 1). But after 72 hrs of P.H., the U3 snRNA's level again increased sharply (4 fold) compared to other UsnRNA's level. Thus, it is evident from Fig. 1, that the level of all the six UsnRNAs decreased sharply during 48 hrs of P.H. compared to the first 24 hrs. So the changes in the total UsnRNAs' pool, during the proliferative phase of liver regeneration might be either due to alteration in the rate of synthesis or in the turn over rate.

It is evident from Fig. 2 that the kinetics of spliceosomal UsnRNAs' synthesis (in terms of labelling) is highly correlated with that of the cytoplasmic poly A^+ mRNA synthesis during liver regeneration [38]. However, the rate of labelling of the five UsnRNAs in first 24 hrs regenerating hepatocytes (R.H.) increased nearly 20–30%, yet the total cellular contents of these UsnRNAs was low ($\sim$ 20%)(Fig. 1) as compared to that in normal liver. This might be due to the faster turnover rate of these UsnRNAs during this phase of liver regeneration. Whereas, during the next 24 hrs of liver regeneration i.e 48 hrs of P.H. the rate of labelling in R.H. decreased approximately 30–50% along with the decrease ($\sim$ 30–50%) in total cellular content of these UsnRNAs. Thus, in this phase of liver regeneration, the turnover rate of these UsnRNAs was not exactly same as that in first 24 hrs of liver regeneration. Because, if the turnover rate of these UsnRNAs in 48 hrs regenerating liver was same as that in 24 hrs, then the total cellular contents of these UsnRNAs in 48 hrs regenerating liver would be much lower than the level we have observed. But during regeneration after 72 hrs of P.H. both the rate of labelling and total cellular content of these UsnRNAs were nearly comparable as that in first 24 hrs sample. This might be due to the fact that during these phases of liver regeneration, major portion of the hepatocytes might be in the same stage of cell division [23, 24]. Higher abundance of U3 snRNA, which is involved in pre-rRNA processing [7–9] has been well documented in two developmental phases of xenopus: a) during Oocyte maturation and b) late embryogenesis [43]. We have shown that total pool

of U3 snRNA at 24 and 72 hrs of P.H. was different from that of all the five spliceosomal UsnRNAs though their rates of labelling were similar (Fig. 1 and Fig. 2). It seems likely, that during these phases of liver regeneration the rate of turn over of U3 snRNA may be low as compared to that of other nucleoplasmic UsnRNA. However, after 48 hrs of P.H. the total cellular pool of U3 snRNA decreased to the normal level in comparison to that in first 24 hrs regenerating liver. This might be either due to lowering (30–50%) in rate of labelling in 48 hrs R.H. or along with it a higher turn over rate in this phase of liver regeneration.

It is evident from our data that the UsnRNAs were accumulated in different ways in different phases of liver regeneration, suggesting that the UsnRNAs may be differentially regulated according to specific cellular requirements or function. It has also been reported that some of the UsnRNAs' proportions are altered differentially in both stationary phase and terminally differentiated cells [14]; certain UsnRNAs may be involved in the production of mRNA and are coordinately regulated with respect to the alterations in resting/differentiating cellular-gene activity besides other factors involved [44]. The UsnRNAs are transcribed by two different RNA polymerases: U1 to U5 snRNAs by pol II and U6 by pol III [4], although their mode of synthesis appears to be similar. The promoter region of these UsnRNAs are different from other pol II and pol III transcribed RNA promoters [45]. Several cis acting elements (e.g Sph motiff, Octamer motiff, GC box, LINES sequence etc.) similar to that of mRNA genes have been characterised in different UsnRNAs along with some trans-acting factors [46]. It is quite evident that these cis-acting elements have some role in cellular proliferation, differentiation as well as tissue specific gene expression [47]. Besides, it has been shown that these UsnRNA enhancer elements can act in a variety of cis-linked configurations to activate gene transcription synergistically [46]. Thus, the differential regulation of these UsnRNAs' transcription during the proliferative phase of liver regeneration might be controlled by all these elements.

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