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Interaction of Albumin mRNA with Proteins from Rat Liver with CCl_4-Induced Injury

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Acute phase responses to intragastric administration of a single dose of CCl_4 were examined with albumin mRNA turnover as a marker. Based on the combination of the changes in stability of albumin mRNA and activity of transcription of its gene, the entire course of liver injury was classified into three stages, the first stage for aggravation of injury until 9 h, the second from 9 to 24 h, and the third for repair of injury or regeneration of liver after 48 h. Liver S100 fractions from normal and CCl_4-treated rats contained, in total, 11 polypeptides cross-linked with part of albumin mRNA, although they did not appear to be specific to this mRNA. Their profiles were altered together with the changes in stability of albumin mRNA in different stages. These findings suggest that the polypeptides with distinct properties play roles in physiologically significant processes involved in utilization and turnover of albumin mRNA, apparently altering its stability.

Key Words: **albumin mRNA; RNA binding protein; CCl_4-induced liver injury.**

CCl_4 eventually induces fatty liver and liver fibrosis in rats that repeatedly receive it over 10 weeks (1). In contrast, [^{14}C]CCl_4 was located in endoplasmic reticulum (ER) in hepatocytes of rats 1 h after oral administration. Simultaneous dissociation of granules on ER occurred. These findings suggested that critical changes occurred at the molecular level in liver soon after CCl_4 administration (2). Since the granules could be assigned to membrane-bound polysomes, the above responses of hepatocytes suggested that the physiological supply of proteins destined for ER and plasma membranes, blood plasma, and extracellular space was to a certain extent interrupted. These proteins should be degraded at individual rates, leading to shortage of them due to the aberrant dissociation of polysomes from rough ER. This dysfunction of hepatocytes might be a principal cause of aggravation of CCl_4-induced injury. On the other hand, although the fate of mRNA molecules included in polysomes dissociated from ER is unknown, they were thought to enter degradation pathways for the physiological turnover of mRNA. The pathological conditions induced by CCl_4 thus appear useful for study of mechanisms for regulation in the physiological decay of polysomal mRNA. In this regard, the level of albumin mRNA ubiquitous in and specific to hepatocytes was reportedly decreased and then restored at 24 and 120 h after CCl_4 administration, respectively (3). DBP, a factor required for transcription of the albumin gene, was shown to disappear and then appear under similar conditions (4). Taking advantage of the fact that these were hepatocyte-specific events, in the present study factors involved in albumin mRNA turnover in rats that received a single dose of CCl_4 were examined, focusing in particular on proteins that interact with this mRNA. The present paper describes the classification of the pathological course of the liver injury into three stages according to changes in stability of albumin mRNA and activity of albumin gene transcription, and demonstrates the stage-specific profiles of polypeptides that interact with albumin mRNA.

MATERIALS AND METHODS

Materials. Rat albumin cDNA, pAct-Alb, was from the Japanese Cancer Research Resources Bank, Tokyo. Rat glyceraldehyde-3-phosphate dehydrogenase cDNA, pRGAPDH, was a generous gift from Dr. Fort (5). Vectors were replaced with Bluescript SK minus plasmid (Stratagene) and an artificial sequence inserted for the expression in mammalian cells was removed from pAct-Alb. The two resultant constructs were designated pAlb and pGAPDH. Other materials including radioactive nucleotides were purchased through local distributors.

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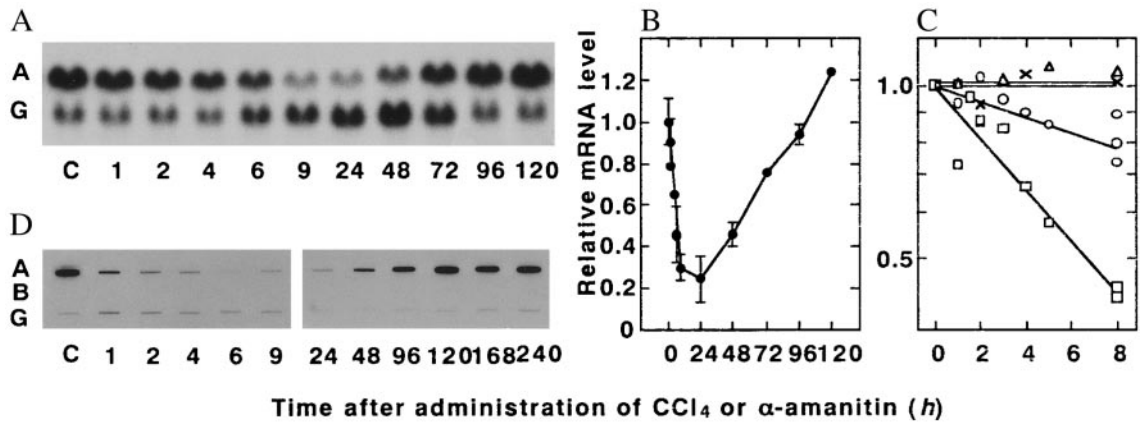

