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Cytoplasmic protein binds *in vitro* to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs

(RNA–protein complexes/iron/translational control)

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ABSTRACT **The mRNAs for the heavy and light subunits of the iron-storage protein ferritin occur in cells largely as inactive ribonucleoprotein particles, which are recruited for translation when iron enters the cell. Cytoplasmic extracts from rat tissues and hepatoma cells were shown by an electrophoretic separation procedure to form RNA–protein complexes involving a highly conserved sequence in the 5′ untranslated region of both ferritin heavy- and light-subunit mRNAs. The pattern of complex formation was affected by pretreatment of rats or cells with iron. Crosslinking by UV irradiation showed that the complexes contained an 87-kDa protein interacting with the conserved sequence of the ferritin mRNA. We propose that intracellular iron levels regulate ferritin synthesis by causing changes in specific protein binding to the conserved sequence in the ferritin heavy- and light-subunit mRNAs.**

Ferritin, an iron-storage protein found in animal, plant, fungal, and bacterial cells (1), has a protein shell ($M_r \approx$ 500,000) consisting in vertebrates of 24 subunits of two kinds, heavy (H; $M_r \approx$ 20,000) and light (L; $M_r \approx$ 19,000). Iron administration to rats (2, 3), bullfrogs (4), or cells in culture (5–7) stimulates subunit synthesis by an actinomycin-resistant mechanism (8) involving mobilization of H- and L-subunit mRNAs from a cytoplasmic pool of inactive messages onto polyribosomes (7–9).

Ferritin genes for rat L (10) and H (11) subunits, human L (12) and H (13) subunits, chicken H subunit (14), and a bullfrog cDNA (15) all have a highly conserved 28-base sequence in their 5′ untranslated regions (5′ UTR) that is essential for the translational regulation of the ferritin L-subunit (16) and H-subunit (17) mRNAs by iron. The response to iron was eliminated by deleting part of the 5′ UTR containing the conserved 28-base sequence (16), thus suggesting that iron-sensitive factor(s) in the cytoplasm may bind to this sequence and regulate the availability of both ferritin messages for translation. Here we report the use of an electrophoretic separation method to identify cytoplasmic proteins forming complexes with the conserved 28-base sequence. Formation of these complexes is affected by pretreatment of the rats or cells with iron.

MATERIALS AND METHODS

Tissue and Cell Extract Preparation. Tissues were taken from male Sprague–Dawley rats (120–150 g). Rat hepatoma cells (FT0-2B) provided by R. W. Hanson (Cleveland) were cultured at the MIT Cell Culture Center to a density of 5×10^7 cells per 150-cm^2 flask. When appropriate, the rats were injected i.p. with a solution of ferric ammonium citrate (pH 7.4) providing 2.3 mg of iron per 100 g of body weight; control rats were injected with 5 mg of ammonium citrate. Cells in culture were iron-loaded with 120 μM ferric ammonium citrate (pH 7.4). S100 cytoplasmic extracts (18) from rat liver, heart, and hepatoma cells were stored at −80°C following protein determination by the Bio-Rad assay.

Preparation of RNA Transcripts. The [^{32}P]RNA transcripts (Fig. 1*A*) were prepared as follows: *a*, "5′-LRNA," a 118-base sense transcript prepared from *Sma* I-digested pGL-66, a rat L-subunit pseudogene (10), containing the first 65 bases of the 5′ UTR (including the conserved sequence), 33 bases of the 5′ flanking sequence, and 20 bases from pGEM2; *b*, "RLFL-RNA," a 198-base sense transcript prepared from *Hin*cII-digested rat L-subunit cDNA (19) containing the next 120 bases of the 5′ UTR and the first 63 bases of the coding region with 15 bases from spP64; *c*, "LCOD-RNA," a 247-base sense transcript prepared from *Nco* I-digested rat L-subunit cDNA (19) containing 227 bases of coding region and 20 bases from pGEM-blue; *d*, "L3′UTR-RNA," a 190-base sense transcript prepared from *Hin*dIII-digested rat L-subunit cDNA (19) containing 148 bases of 3′ UTR and 42 bases from pGEM2; *e*, "L66AS-RNA," a 159-base antisense transcript prepared from *Stu* I-digested pG-L66, a rat L-subunit pseudogene (10), containing 149 bases of 3′ UTR and 10 bases from pGEM2; and also "pGEM-RNA," a 172-base transcript prepared from the Riboprobe Positive Control Template (Promega Biotec, Madison, WI) by using SP6 polymerase. "5′-HRNA" is a 166-base sense transcript containing the 28-base conserved sequence prepared from *Dde* I-digested pG-H234, a subclone of the rat H-subunit gene (11), containing the first 101 bases of 5′ UTR, 33 bases of 5′ flanking region, and 27 bases from pGEM2. Transcription reactions were performed in the presence of 100 μCi (1 Ci = 37 GBq) [α-^{32}P]ATP, -CTP, -GTP, or -UTP (Amersham, >400 Ci/mmol); 50 μM of unlabeled nucleotide (corresponding to the limiting labeled nucleotide); 2.5 mM of the other three unlabeled nucleotides; and either phage SP6 or T7 RNA polymerase. After electrophoresis on a 5% denaturing polyacrylamide gel, the transcripts were eluted overnight at 25°C from gel slices in 0.5 M ammonium acetate/1 mM EDTA, concentrated in a Speed Vac (Savant), ethanol-precipitated, and resuspended in water to 10,000 cpm/μl (about 10,000 cpm/0.3 ng of RNA). Unlabeled RNA transcripts were synthesized by using 2.5 mM unlabeled nucleotides, including tracer amounts (0.1 μCi) of uridine 5′-[α-(^{35}S)thio]triphosphate (Amersham, >400 Ci/mmol) to quantitate the yield of RNA. The unlabeled transcripts were isolated after electrophoresis in 1.6% low-melting-point agarose gels (20).

