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The iron-responsive element binding protein: A method for the affinity purification of a regulatory RNA-binding protein

(translational control/mRNA stability/posttranscriptional regulation/transacting factors)

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ABSTRACT **A method of affinity purification of a regulatory protein that binds specific RNA sequences is described. RNAs containing the regulatory sequences are transcribed *in vitro* from oligonucleotide templates, biotinylated, and incubated with unfractionated cytosol. Specific RNA–protein complexes are bound in solution to avidin, and the resulting complex is bound to biotin-agarose beads. The cytosolic binding protein is released from the RNA in high salt, and a second round of purification yields an essentially homogeneous protein. Using this method, we have identified the protein in human liver that binds iron-responsive RNA regulatory sequences. Iron-responsive elements (IREs) are RNA stem–loops present in the mRNAs encoding ferritin and the transferrin receptor. IREs form the basis for the translational regulation of ferritin gene expression and the regulation of transferrin receptor mRNA degradation rates. The IRE binding protein purified by this technique migrates as a 90-kDa polypeptide on SDS/PAGE. The interaction of the purified protein with IRE-containing RNAs can be detected by gel-mobility shift assays or by covalent crosslinking induced by UV irradiation.**

Our understanding of the molecular basis of gene regulation has increased markedly with the identification of cis-acting nucleic acid regulatory sequences and corresponding trans-acting regulatory proteins. Most of this work has focused on transcriptional control elements. Sites for the binding of specific proteins have been assessed by a number of techniques including the protection of the cognate DNA sequences against nuclease digestion or chemical modification or by assays that detect specific protein–DNA complex formation. The purification of trans-acting DNA binding proteins is essential to the description of a transcriptional regulatory system. A variety of approaches including affinity purification (1) and direct cloning from expression libraries (2) has led to the successful identification of these factors. In general, characterization of the molecular components involved in the posttranscriptional regulation of specific genes has lagged behind. Nonetheless, there is a growing list of examples of the regulation of gene expression at the RNA level. Recently, we and others have begun to define the elements involved in the posttranscriptional regulation of two genes central to cellular iron homeostasis in higher eukaryotes (3–14).

Iron is an essential component of numerous cellular enzyme systems including those that participate in oxidative metabolism and in the replication of DNA. Iron is indispensable to the function of these enzymes, but excess iron causes significant cellular toxicity. Though many important details in the uptake and distribution of iron are not yet understood, some of the proteins that play critical roles in these processes have been extensively studied. The transferrin receptor (TfR) regulates uptake of iron from transferrin and ferritin serves to sequester intracellular iron. The expression of genes encoding each of these proteins is primarily regulated posttranscriptionally. Regulation of expression of the TfR is achieved through differential degradation of mRNA according to cellular iron needs (3). The expression of ferritin is regulated at the level of translation initiation (4). Similar stem–loop structures exist in the 3′ untranslated region of the TfR mRNA and in the 5′ untranslated region of ferritin mRNA (5–8). These stem–loop structures are responsible for iron-dependent regulation of both of these mRNAs and have been termed iron-responsive elements (IREs). *In vitro* gel-retardation assays using radiolabeled RNA probes containing IRE sequences demonstrate that IREs from both the TfR mRNA and ferritin mRNA bind the same cytosolic protein (9, 10). Binding activity of this IRE-binding protein (IRE-BP) is increased in lysates from cells that have been pretreated with desferrioxamine, an iron chelator (9, 11). When binding activity is activated by iron starvation, the protein may function simultaneously as a repressor of ferritin translation and as an inhibitor of TfR mRNA degradation (6–8, 13). Inactive IRE-BP found in the cytosol can be activated *in vitro* with 2-mercaptoethanol (11). Oxidation–reduction of critical sulfhydryl groups of the IRE-BP appears to be the means by which iron regulates the RNA binding activity of the IRE-BP.