FIG. 1. Factors affecting albumin mRNA turnover in liver with the CCl_4-induced injury. (A) The levels of mRNAs each encoding albumin (A) and GAPDH (G) were determined for the indicated periods after CCl_4 administration by Northern blot analysis. (B) The level of albumin mRNA was determined from signal intensities shown in A, and expressed as values (mean ± SD) relative to normal level. (C) $t_{1/2}$ determined for normal (circle) rat, and rats 1 (square), 9 (cross), or 96 (triangle) h after CCl_4 administration. (D) Run-on transcription products at the indicated periods of the liver injury were trapped with pAlb (A), pGAPDH (G), and vector alone (B). Uppercase letter, C, in A and D denotes control rat.

CCl_4 administration, total RNA preparation, and Northern blot analysis. Male Wistar rats (7 weeks old) fed *ad libitum* with normal laboratory chow and weighing ≈250 g were fasted overnight. The following morning, rats received 2 ml/kg body weight of 50% CCl_4 in olive oil by gavage. They were anesthetized and killed to excise liver at suitable intervals. Unless otherwise stated, rats that received olive oil alone were used as controls. Liver total RNA (10 μg) prepared by the method of Chomczynski and Sacchi (6) was subjected to Northern blot analysis (7) using Hybond N^+ and probes for albumin mRNA or GAPDH mRNA. Abundance of mRNA was determined using an imaging analyzer, BAS2000 (Fuji Photo Film Co., Ltd., Tokyo). Kodak XAR5 film was used to prepare autoradiograms.

Half-life of mRNA. Rats at 1, 9, 24, 48, and 96 h after CCl_4 administration received α-amanitin intraperitoneally (1 mg/kg body weight). Total RNA was isolated 1, 2, 3, 4, 5, and 8 h after α-amanitin injection and examined for mRNA levels, the half-life ($t_{1/2}$) of which was determined.

Interaction of RNA with proteins. Three riboprobes (RIT14, T14H, and HRI) obtained by *in vitro* transcription using T7 or T3 RNA polymerase in the presence of [α-^{32}P]CTP (5 TBq/mmol) or CTP respectively encompassed nucleotides 11 to 1078, 1079 to 1302, and 1303 to 2014 of rat albumin mRNA (8) (cf. Fig. 2A), and T14H corresponded to the riboprobe used to label rat lung glutathione peroxidase mRNA binding protein (GPx-BP) by Clerch *et al.* (9). Another riboprobe, GAT14, comprised nucleotides 877 to 1067 of GAPDH mRNA (5). Electrophoretic mobility shift assay (EMSA) and cross-linking by irradiation with UV light (10, 11) were performed using the probes. In addition to normal rats, rats 2 and 72 h after CCl_4 administration were used for preparation of S100 fractions, since these periods were representative with respect to albumin mRNA decay (see Results). Liver S100 fraction was prepared from 20% liver homogenate containing 25 mM Tris–HCl buffer, pH 7.4, 40 mM KCl, 0.1 mM EDTA, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin A and leupeptin (9). Ten μg of protein (12) were incubated with one of the riboprobes (about 500,000 cpm, 30 fmol) at 25°C for 30 min in a final volume of 15 μl of a reaction mixture containing 10 mM Hepes–NaOH buffer (pH 7.5), 40 mM KCl, and 3 mM $MgCl_2$. Excess RNA fragments were removed by sequential reactions with RNase T1 (120 units) and heparin (5 mg/ml) for 10 min for each, and then RNase A (0.6 mg/ml) for 30 min. For EMSA, the mixtures were subjected to electrophoresis using a 4% polyacrylamide gel (PAGE). Aliquots of this mixture but digested with proteinase K in the presence of SDS were examined for protection of RNA. To cross-link proteins with the probes, another aliquot of the mixture for EMSA was irradiated with light at 254 nm and 0.75 J/cm^2 using a FUNA UV-Linker (Funakoshi, Ltd. Tokyo, Japan). Cross-linked products were detected by polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) followed by autoradiography. For competition, prior to incubation with a [^{32}P]riboprobe, the S100 fraction was incubated with a 100 times molar excess of a cold probe for 30 min at 25°C.