RNA–Protein Binding Reaction and Electrophoresis of Complexes. Binding reactions were carried out with S100

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Abbreviations: UTR, untranslated region; H, heavy; L, light.

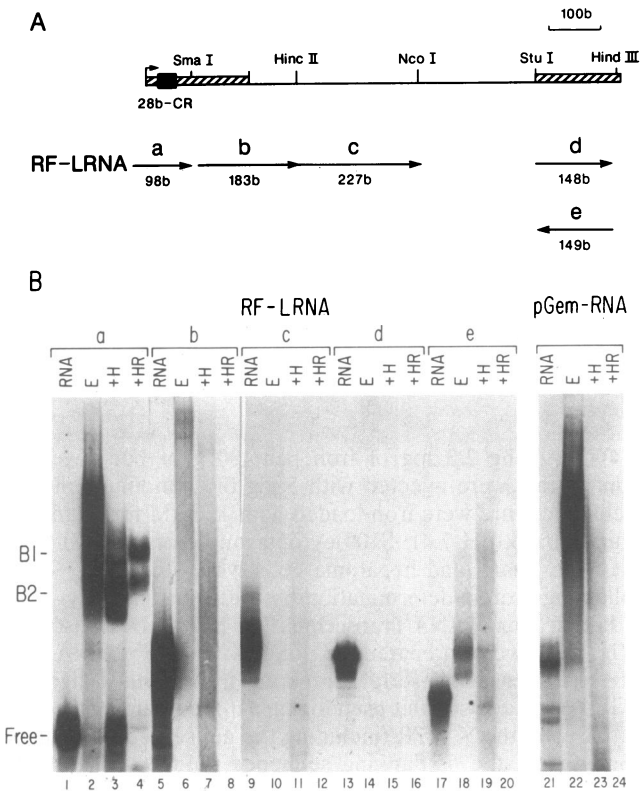

FIG. 1. Formation of specific RNA–protein complexes between 5′-LRNA and rat liver S100 cytoplasmic extracts. (*A*) Restriction map of rat liver ferritin L-subunit mRNA showing the 5′ UTR and 3′ UTR (hatched bars) and the coding region (line). Arrows indicate the orientation of [^{32}P]RNA transcripts from portions of this mRNA, with the nucleotide sizes indicated beneath each arrow. 28b-CR is the conserved region. (*B*) [^{32}P]RNA transcripts *a* through *e* in *A* and pGEM-RNA (control) were incubated at 25°C in a S100 rat liver extract. The products were analyzed by electrophoresis in a nondenaturing polyacrylamide gel either as the free RNA transcript [lanes 1, 5, 9, 13, 17, and 21 (lanes RNA)] or after incubation in the extract only [lanes 2, 6, 10, 14, 18, and 22 (lanes E)] or after heparin treatment [lanes 3, 7, 11, 15, 19, and 23 (lanes +H)] or after sequential addition of RNase T1 followed by heparin [lanes 4, 8, 12, 16, 20, and 24 (lanes +HR)]. B1 and B2 are complexes resolved in lane 4. Differences in gel mobility between transcripts d and e are due to the inclusion of different amounts of pGEM2 sequence.

cytoplasmic extract (40 μg) and 10,000 cpm (0.2–0.5 ng) of [^{32}P]RNA in 10 mM Hepes, pH 7.6/3 mM $MgCl_2$/40 mM KCl/5% glycerol/1 mM dithiothreitol in a volume of 10–15 μl. After incubation at 0°C or at 25°C for 30 min, 1 unit of RNase T1 (Calbiochem) was added for 10 min, followed by 5 mg of heparin per ml for 10 min, both at the temperature of the binding reaction. Electrophoresis of RNA–protein complexes was carried out as described by Konarska and Sharp (21) with modifications. A 4% nondenaturing polyacrylamide gel (acrylamide/methylene bisacrylamide ratio, 60:1) was preelectrophoresed for 30–60 min at 8 V/cm, followed by electrophoresis of the complexes for 3 hr at the same voltage. The dried gel was autoradiographed (−80°C).

