Iron Regulatory Proteins 1 and 2 Bind Distinct Sets of RNA Target Sequences*

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Iron regulatory proteins (IRPs) 1 and 2 bind with equally high affinity to iron-responsive element (IRE) RNA stem-loops located in mRNA untranslated regions and, thereby, post-transcriptionally regulate several genes of iron metabolism. In this study we define the RNA-binding specificities of mouse IRP-1 and IRP-2. By screening loop mutations of the ferritin H-chain IRE, we show that both IRPs bind well to a large number of IRE-like sequences. More significantly, each IRP was found to recognize a unique subset of IRE-like targets. These IRP-specific groups of IREs are distinct from one another and are characterized by changes in certain paired (IRP-1) or unpaired (IRP-2) loop nucleotides. We further demonstrate the application of such sequences as unique probes to detect and distinguish IRP-1 from IRP-2 in human cells, and observe that the IRPs are regulated similarly by iron and reducing agents in human and rodent cells. Importantly, the ability of each IRP to recognize an exclusive subset of IREs was conserved between species. These findings suggest that IRP-1 and IRP-2 may each regulate unique mRNA targets in vivo, possibly extending their function beyond the regulation of intracellular iron homeostasis.

The regulation of cellular iron homeostasis is under the post-transcriptional control of iron regulatory protein-1 (IRP-1), a cytoplasmic RNA-binding protein with specificity for mRNA stem-loop structures known as iron-responsive elements (IREs) (reviewed by Klausner et al. (1993) and Kühn (1994)). IRP-1, formerly referred to as IRE-binding protein (Rouault et al., 1988; Lebold and Munro, 1988), iron regulatory factor (IRF; Mülner et al., 1989), or ferritin repressor protein (Walden et al., 1988), has been identified as the cytosolic counterpart of the citric acid cycle enzyme, aconitase (Hentze and Argos, 1991; Rouault et al., 1991; Kaptain et al., 1991; Haile et al., 1992a; Kennedy et al., 1992). IRP-1 is now regarded as a bi-functional “sensor” of iron, switching between RNA binding and enzymatic activities depending on cellular iron status (Haile et al., 1992a, 1992b; Constable et al., 1992; Emery-Goodman et al., 1993; Basilion et al., 1994). In iron-depleted cells, IRP-1 inhibits translation of ferritin and erythroid 5-aminolevulinic acid synthase mRNAs by binding to IREs located in their 5'-untranslated region (UTR) (Aziz and Munro, 1987; Hentze et al., 1987; Bhasker et al., 1993; Melfors et al., 1993). Binding of IRP-1 to a cluster of five IREs in the 3'-UTR of transferrin receptor mRNA stabilizes this transcript (Casey et al., 1988; Mülner and Kühn, 1988; Mülner et al., 1989; Koehler et al., 1989). The net result of this RNA-protein interaction is thus increased cellular iron uptake and availability. The inverse effect ensues when iron is high, as IRP-1 no longer binds well to the IRE hairpins (reviewed by Kühn (1994)).

A second IRE-binding protein has been characterized in rodents (Henderson et al., 1993; Guo et al., 1994) and in human cells (Samaniego et al., 1994), and is now commonly referred to as IRP-2. IRP-2 is a 105-kDa protein that binds specifically to all known mRNA IREs with an affinity equally as high as that of IRP-1 (Henderson et al., 1993; Guo et al., 1994). The two proteins are encoded by separate genes (Rouault et al., 1993), and their cDNA sequences (Rouault et al., 1992; Guo et al., 1995a) reveal conservation of cysteine residues known to ligate a [4Fe-4S] cluster in IRP-1 (Philippot et al., 1993; Hirling et al., 1994), although IRP-2 is apparently enzymatically inactive (Guo et al., 1994). The IRPs both respond to iron, but via different pathways. IRP-1 is post-translationally converted between active and inactive RNA-binding forms (reviewed by Kühn (1994)). IRP-2, however, is induced following iron starvation through renewed synthesis of stable IRP-2 protein (Henderson and Kühn, 1995; Pantopoulos et al., 1995), and its inactivation by iron reflects degradation of IRP-2 protein (Guo et al., 1994; Samaniego et al., 1994) by a translation-dependent mechanism (Henderson and Kühn, 1995). The IRPs are able to inhibit the translation of IRE-containing mRNAs in vitro (Guo et al., 1994; Kim et al., 1995), and therefore both proteins are potential mRNA regulators in vivo.

The possibility that the IRPs may bind additional IRE-like hairpins, and thus regulate an extended repertoire of mRNAs, is supported by a recent study in which a wide range of suboptimal IRP-1 binding sequences were selected from a partially degenerate pool of ferritin IRE RNAs (Henderson et al., 1994). Interestingly, several of these bound exclusively to IRP-1 and not to IRP-2. These findings suggest that the RNA-binding specificities of these two related proteins are overlapping, but different. We postulated that IRP-2 might also bind not only to wild-type IREs, but exclusively to a unique set of RNA targets. In this study we investigated this premise, using the ferritin IRE as a model for mutagenesis, as this sequence is the optimal IRP target and a range of well characterized mutants is already established for comparison (Henderson et al., 1994). By focusing on sequential mutagenesis of different IRE loop bases, we directly define and compare the RNA-binding specificities of mouse IRP-1 and IRP-2. Our new findings demonstrate the favorable effect that a structured IRE loop has on RNA binding.
by both IRPs and identify a novel set of four RNAs that bind exclusively to IRP-2. Furthermore, an assay is described for the discrimination of human IRP-1 from IRP-2, using IRP-specific RNA probes. Finally, we show that several features that distinguish the regulation of rodent IRP-1 and IRP-2 are well conserved in humans.