Further characterization of the function of this protein requires purification of the IRE-BP. In this paper we outline a purification scheme that is based on the high affinity of the IRE-BP for RNAs containing IRE sequences and on the demonstrated ability to utilize biotinylated RNA to purify RNA–protein complexes involved in RNA splicing (15, 16). RNA transcripts containing an IRE 5′ to a biotinylated unstructured tail are added in solution to unfractionated lysates. The high affinity interaction between biotin and avidin is used to attach the IRE/IRE-BP complex to a solid matrix, and the IRE-BP is subsequently eluted from the RNA with 2 M KCl. One round of affinity purification yields clearly identifiable IRE-BP in the presence of numerous contaminating bands, and a second round purifies the IRE-BP to near homogeneity. This method yields purified protein within hours. The purified IRE-BP migrates as a 90-kDa polypeptide on SDS/PAGE. When the eluates of the affinity resin are subjected to covalent crosslinking with an IRE-containing ^{32}P-labeled RNA, the resultant radiolabeled protein–RNA complex comigrates with the single protein that can be similarly crosslinked to IREs in unfractionated cellular lysates. Affinity purification of an RNA regulatory protein is an approach that may be applicable for purification of other RNA-binding proteins.

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Abbreviations: IRE, iron-responsive element; IRE-BP, iron-responsive element binding protein; TfR, transferrin receptor.

MATERIALS AND METHODS

Generation of *in Vitro* Transcripts. Oligonucleotide templates were generated on an Applied Biosystems model 381A oligonucleotide synthesizer, and full-length oligonucleotides were purified on a 10% acrylamide/8 M urea denaturing gel. A 17-base 3′ sequence (underlined below) was included to generate a T7 polymerase promoter after annealing with a complementary oligonucleotide. T7 polymerase was obtained from David Draper and Sarah Morse (Johns Hopkins University). Transcripts were generated according to Milligan *et al.* (17) with 2 mM allylamineuridine triphosphate (Enzo Diagnostics), 2 mM UTP, 4 mM ATP, 4 mM GTP, and 4 mM CTP (Pharmacia). Transcripts were trace-labeled (200 cpm/μg) by the inclusion of [^{32}P]CTP (ICN). Full-length transcripts were eluted from a 10% acrylamide/8 M urea denaturing gel in 1 mM EDTA/0.1% SDS/5 mM ammonium acetate followed by phenol extraction and ethanol precipitation. Biotinylation of allylamine-labeled transcripts was performed by using biotin-XX-NHS ester (Clontech) according to the manufacturer's guidelines. After biotinylation, RNA was ethanol precipitated and quantitated by spectrophotometry (OD at 260 nm). The oligonucleotide sequence for the template of the IRE-containing RNA was 5′ AAGAAGAAGAAGAAGAAGAAGAAGAGGAGGGGGAGGACATTATGGAAGACACTGCTCCCGATAATGTCCTCCCTATAGTGAGTCGTATTA 3′. The sequence of the template for the control non-IRE RNA was 5′ AAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAGAGAGATATTAATAGCCTTCGTCACGGAAGGTATTAATACCTCCCTATAGTGAGTCGTATTA 3′. The sequence of the template for the ferritin IRE used as a probe in the gel-shift assays and for UV crosslinking was 5′ GGGTTCCGTCCAAACACTGTTGAAGCAGGAAACCCTATAGTGAGTCGTATTA 3′. The predicted structures of the RNAs used for the affinity resins are shown in Fig. 1*A*.

Preparation of Lysate. Surgically removed samples of human liver were frozen at −70°C. Frozen liver was pulverized under liquid nitrogen and homogenized for 1 min at top speed in a Sorvall Omnimixer model 17105. Homogenization buffer (150 mM KCl/1.5 mM MgCl/10 mM Tris·HCl, pH 7.4/0.5 mM dithiothreitol/25 μM *p*-nitrophenyl *p*′-guanidinobenzoate/10 μg of leupeptin per ml) was added to the frozen liver (1.5 ml of buffer per gram of liver) to give a final protein concentration of lysate of 35–70 mg/ml. The lysate was clarified by centrifugation (10 min at 2000 × g followed by 1 hr at 100,000 × *g*). Protein concentrations were determined by BCA (bicinchoninic acid) assay (Pierce).