Isolation of RNA–Protein Complexes and RNase T1 Mapping. To determine the RNA sequence protected by the complexes, 5′-LRNA transcripts were labeled with [α-^{32}P]CTP or [α-^{32}P]GTP or [α-^{32}P]UTP and added to either a control (0 hr) or to a 4-hr iron-treated rat liver S100 extract. Complexes B1 and B2 from the 0-hr extract and complex B1 from the 4-hr extract were cut out of the gel, transferred to dialysis bags and electroeluted in 45 mM Tris·HCl/45 mM boric acid/1 mM EDTA, pH 8.3, at 150 V for 2–3 hr, the current being reversed for the last 5 min of electroelution. After carrier yeast tRNA (15 μg/ml) was added, the isolated RNA was precipitated with ethanol, extracted with phenol/chloroform, reprecipitated with ethanol, and finally taken up in water to give 600–1000 cpm/μl. For RNase T1 mapping, ^{32}P-labeled protected RNAs (500–1000 cpm) were incubated in 10 mM Tris·HCl, pH 7.4/1 mM EDTA containing 10 units of RNase T1 for 30 min at 37°C. For comparison, intact 5′-LRNA labeled with [α-^{32}P]ATP, -CTP, -GTP, or -UTP was also digested with RNase T1. The samples were boiled for 2 min in loading dye (10 M urea/0.05% xylene cyanol/0.05% bromophenol blue) and electrophoresed on a 20% denaturing polyacrylamide gel. The gel was run at 2000 V until the xylene cyanol migrated 9 cm and was autoradiographed at −80°C. [^{32}P]RNA size markers (42 and 28 bases) were synthesized from *Hin*cII-linearized pGEM1 plasmid with SP6 polymerase (42-base RNA) or with *Hin*cII-linearized pGEM2 with T7 polymerase (28-base RNA).

Crosslinking of RNA–Protein Complexes by UV Light (UV-Crosslinking). RNA–protein binding reactions containing 0.3 ng of [^{32}P]RNA and 40 μg of S100 extract protein in a 15-μl volume were incubated at 0°C, followed by treatment with RNase T1 and heparin as described above. The Microfuge tubes containing the reaction products were covered with Saran Wrap and placed on ice, followed by irradiation 3 cm below a UV light for 30 min (Ultraviolet Products, San Gabriel, CA, 300-nm UV bulb; maximum intensity, 8000 μW/cm^2). The samples were then boiled for 5 min in $NaDodSO_4$ sample buffer, subjected to $NaDodSO_4$/8% PAGE, dried, and autoradiographed.

RESULTS

Demonstration of Specific RNA–Protein Binding. An electrophoretic method for detecting RNA–protein interactions based on the technique of Konarska and Sharp (21) was used to demonstrate that cytoplasmic proteins bind to specific sequences in the 5′ UTR of rat ferritin L- and H-subunit mRNAs. A S100 cytoplasmic extract prepared from rat tissues or cultured cells was incubated with a ^{32}P-labeled transcript of part of the 5′ UTR of rat ferritin L-subunit mRNA containing a highly conserved 28-base region (5′-LRNA, transcript *a* in Fig. 1*A*). RNase T1 and heparin were then added sequentially, and the [^{32}P]RNA–protein complexes were resolved in a nondenaturing low-crosslinked polyacrylamide gel. Heparin displaces proteins bound nonspecifically to the RNA, while RNase T1 treatment degrades 5′-[^{32}P]RNA bound nonspecifically to proteins. Concentrations of $MgCl_2$, KCl, and heparin and also temperature (<30°C) were adjusted to give optimal conditions for specific RNA–protein binding.