Run-on transcription. Products of run-on transcription (13) using 7.4 MBq of [α-^{32}P]UTP (30 TBq/mmol) and 1.5×10^7 nuclei (14) were trapped with pAlb, pGAPDH, and Bluescript vector each linearized and immobilized on a single sheet of Hybond C, and quantitated with BAS2000.

RESULTS

Levels of albumin mRNA in rats receiving CCl_4. As shown in Figs. 1A and 1B, the level of albumin mRNA had begun to decrease 2 h after CCl_4 administration and reached about 25% of control level at 9 h, indicating that dissociation of polysomes (2) and albumin mRNA decay occurred at similar periods, and that albumin mRNA reached minimal level earlier than previously reported (24 h) (3). From this minimal level, which persisted over 10 h, albumin mRNA began to increase slowly to normal level, which was observed later than 120 h. To determine which of possible factors caused the changes observed, $t_{1/2}$ of albumin mRNA was determined within the above time course. In rats that received α-amanitin 1 h after CCl_4 administration, $t_{1/2}$ was 6 h, and one-fourth that in normal rats (Fig. 1C). However, the $t_{1/2}$ values determined for 9 and 96 h rats were infinite (Fig. 1C). That this mRNA was quite stable was confirmed in 24 and 48 h rats (not shown). Therefore, three different conditions under which albumin mRNA decayed with normal, increased, or suppressed rate were clearly distinguishable. On the

other hand, GAPDH mRNA used as an internal control and probed in parallel on an identical filter increased to twice normal level at 48 h (Fig. 1A, lower row). Its normal $t_{1/2}$ of approximately 12 h was temporarily extended to over 30 h in 9 and 24 h rats (not shown), indicating that the two mRNA molecules were stabilized later than 9 h, but in a manner intrinsic to individual mRNA molecules.

Transcription of the albumin gene. As shown in Fig. 1D (upper row), run-on transcription of the albumin gene was 55% of control activity at 1 h and nearly undetectable at 6 h. This arrest of transcription was also indicated by the finding that $t_{1/2}$ determined from the albumin mRNA levels in Fig. 1B for rats treated with only CCl_4 was quite similar to that determined with α-amanitin. Since nearly 80% of albumin mRNA was degraded within the initial 9 h during which its gene was minimally transcribed, it is likely that albumin mRNA in the polysomes dissociated from ER (2) is a target for a regulatory machinery controlling mRNA degradation. In turn, the suppression of transcription that lasted until 24 h appeared no longer to be present later than 48 h (Fig. 1D), whereas albumin mRNA was still stable (Fig. 1C). Consequently, albumin mRNA may be increased after 48 h. Since GAPDH gene transcription appeared less active around 48 h (Fig. 1D), the increase in GAPDH mRNA level was ascribed to suppression of its degradation.

Interaction of albumin mRNA with proteins. It is, in general, conceivable that the stability of mRNA is determined by its interaction with proteins. To gain more insight into the distinct degradation rates of albumin mRNA, proteins that interact with this mRNA were surveyed in liver S100 fraction by EMSA and cross-linking analysis. In EMSA using [^{32}P]T14H, a normal S100 fraction revealed five signals, A to E, with retarded mobilities (Fig. 2B). The signals with [^{32}P]T14H revealed reduced intensities following incubation of the liver extract with T14H in advance, indicating significant competition between the hot and cold riboprobes. Although the competition differed in extent from signal to signal, this finding implied that rat liver contained proteins that interact with part of albumin mRNA. The five signals were, however, located at basically identical positions in lanes for 2 and 72 h rats. Since signals A and D were each intensified at 72 h, it was likely that these proteins underwent alteration in amount or ability to interact with [^{32}P]T14H in different stages of the CCl_4-induced liver injury.