Solution Binding and Protein Purification. Lysates were pretreated for 15 min with Inhibit-ACE at 333 μg/ml (5 Prime → 3 Prime) and 2% 2-mercaptoethanol. RNA transcripts were added at a final concentration of 100 nM. Binding at room temperature for 20 min was followed by the addition of succinylated avidin (EY Laboratories) at a final concentration of 1 μM for 10 min. Biotin-agarose (Vector Laboratories) was added (1 ml of a 50% slurry per 10 ml of lysate) after prewashing the beads with 2 M KCl followed by three washes with lysis buffer. After 45 min of tumbling at room temperature, the beads were centrifuged and washed five times with lysis buffer (each wash was 25 times the bead volume). Protein was eluted from the beads in 2 M KCl/0.75 mM $MgCl_2$/5 mM Tris·HCl, pH 7.4 (elution buffer). This material was then adjusted to a final concentration of 150 mM KCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$, and 2-mercaptoethanol was added to a final concentration of 2% (final volume was 5–10 ml). RNA (100 nM) was added to the diluted eluate of the first column, and binding to the probe was followed by addition of avidin and biotin-agarose as described above. The

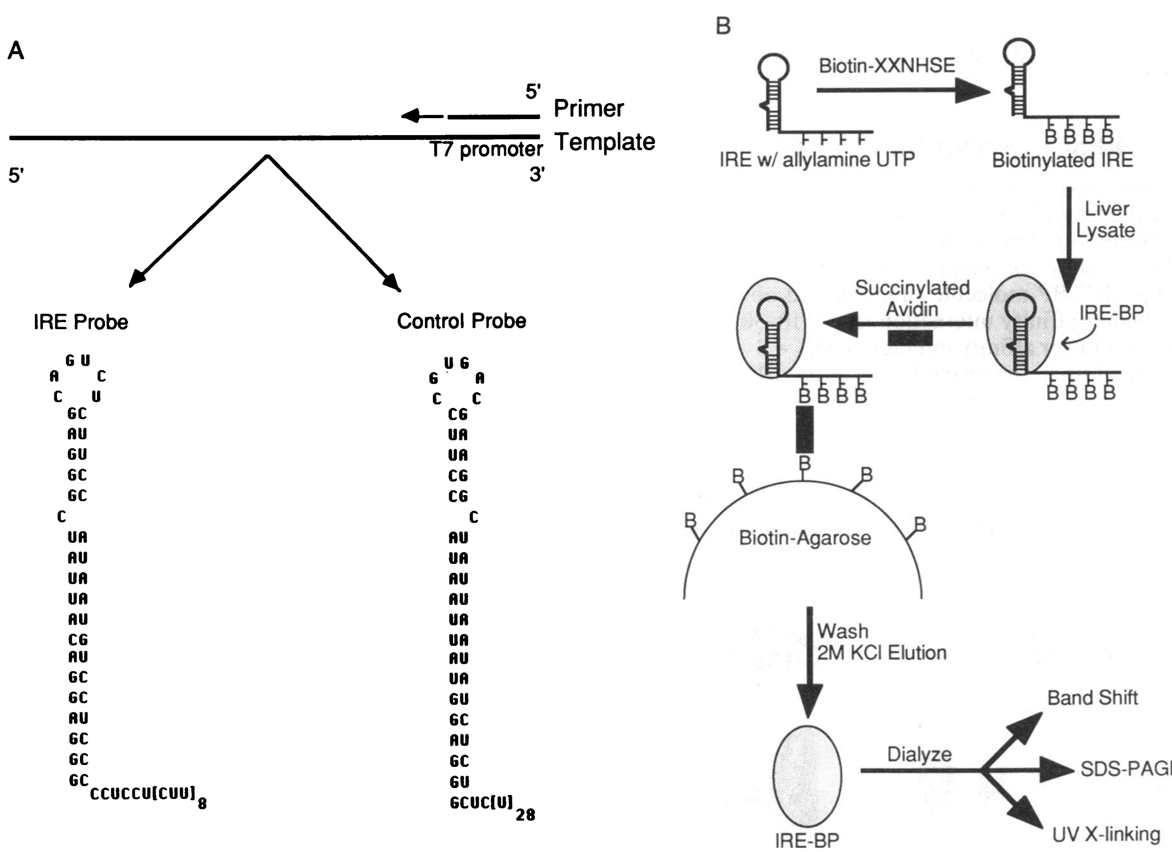

FIG. 1. (*A*) Illustration of the predicted secondary structure for the IRE and control probes. (*B*) Schematic of a single round of the affinity purification scheme. The allylamineuridine triphosphate is incorporated at random throughout the entire transcript. X-linking, crosslinking.

beads were then washed as described above. Binding protein was eluted in 600 μl of elution buffer over a period of 30 min in two successive elutions, and the pooled eluates were dialyzed against 250 volumes of standard lysis buffer for 1 hr on VS Millipore filters with a pore size of 0.025 μm (Millipore). Samples were used for RNA gel-retardation assays (see below) or were loaded onto 7% SDS/PAGE gels after boiling for 10 min in Laemmli sample buffer (18). Proteins were visualized by silver staining (Bio-Rad) or with Coomassie blue G-250 (Bio-Rad). Proteins were quantitated by BCA assays. A single round of the protein purification scheme is shown in Fig. 1*B*. In some experiments, RNA was omitted from the control sample. Coomassie blue-stained gels from these controls did not appear to differ from control samples that were incubated only with avidin plus biotin-agarose.