Fig. 1*A* displays RNA transcripts labeled with [^{32}P]GTP representing portions of the rat liver ferritin L-subunit mRNA (RF-LRNA, Fig. 1*A*; size and orientation of transcripts are shown by arrows *a–e* and described in *Materials and Methods*). Fig. 1*B* shows that, compared with free [^{32}P]5′-LRNA (Fig. 1*B*, lane 1), addition of S100 extract retarded its mobility in the form of a smear (Fig. 1*B*, lane 2), which was partially resolved into bands with heparin (Fig. 1*B*, lane 3). Addition of RNase T1 prior to heparin resulted in the resolution of two RNA–protein complexes, B1 and B2 (Fig. 1*B*, lane 4). A similar analysis of the other [^{32}P]RNA transcripts from RF-LRNA (Fig. 1*A*, arrows b–d), from pG-L66 (Fig. 1*A*, arrow e), and from pGEM-RNA showed that, although some formed smears with the extract or extract plus heparin, RNase T1 eliminated the binding completely, indicating that complexes B1 and B2 are specific to the 5′-LRNA transcript *a* (Fig. 1*B*, lane 4). Specific binding occurred within 1 min of addition of the 5′-LRNA to the S100 extract (data not shown) and is due to protein(s) in the extract, since B1 and B2 no longer form when the extract is pretreated with proteinase K (500 μg/ml) at 37°C for 1 hr.

Competition Studies. To show that B1 and B2 are specific complexes formed with the [^{32}P]5′LRNA, competition studies were performed. With 30- to 1330-fold molar excess of unlabeled 5′-LRNA as competitor (Fig. 2*A*, lanes 5–8), B1 and B2 began to disappear in the presence of 25 ng of RNA (80-fold molar excess) or more. In contrast, the nonspecific competitor L66AS RNA (Fig. 1*A*, transcript e) was not competitive even at high concentrations (Fig. 2*A*, lanes 9–14), nor was a 172-base pGEM-RNA or a yeast tRNA (data not shown).

To demonstrate that binding is specific for the conserved 28-base sequence, a ^{32}P-labeled transcript (5′-HRNA) was prepared from the rat H-subunit gene (11) consisting of 33 bases of 5′ flanking region and 101 bases of adjacent 5′ UTR, the only sequence common to the 5′-HRNA and the 5′-LRNA being the conserved 28-base sequence. When 5′-HRNA was incubated with the S100 extract, B1 and B2 appeared (Fig. 2*B*, lane 16). When unlabeled 5′-LRNA was added to the reaction, competition with 5′-HRNA was observed at 15 ng (30-fold molar excess) (Fig. 2*B*, lane 17) with complete loss of B1 and B2 observed at >60 ng (Fig. 2*B*, lanes 20 and 21). This suggests that B1 and B2 are specific complexes formed with the 28-base conserved sequence common to 5′-LRNA and 5′-HRNA.

Complex Formation Occurs with Extracts from Other Tissues and Cells and Is Affected by Iron. Iron administration to rats increases liver ferritin synthesis (2, 3) through mobilization of inactive ferritin H- and L-subunit mRNAs to the polysomes (8, 9). To determine whether previous iron administration affects formation of B1 and B2, rats were injected i.p. with ferric ammonium citrate, and S100 extracts were prepared from their livers and hearts at 0, 1, 4, 8, or 16 hr after injection of iron. When liver S100 extract was incubated with [^{32}P]5′-LRNA, B2 diminished at 1 hr after iron, was absent at 4 hr, and reappeared at 8 and 16 hr (Fig. 3*A*, lanes 2–6). This pattern was also observed with 5′-HRNA (Fig. 3*B*, lanes 19–23). The times of loss and reappearance of B2 coincide with the iron-induced mobilization of inactive ferritin L- and H-subunit mRNA (9). When heart