Polypeptides cross-linked with [^{32}P]T14H RNA in a stage-specific manner. UV irradiation of a reaction mixture containing both a normal liver S100 fraction and [^{32}P]T14H yielded nine signals cross-linked (Fig. 2C, lane A for CTR). No meaningful signal was found without liver extract. All signals were lost following treatment of the reaction mixture with proteinase K

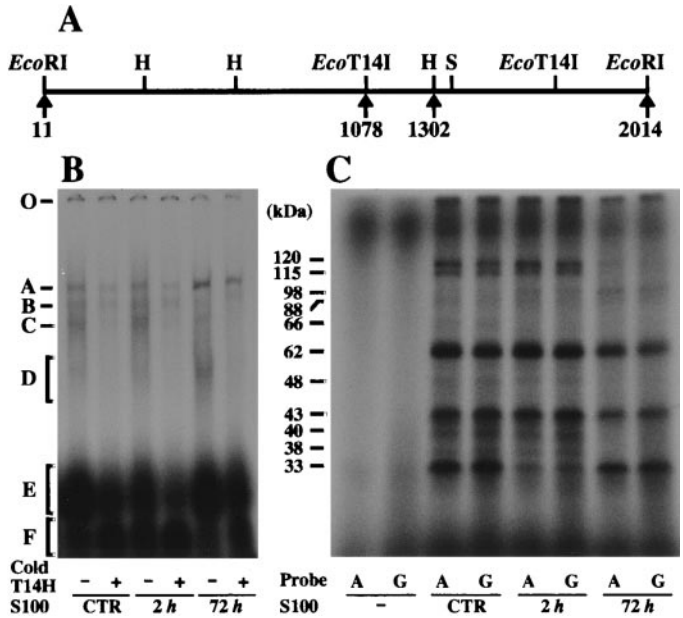

FIG. 2. Interaction of proteins with T14H and GAT14. (A) Positions of RIT14 (nucleotides 11 to 1078), T14H (1079–1302), and HRI (1303–2014) are shown with recognition sites for *Hin*dIII (H) and *Sal*I (S) in albumin cDNA. The protein coding region encompasses nucleotide 38 to 1861. (B) EMSA using [^{32}P]T14H and S100 fractions from normal (lanes CTR), 2, and 72 h rats. Cold T14H was used as a competitor for [^{32}P]T14H. Uppercase letters, O, A to E, and F respectively indicate the origin of electrophoresis, five signals revealed, and the free probe. (C) Cross-linking of polypeptides in S100 fractions from normal, 2, and 72 h rats with [^{32}P]T14H (A) or [^{32}P]GAT14 (G). Sizes of polypeptides revealed were indicated in kDa. Autoradiography was performed for 10 h for B and 8 days for C.

before or after cross-linking (not shown). Thus, it is clear that liver S100 fraction from normal rat contain nine polypeptides that interact with T14H. The polypeptides detected are henceforth expressed by their sizes, as p120, p115, p66, p62, p48, p43, p40, p38, and p33. Rats 2 h after CCl_4 administration displayed signals quite similar in size and intensity to those found in normal rats, but with weak labeling of p66 and p33. In addition, 2 h rats had a weak but novel p98 signal (Fig. 2C, lane A for 2 h). In this context, 72 h rats expressed a more distinct profile of products cross-linked: signals of p120, p115, p48, p40, and p38 disappeared, while those of p62 and p43 were weaker than in normal and 2 h rats, and p98 and p88 signals were intensified (Fig. 2C, lane A for 72 h). These findings demonstrated that the eleven polypeptides, in total, in liver S100 fraction could interact with part of albumin mRNA, and that their population was altered in a manner specific to the stages of the liver injury tested. The present procedure, but in the presence of 2% 2-mercaptoethanol, revealed no signal (not shown), indicating that none of the polypeptides presently detected met the requirement to identify GPx-BP labeled with the re-