Gel-Retardation Assays. Assays were performed by using various amounts of ^{32}P-labeled RNA probes and protein in 20-μl volumes containing 2% 2-mercaptoethanol and Inhibit-ACE (11). Probes used in these assays were 35-base transcripts corresponding to the ferritin 5′ IRE sequence as shown above. In contrast to the previous descriptions of this assay, neither heparin nor RNase T1 was included. Binding at room temperature for 20 min was followed by addition of sample buffer containing glycerin and bromphenol blue, and the complexes were resolved on a 4% acrylamide nondenaturing gel as previously described (9–11, 14).

***In Vitro* Crosslinking.** Eluates from the IRE resin or control resin were added to a high-specific-activity ferritin IRE probe (500,000 cpm/ng) at a final concentration of 500 pM in the presence of 2% 2-mercaptoethanol. After a 30-min room temperature binding reaction, RNase T1 (1 unit; Calbiochem) was added for 10 min followed by addition of heparin (5 mg/ml; Sigma) for 10 min. Crosslinking was performed in microfuge tubes for 5, 10, and 30 min with a short wavelength UV lamp (Ultraviolet Products, San Gabriel, CA) held at 3 cm according to the method of Leibold *et al.* (14). Samples were boiled in Laemmli sample buffer and analyzed on 7% SDS/PAGE gels.

RESULTS AND DISCUSSION

We have previously demonstrated that IRE stem–loops interact specifically with one or more cytosolic proteins (9, 10). We recently measured the affinity of this interaction and have identified two classes of binding sites that we believe reside on the same IRE-BP molecule (unpublished observations). There is a higher affinity interaction (K_d = 10–30 pM) and a more abundant lower affinity interaction (K_d = 3–5 nM). The K_d is dependent upon the exact details of the IRE sequence. For use in affinity purification, we chose a synthetic IRE sequence that demonstrates high-affinity binding to IRE-BP and was derived from the sequence of one of the IRE stem–loops found in the 3′ untranslated region of the TfR. A control affinity matrix was synthesized by inverting the sequence of this synthetic IRE (Fig. 1*A*). Direct binding and binding competition studies demonstrate that this control RNA transcript has no detectable affinity for the IRE-BP despite the fact that it is capable of assuming a similar stem–loop structure. We reasoned that the high affinity of the IRE/IRE-BP interaction as measured by the gel-retardation assay would be sufficient for affinity purification.