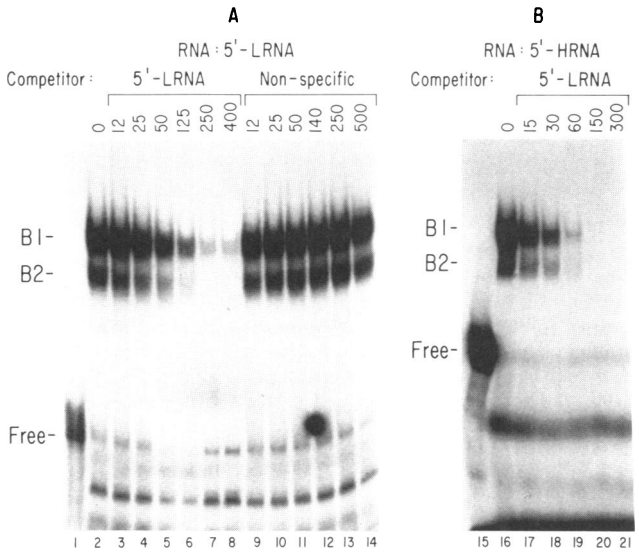

FIG. 2. RNA–protein complexes B1 and B2 form specifically with both 5′-LRNA and 5′-HRNA. (*A*) RNA–protein binding reactions were carried out as described in the legend to Fig. 1. [^{32}P]5′-LRNA (0.3 ng, 10,000 cpm; lane 1) was incubated with rat liver S100 extract alone (lane 2) or in the presence of either unlabeled 5′-LRNA (lanes 3–8) or nonspecific L66AS-RNA (lanes 9–14). (*B*) [^{32}P]5′-HRNA (0.3 ng, 10,000 cpm; lane 15) was incubated with a rat liver S100 extract alone (lane 16) or with unlabeled 5′-LRNA (lanes 17–21). Unlabeled competitors = ng per reaction mixture.

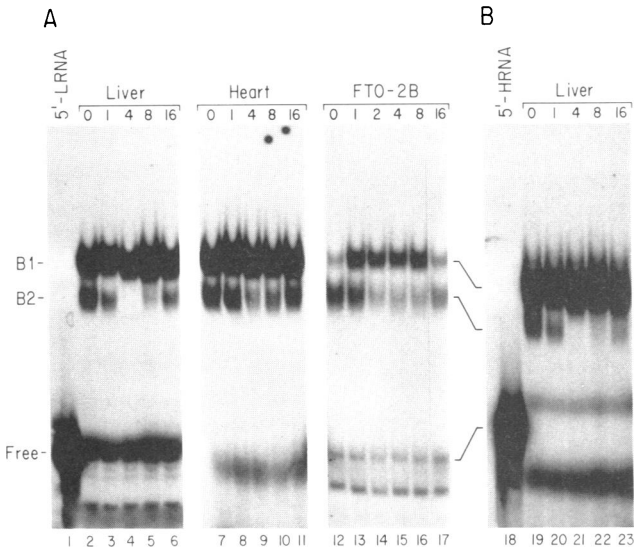

FIG. 3. RNA–protein complex formation with S100 extracts from tissues and hepatoma cells before and after iron treatment. (*A*) [^{32}P]5′-LRNA was incubated at 0°C with liver or heart S100 extracts from rats injected i.p. with ferric ammonium citrate for 0, 1, 4, 8, or 16 hr. S100 extracts were also prepared from hepatoma cells (FT0-2B) treated with ferric ammonium citrate for 0, 1, 2, 4, 8, and 16 hr. Complexes B1 and B2 were resolved as described in the legend to Fig. 1. (*B*) [^{32}P]5′-HRNA was incubated with liver S100 extract as described in *A*. Positions of complexes B1 and B2 and free RNA are indicated.

extracts from iron-injected rats were incubated with 5′-LRNA, B1 and B2 were again observed at 0 hr (Fig. 3*A*, lane 7), with B2 undergoing a moderate decrease at 4 and 8 hr after injection of iron (Fig. 3*A*, lanes 9–10).

To determine if B1 and B2 formed with S100 extracts from cells in culture, a rat hepatoma cell line FT0-2B was chosen because these cells show the translational shift from H and L ferritin messenger ribonucleoproteins to polysomes in response to iron (7). S100 extracts prepared from confluent FT0-2B cells treated with ferric ammonium citrate for 0, 1, 2, 4, 8, or 16 hr were incubated with [^{32}P]5′-LRNA. Complexes B1 and B2 were formed (Fig. 3*A*, lanes 12–17), but in contrast to liver and heart, the amount of B1 relative to B2 was greater at 0 hr and 16 hr. Like liver and heart, however, iron administration caused a reduction of B2 at 1, 2, 4, and 8 hr after iron injection with a reciprocal gain in the amount of B1 (Fig. 3*A*, lanes 13–16).

Identification of the Protected RNA Sequences by RNase T1 Mapping. Since the above data suggest interaction of protein(s) with the 28-base conserved sequence of [^{32}P]5′-LRNA, the length and sequence of the protected RNA were determined (Fig. 4). 5′-LRNA labeled with [^{32}P]GTP was incubated with S100 extracts from either 0-hr control rats (B1 and B2) or from 4-hr-iron-treated rats (B1). The ^{32}P-labeled protected RNAs isolated from the complexes were electrophoresed on a 20% denaturing polyacrylamide gel (Fig. 4*A*, lanes 2–4). RNA fragments of ≈40–50 bases were obtained for all three complexes. This heterogeneity in fragment lengths may be due to variable RNase digestion at the boundaries of the RNA–protein complex.