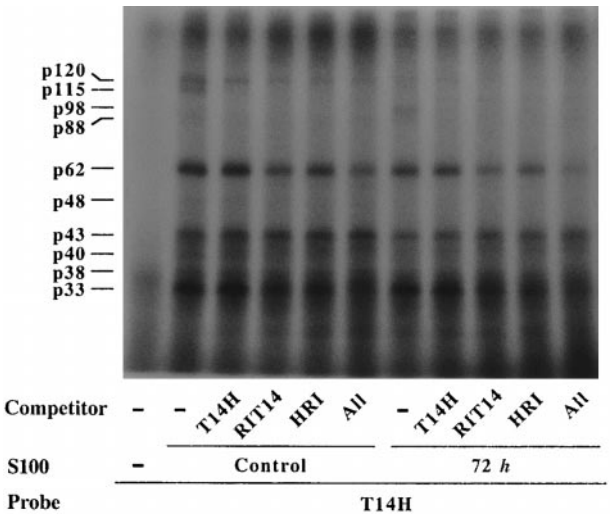

FIG. 3. Competition of the three regions derived from albumin mRNA with [^{32}P]T14H. S100 fractions were treated with one or all of the three competitors RIT14, T14H, and HRI prior to incubation with [^{32}P]T14H.

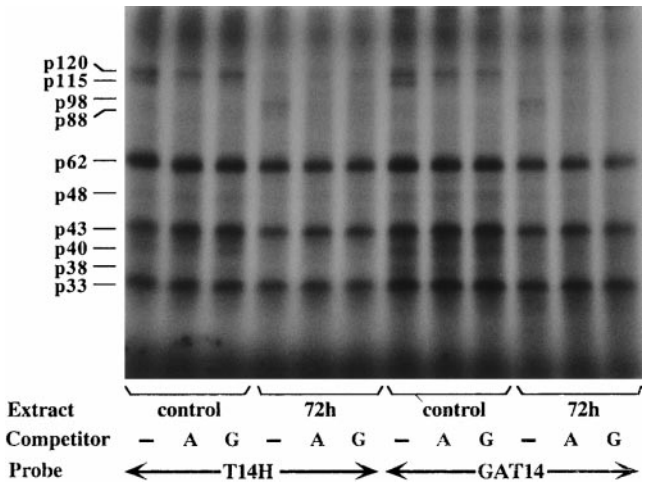

FIG. 4. Competition of GAT14 with T14H. S100 fractions from normal and 72 h rats were treated with T14H or GAT14 prior to incubation with [^{32}P]T14H or [^{32}P]GAT14 to test effects of competitor RNA with structures heterologous to hot probes.

gion identical to T14H, but in the presence of 2-mercaptoethanol (9).

Polypeptides cross-linked with different RNA fragments. To distinguish whether T14H is the only region that can interact with this set of polypeptides, additional probes, [^{32}P]RIT14 and [^{32}P]HRI, were prepared for the regions up- and downstream from T14H in albumin mRNA (cf. Fig. 2A). The signals revealed with them for normal, 2 h, and 72 h rats were comparable to those revealed with [^{32}P]T14H for rats in comparable stages (not shown). Likewise, part of GAPDH mRNA, [^{32}P]GAT14, displayed a set of signals identical to that revealed with [^{32}P]T14H (Fig. 2C, lanes G for CTR, and 2 and 72 h), although p38 was more strongly labeled with [^{32}P]GAT14 than with [^{32}P]T14H, indicating that all of the polypeptides detected with [^{32}P]T14H could interact with the three different RNA fragments tested.

Competition with homologous and heterologous competitors. Figure 2B demonstrates that cold T14H competitor significantly reduced the intensities of the signals revealed by EMSA using [^{32}P]T14H. To further characterize the polypeptides cross-linked with [^{32}P]T14H, competition in cross-linking was examined using both homologous and heterologous competitors. As can be seen from Fig. 3, of the signals revealed with [^{32}P]T14H, the signals of p115 in normal rat and of p98 and p88 in 72 h rat were abrogated with the homologous competitor, T14H. This competitor less effectively reduced the intensity of p120 signal. The degree of competition by RIT14 and HRI, when each separately used as a competitor, was similar to those of T14H. Concerning the p62 signal, however, the competitive reaction of RIT14 with [^{32}P]T14H appeared stronger than that of T14H with [^{32}P]T14H, suggesting that p62 interacted with a higher specificity for RIT14 than for T14H. Since simultaneous use of the three regions enhanced the degree of competition with [^{32}P]T14H on p62 (lanes All, in Fig. 3), it was also likely that a three times larger amount of the competitor successfully reduced signal intensity of p62. Furthermore, as would be expected from the series of results obtained, T14H and GAT14 could compete with each other (Fig. 4). To tentatively explain why the four riboprobes with different structures could be bound with the identical set of polypeptides, these RNA fragments were finally examined for homologous regions by GENETYX-SV/RC (Software Development Co., Ltd., Tokyo, Japan). Their nucleotide sequences confined three homologous stretches each comprising hexanucleotides (Table 1). It is unclear at present whether these were the sites responsible for interaction with the polypeptides presently detected, although short fragments were protected (not shown).