One of the problems associated with attempting to isolate an RNA-binding protein by RNA affinity chromatography is the degradation of the RNA probe by endogenous RNases contained with the unfractionated lysate. We found that, in the presence of appropriate concentrations of Inhibit-ACE, the RNA transcripts that we used remained essentially intact. After interacting with the lysate, 40–80% of the probe was bound to the biotin-agarose beads. After binding of the RNA–protein complex to the beads, the beads were extensively washed. We screened different elution techniques by examining which treatments could dissociate an IRE/IRE-BP complex in solution and still allow recovery of binding activity after removal of the elution agent. This screening led us to choose 2 M KCl as an effective and reversible (by dialysis) eluant.

Previous studies (11) have shown that free sulfhydryl groups in the IRE-BP are critical for binding to the IRE. We treated unfractionated liver lysate with 2% 2-mercaptoethanol to recruit maximal amounts of IRE-BP into an active binding form and similarly treated the eluate from the first round to fully activate binding activity prior to the second round of affinity chromatography.

We analyzed two sequential rounds of this affinity purification. For this purification, we began with 450 mg of unfractionated clarified liver lysate protein. Eluates from control and IRE-containing beads were assayed for IRE-BP activity by a gel-shift assay. These assays were performed after dialysis to lower the KCl concentration to 150 mM. The results are shown in Fig. 2. Yields of IRE-BP from the first and second rounds of purification were calculated by using titration curves of eluates with various concentrations of the ferritin IRE probe. Eluates from the second round of the control resin demonstrated no binding activity, and eluates from the first round usually demonstrated no binding activity, though in some preparations there was detectable activity in the first round control eluate (always less than 10% of the binding activity obtained from the IRE resin). Protein concentrations of eluates were measured, and the extent of purification was calculated. A Coomassie blue-stained gel of eluates from the control and IRE resins after one round of

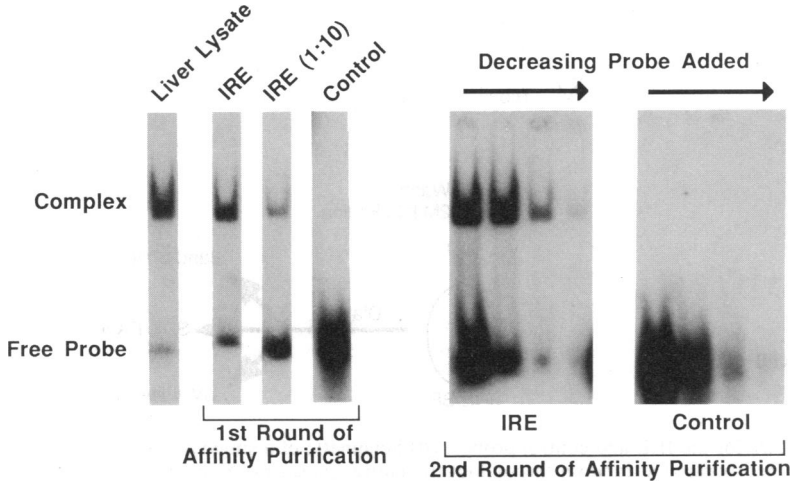

FIG. 2. The ferritin IRE probe (50,000 cpm/ng) was added to unfractionated liver lysate and to eluates from the IRE and control resins after the first and second round of affinity purification in the presence of 2% 2-mercaptoethanol and Inhibit-ACE. Gel-retardation assays were performed. (*Left*) Assays in which the ferritin IRE probe (20,000 cpm) was added to unfractionated liver lysate (5 μg of protein assayed of 450 mg of total starting material) or to 0.1% of the first eluate from the IRE resin (IRE), 0.01% of the IRE resin material (IRE 1:10), or 0.1% of the eluate from the control resin (Control). (*Right*) Complexes obtained with the addition of 20,000, 5,000, 1,250, and 600 cpm of probe, respectively, to 0.4% of the eluate from the IRE resin or from the control resin after a second round of affinity purification.