To determine the sequences of the protected 40- to 50-base fragments in lanes 2–4 of Fig. 4, each was subjected to a complete digestion with RNase T1, which cleaves after guanosine residues. The resulting fragments were compared to a complete RNase T1 digest of the 118-base [^{32}P]GTP-labeled 5′-LRNA (Fig. 4, lane 5), which yielded oligonucleotides varying from 12 through 2 bases. The RNase T1 digests of the protected 40- to 50-base RNAs showed a

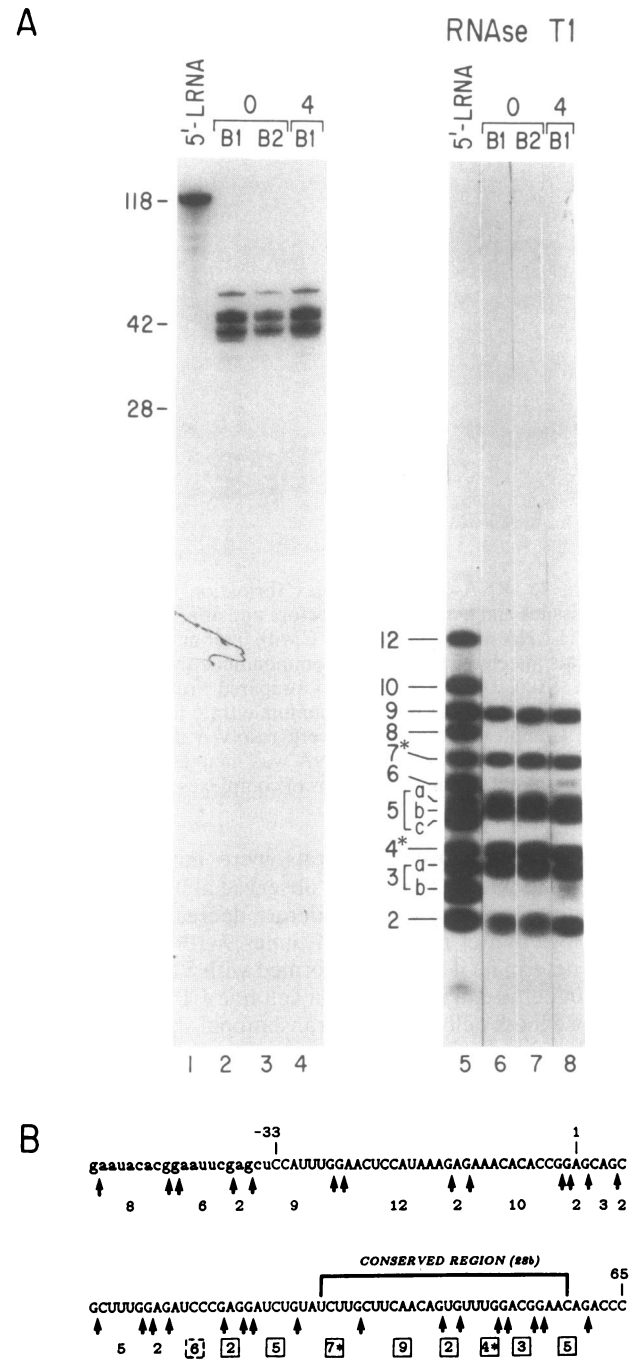

FIG. 4. Identification of the protected sequence in complexes B1 and B2. (*A*) Protected 5'-LRNA labeled with [^{32}P]GTP was isolated from complexes B1 and B2 (0-hr control) and complex B1 (4 hr after iron injection), electrophoresed on a 20% denaturing polyacrylamide gel (lanes 2–4), and compared to the size of intact [^{32}P]5'-LRNA (lane 1). RNA size markers are indicated in nucleotides. Protected [^{32}P]RNAs from the complexes (lanes 6–8) and intact [^{32}P]5'-LRNA (lane 5) were each digested with RNase T1 and also electrophoresed on a 20% denaturing polyacrylamide gel. Oligonucleotides are indicated according to size; asterisks on fragments 7 and 4 indicate that they are unique to the conserved region (see *B*). Multiple RNA fragments of 5 and 3 bases from 5'-LRNA migrate slightly differently (lane 5). (*B*) The sequence of the 5'-LRNA transcript is shown with arrows indicating guanosine residues cleaved by RNase T1. Numbers beneath indicate sizes of the predicted oligonucleotide fragments, boxed numbers indicate fragments obtained from RNase T1 digests of B1 and B2, asterisks indicate oligonucleotide fragments 4 and 7 unique to the protected sequence, and the dotted box indicates variable yields of the 6-base fragment.