TABLE 1

Homologous Sequences in RIT14, T14H, HRI, and GAT14

RNA	Sequence 1	Sequence 2	Sequence 3
RIT14	746 gCCUGGGc	412 uUUCCUGc 1001 aUUCCUGc	648 uGAAGCUc
T14H	1085 uCCUGGGc	1084 cUUCCUGg	1296 aGAAGCUu
HRI	1395 aCCUGGGa	1427 cUUCCUGa	1432 uGAAGCUc
GAT14	882 uCCUGGGc	997 uUUCCUGg	988 uGAAGCUc

Note. The sequences conserved in the four riboprobes were listed with uppercase letters. The positions of the first nucleotides were indicated with the nucleotide numbers in albumin mRNA (RIT14, T14H, and HRI) and GAPDH mRNA (GAT14).

DISCUSSION

The present study first demonstrated that liver S100 fractions from normal and CCl_4-treated rats contain the eleven polypeptides cross-linked with T14H derived from the protein coding region of albumin mRNA, although they did not appear to be specific to this mRNA. On the other hand, albumin mRNA was found to be a sensitive marker of CCl_4-induced liver injury, in that its production and degradation promptly responded to pathological conditions developing following CCl_4 administration. For instance, while albumin mRNA was initially degraded at an enhanced rate but quite stabilized after 9 h, its production was conversely inhibited until 24 h and restored after 48 h. Based on the combination of these distinct molecular properties of albumin mRNA turnover, the entire course examined was tentatively classified into three stages, the first stage until 9 h, the second from 9 to 24 h, and the third after 48 h.

In addition to the changes listed above, the aberrant dissociation of membrane-bound polysomes and suppressed biosynthesis of proteins were reported to occur at periods comparable to the first stage (1, 2, 4). Production of HGF (15) and incorporation of thymidine into DNA (3) precede the third stage. Therefore, the first and third stages are assigned to the periods for aggravation of injury and for repair of liver injury and regeneration of liver, respectively. Interestingly, as represented by p33 in the first stage and p120, p115, p66, p48, p40, and p38 in the third stage, profiles of the polypeptides detectable with T14H were altered together with the changes in stability of albumin mRNA, although the relationships among the mechanisms underlying these events are unclear at present.

RNA binding proteins generally function in various processes involved in biogenesis, transport, storage, translation, and degradation (16, 17), all of which are affected in CCl_4-induced liver injury. For instance, it is conceivable that polysomes degraded in the first stage are restored in the third stage. In the first stage, therefore, albumin mRNA in polysomes dissociated from ER appeared to enter pathways for degradation. Conversely, in the third stage accumulated albumin mRNA might be utilized to restore the level of polysomes for albumin production, apparently stabilizing albumin mRNA.

As a protein that can bind albumin mRNA, a 32 kDa protein required for transport of mRNA including albumin mRNA was purified from rat liver (18). A polypeptide with a similar size, p33, was detectable with T14H. On the other hand, the only reported study of an RNA binding protein in rats treated with CCl_4 (19) showed that iron regulatory protein (IRP)-2, which controls both the translation activity of ferritin mRNA and the stability of transferrin receptor mRNA (20, 21), is induced as a regulator in iron homeostasis in regenerating liver. Taking into account the present and previous findings, the polypeptides we detected appear to play roles in physiologically significant processes involved in utilization and turnover of albumin mRNA. Structural and functional analyses of the individual polypeptides would clarify their roles in normal and injured livers.

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