affinity purification is shown in Fig. 3. The arrowhead points to a clearly defined band at 90 kDa that is present in the eluate from the IRE resin, but is not visible in the eluate of the control resin. All other bands are present in the eluate from both resins. After one round of purification, the purification is estimated to be ≈200-fold, and the yield of IRE-BP as assessed by binding activity is 2–5%.

The second round of purification yields a highly purified 90-kDa polypeptide as assessed by silver staining of SDS/PAGE gels (Fig. 4). A band at 50 kDa most likely represents the 50-kDa subunit of the Inhibit-ACE that is added to the reaction mixtures. The amount of this 50-kDa polypeptide varied from preparation to preparation. IRE binding activity is present in these second round eluates as assessed by activity in gel-shift assays (Fig. 2). By using 450 mg of protein as starting material, the total amount of IRE-BP obtained after the second round of affinity purification is ≈200 ng based on the intensity of silver staining, and the final yield of IRE-BP is ≈0.2% as assessed by functional binding activity. Purified protein loses its binding activity over time in the buffers described and yield calculations that are based on binding activity, therefore, represent an underestimate of the chemical yield.

To assess whether the single 90-kDa protein specifically purified by the IRE-containing resin is identical to the IRE-BP that can be identified in the total liver lysate, eluates from the control resin and the IRE resin were UV crosslinked to a high specific activity. The IRE-containing probe and the ^{32}P-labeled protein were analyzed by SDS/PAGE. As shown in Fig. 5, the sole protein that is labeled is present in the eluate from the IRE column, and it comigrates with the complex that is obtained from unfractionated liver lysates. The relatively small change in migration of the protein after covalent linkage to the labeled RNA may reflect the removal of some of the RNA by the RNase. The finding of an identical crosslinked band in the liver lysate and in the affinity-purified material from both the first and second round of purification indicates that this protocol is isolating the major, if not the only, IRE-BP in the liver lysate. The photoaffinity labeling of the IRE-BP is specifically blocked by addition of excess unlabeled IRE-containing RNA and is not affected by the presence of nonspecific RNA competitor (data not shown).

This study demonstrates that the principle of affinity chromatography can be applied to purify a trans-acting RNA regulatory protein from cytosol. We chose to initiate the affinity purification by interacting the RNA with the lysate in solution in order to avoid the possibility that a biotinylated RNA bound to a resin would be sterically inaccessible. Once the RNA–protein interaction was established in solution, the addition of soluble avidin did not disrupt the interaction.

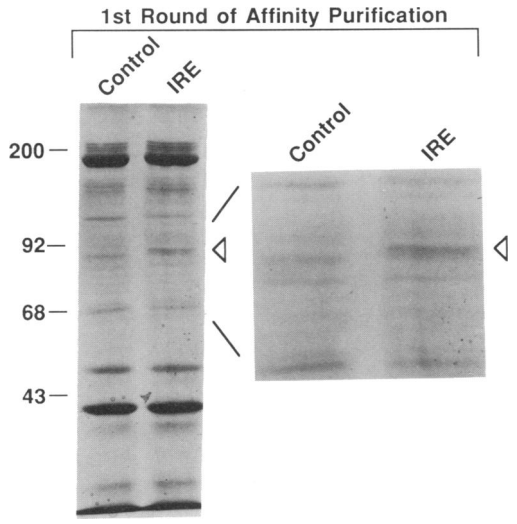

FIG. 3. Eluted material from the first round of affinity purification was stained with Coomassie blue after resolution of proteins by 7% SDS/PAGE. The arrowhead points to a 90-kDa band that is specific to the eluate from the IRE resin, and a 2-fold magnification of the 90-kDa region is shown to the right.