subset of fragments of 9, 7, 5, 4, 3, and 2 bases for both B1 and B2 at 0 hr (control) and for complex B1 4 hr after iron injection (Fig. 4*A*, lanes 6–8). Importantly, in the 118-base 5'-LRNA labeled with [^{32}P]GTP, there is only one unique 7-base fragment and one unique 4-base fragment, both of which occur in the 28-base conserved region. These are accompanied by 9-, 5-, 3-, and 2-oligonucleotide fragments (Fig. 4*A*, lanes 6–8). In addition, a 6-base oligonucleotide is faintly visible in the digest of the protected sequence and may represent variable digestion at the 5' end of the protected region. These findings were confirmed by RNase T1 mapping with the intact 118-base 5'-LRNA as well as the isolated complexes B1 and B2 labeled with [^{32}P]ATP, -CTP, or -UTP (data not shown). Thus, RNase T1 mapping of the protected RNA can account for 40 bases, consisting of the 28 bases of the conserved region in addition to 10 bases upstream and 2 bases downstream from the conserved sequence (Fig. 4*B*). Included are three single guanosine residues that are not labeled and, therefore, not seen in Fig. 4*A*. At the 5' and 3' boundaries of the protected fragment, a faintly labeled 6-base fragment at the 5' end and an unlabeled 4-base fragment at the 3' end increase the length of the protected fragment to 50 bases, which may account for the variability in fragment sizes (Fig. 4*A*, lanes 2–4).

UV-Crosslinking of ^{32}P-Labeled 5'-LRNA to Protein in the Complexes. To identify protein(s) binding to the conserved 28-base sequence, [^{32}P]5'-LRNA was incubated with a control 0-hr liver extract and UV-crosslinked for 0, 1, 5, 15, or 30 min, and the products were analyzed on a 8% NaDodSO$_4$ gel. A 100-kDa RNA–protein complex appeared on the gel after 5 min and increased with longer exposure (Fig. 5*A*, lanes 1–5). When the binding reaction products were treated with proteinase K before UV-crosslinking, the 100-kDa RNA complex did not appear (Fig. 5*A*, lane 6), suggesting that protein was crosslinked with the RNA. The 100-kDa complex also did not occur when excess (125 and 250 ng)

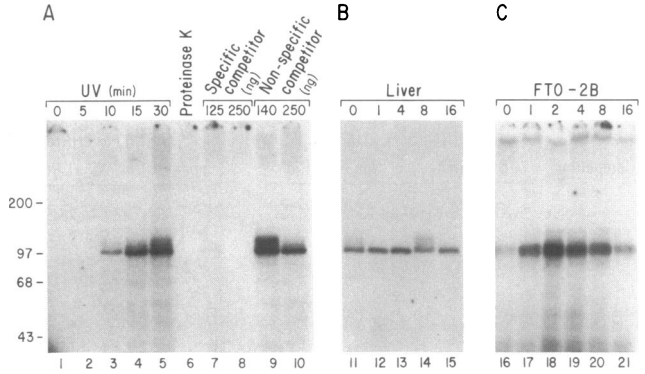

FIG. 5. Identification by UV-crosslinking of a 100-kDa RNA–protein complex formed with 5'-LRNA and S100 extracts from rat liver and hepatoma cells. UV-crosslinking of [^{32}P]5'-LRNA with S100 rat liver and hepatoma cell extracts was performed as described. The crosslinked products were subjected to NaDodSO$_4$/8% PAGE, dried, and autoradiographed. (*A*) UV-crosslinking was carried out for the indicated times (lanes 1–5). In lane 6, proteinase K (100 μg/ml) was added to the binding reaction after addition of RNase T1 and heparin, followed by incubation for 30 min at 25°C and UV exposure for 30 min. RNA–protein binding reactions were then carried out in the presence of either 125 or 250 ng of unlabeled 5'-LRNA (lanes 7 and 8) or with either 140 or 250 ng of unlabeled nonspecific L66AS-RNA (lanes 9 and 10), followed by UV-crosslinking for 30 min. Prestained high molecular mass markers (Bethesda Research Laboratories) are shown in kDa. (*B* and *C*) UV-crosslinking (30 min) of [^{32}P]5'-LRNA with the S100 extracts from rat liver at 0, 1, 2, 4, 8, and 16 hr after injection with ferric ammonium citrate (*B*) or with the S100 extract from rat hepatoma cells treated with ferric ammonium citrate for 0, 1, 2, 4, 8, and 16 hr (*C*). Exposures for *B* and *C* are for different times.

unlabeled 5′-LRNA was incubated in the binding reaction (Fig. 5A, lanes 7 and 8), whereas excess (140 and 250 ng) of an unlabeled nonspecific competitor (L66AS-RNA) had no effect on UV-crosslinking (Fig. 5A, lanes 9 and 10). Since the RNA fragment protected by S100 extracts is composed of approximately 40–50 bases, its contribution to the RNA–protein complex is about 13–16 kDa, leaving about 84–87 kDa accounted for by the binding protein.