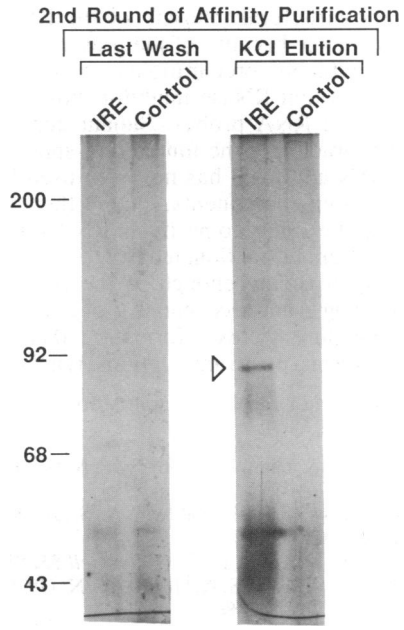

FIG. 4. Silver staining of 20% of the material in the last wash and 20% of the eluate of the second round of affinity purification after resolution by 7% SDS/PAGE.

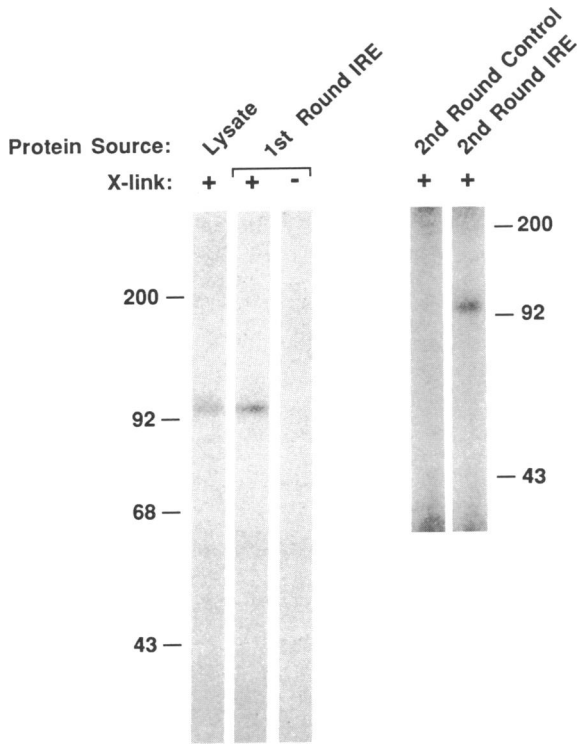

FIG. 5. Crosslinking of unfractionated liver lysate and fractions of eluates from the first and second round of purification from the IRE and control resins. The control samples used for crosslinking from the second round of purification were eluates from a column consisting only of avidin plus biotin-agarose. Aliquots of lysate and eluates were incubated with the ^{32}P-labeled ferritin IRE probe. Crosslinking was performed for 30 min. The ^{32}P-labeled proteins were analyzed by 7% SDS/PAGE. X-link, crosslinked.

Maintaining the structural integrity of the RNA transcript presented a challenge because of the presence of RNases in unfractionated cytosolic preparations. The use of high concentrations of a potent RNase inhibitor, however, successfully protected the RNA probes. Similar approaches have been used to characterize the function of spliceosomes (15, 16), although this approach has not been used for the purification of unknown components. One of the advantages of this approach is the ability to purify an RNA-binding protein to homogeneity from unfractionated lysates within hours. We have been able to isolate enough of the IRE-BP to allow protein sequencing from less than 1 g of cytosolic protein. Thus, this procedure yields information that can lead to successful cloning of a posttranscriptional regulatory protein.

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