When UV-crosslinking was tested with liver extracts of rats that had received iron for 0, 1, 4, 8, and 16 hr, a 100-kDa complex was present in all of the extracts. The 100-kDa complex was also observed in heart extracts from control and iron-injected rats (data not shown). When extracts from rat hepatoma cells treated with iron for 0, 1, 2, 4, 8, and 16 hr were UV-crosslinked with [^{32}P]5′-LRNA, a 100-kDa complex was again observed as in rat liver and increased after iron treatment (Fig. 5C, lanes 16–21). This pattern coincides with the greater amount of B1 in iron-treated cells (Fig. 3A, lanes 12–17), suggesting that the protein involved in crosslinking may be a component of complex B1.

DISCUSSION

Recently, it has been demonstrated that ribonucleoproteins, including splicing complexes (21–24) and complexes associated with 3′-end RNA processing (25), can be resolved by electrophoresis on nondenaturing polyacrylamide gels. We have modified the procedure of Konarska and Sharp (21) to identify RNA–protein complexes formed *in vitro* between cytoplasmic extracts of rat liver, heart, and hepatoma cells and [^{32}P]RNA transcripts from the 5′ UTRs of ferritin H- and L-subunit mRNAs. The inclusion in our procedure of RNase T1 in addition to heparin improved the resolution of the RNA–protein complexes (Fig. 1). This system may have advantages in identifying other sequence-specific cytoplasmic RNA-binding proteins.

Iron stimulates ferritin protein synthesis in rat tissues (2, 3) and cells in culture (5–7) by the translational mobilization of stored inactive ferritin H- and L-subunit mRNAs. Relevant to this activation is a highly conserved region of 28 bases in the 5′ UTR of all ferritin H- and L-subunit mRNAs so far sequenced (10, 14). When either rat L-subunit (16) or human H-subunit (17) 5′ UTRs are fused to the reporter gene encoding chloramphenicol acetyltransferase, expression of that enzyme becomes controlled by iron. Deletion of the first 65 bases of the 5′ UTR including the conserved 28-base sequence results in loss of chloramphenicol acetyltransferase regulation by iron (16), indicating a cis-acting regulatory sequence responsive to iron. Computer programs for predicting RNA secondary structures show a potential stem-and-loop structure formed by the 28-base sequence and adjacent regions in rat and human H- and L-subunit mRNAs (11, 16). Our results indicate that this conserved sequence forms complexes with proteins in the cytoplasmic extracts of rat tissues and cells and that these complexes respond to iron treatment in parallel with the time of translational activation of H- and L-subunit mRNAs (9). Interactions between proteins and RNA secondary structures have been identified as regulating translational repression in some prokaryotic systems (26–28).

To provide an explanation for the behavior of complexes B1 and B2 in response to iron, the following observations have to be reconciled. First, both B1 and B2 are formed with the same 40- to 50-base segment of 5′-LRNA, including the 28-base conserved region. Second, B1 and B2 form within 1 min of addition of [^{32}P]5′-LRNA to the cytoplasmic extract (data not shown), suggesting that either the binding factors have a high affinity for the ferritin mRNA sequence or that they are abundant in the S100 extract. Third, iron treatment of rats and of hepatoma cells (Fig. 3A) results in selective loss of B2, coincident in hepatoma cells with a gain in B1, thus suggesting a reciprocal relationship. However, interpretation of this is complicated because the decreased amount of stored ferritin mRNA after iron treatment may leave more protein free to bind to [^{32}P]5′-LRNA, explaining the increase in bound radioactive transcripts on B1 but not the reduction in B2 after iron treatment. Fourth, UV-crosslinking of [^{32}P]5′-LRNA to protein in rat liver (Fig. 5B), heart (data not shown), and hepatoma cells (Fig. 5C) suggests that only one 100-kDa RNA–protein complex crosslinks. In the case of hepatoma cells, the amount of crosslinking increases with iron treatment (Fig. 5C) in parallel with the amount of B1 (Fig. 3A), implying that the 100-kDa complex is formed with B1 but not with B2. In the case of B2, it is possible that the spatial orientation of the RNA to the binding protein(s) is altered and does not favor crosslinking. The mobility differences between the two complexes may be due to conformational changes or modified forms of the 87 kDa protein or the presence of additional subunits and factors. The binding of cytoplasmic proteins to regulatory regions on mRNAs may serve as modulators of activation/derepression of translationally controlled mRNAs.

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