MATERIALS AND METHODS

Cell Culture and Treatments—Mouse B16.F1 melanoma cells and human HEK-293 fibroblasts and HL60 promyelocytic leukemia cells were grown in a minimal essential medium. Human Molt-4 T-cell leukemia, HeLa cervical carcinoma cells, HFF fetal fibroblasts, TK6 lymphoblasts, and HT-1080 fibrosarcoma cells were cultured in Dulbecco’s modified Eagle’s medium. The human colon carcinoma cell lines SW480 and Col15 were grown in L15 medium. All cell cultures were supplemented with 10% fetal calf serum, except for cell lines SW480, Col15, and HEK-24, which required 15% fetal calf serum. The chelation of intracellular iron was achieved by 20 h of treatment with 100 μM desferrioxamine (Desferal; gift from Ciba-Geigy, Basel, Switzerland). For iron treatments, 60 μg/ml ferric ammonium citrate was added to cells after washing once with phosphate-buffered saline.

Cell Fractionation Procedure—Mouse B16 cells were treated for 20 h with 100 μM desferrioxamine in order to fully induce IRE-binding proteins, and the cells were then harvested at 4°C in lysis buffer containing 10 mM Hepes, pH 7.5, 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 0.3% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. The cytoplasmic protein extract (36 mg) was next diluted to 24 ml in Buffer A (20 mM Tris-HCl, pH 8, 8 mM 2-mercaptoethanol (2-ME), 5% glycerol), resulting in a final salt concentration of 5 mM KCl. The extract was prefiltered through a 0.25-μm Nalgene filter prior to loading onto a Mono-Q anion exchange column (Pharmacia Biotech Inc.) equilibrated in Buffer A. The column was first washed in Buffer A, and flow-through and 10 ml washes were collected. Subsequently, bound proteins were eluted from the column in 40 1.5 ml fractions using a linear salt gradient of 5 mM to 600 mM KCl (in Buffer A). Phenylmethylsulfonyl fluoride was added to 1 mM to each fraction, and samples were stored frozen at −70°C. Each fraction was then tested for IRP binding activity. The fractionation was performed three times at 4°C, giving similar elution profiles for IRP-1 and IRP-2 each time.

Screening Fractionated Extract with Degenerate IRE Probes—Fractions containing IRP-1 or IRP-2 were detected by band-shift assay, using a wild-type ferritin heavy chain IRE probe (transcribed from a pGEM-3Zf(+) plasmid cloned into M13 mp18). The IRE probe (clone 42 RNA; 104 cpm, 0.093 nM) was added to each RNA (further details given by Henderson et al., 1993). The IRE probe was first denatured by heating for 10 min at 70°C. Every second fraction was tested by band-shift assay for IRE-binding activity. Each Series 1 pool maintained a fixed base at loop position 1, while the remaining 4 bases were completely randomized except for the base at position 6, which could not pair with the first loop base. Series 2 RNAs differed from those of Series 1 only in that the base at loop position 5 was constrained to pair with that of position 1. The loop sequence and degeneracy of these RNA pools is shown in Fig. 2. The RNAs were labeled and used to probe B16 fractions. In each case, protein was in excess, and the detection of IRP complexes was limited by the number of actual IRP molecules in each RNA mixture. The detection limit of the band-shift assay was about 100 cpm/complex, and mixed RNA probes were used at levels (5 × 10⁴ to 10⁶ cpm) sufficiently high to detect a protein binding to a single sequence with high affinity. While certain degenerate IRE pools detected IRP-1 or IRP-2, all RNA mixtures detected some additional proteins, none of which were sequence-specific as judged by band-shift competition assays.

Selection of IRE-binding Sequences—Oligonucleotide templates corresponding to the Series 2 RNA pools CG₁ and GC₁ were made double-stranded by annealing a T7 promoter primer and extending with Klenow polymerase (Boehringer Mannheim). The degenerate DNA duplexes were then cut with SalI and BamH1, and directionally cloned into pGEM-3Zf(−) (Promega) as outlined in Fig. 3. About 380 clones of each degenerate pool were screened for binding to mouse IRP-1 or IRP-2. Briefly, DNA was prepared from pools of five bacterial colonies and used as template in vitro transcription of 32P-labeled RNA probes. 5 × 10⁴ cpm of each gel-purified probe was mixed with 12 μg of total cytoplasmic extract and IRP-I/IRE complexes detected by band-shift assay. Individual IRP-binding sequences were subsequently identified by rescreening positive pools, and sequenced by the method of Sanger et al. (1977) using the M13 reverse primer and Sequenase version 2.0 (U. S. Biochemical Corp.).

RNA Preparation—Degenerate oligonucleotide templates were made double-stranded by polymerase chain reaction (see Henderson et al., 1994), to avoid folding of the DNA during transspiration. Plasmid DNA templates (in pGEM-3Zf(−)) were first linearized with BamH1 prior to RNA preparation. In vitro transcription was performed with T7 RNA polymerase (Promega) in a reaction containing 1.5 mM unlabeled ATP, GTP, and UTP, and 40–60 μCi of [α-32P]CTP (800 Ci/mmol; Amersham Corp.) for probes, or 1.5 mM CTP for unlabeled competitor RNAs. After transcription, RNAs were purified after denaturation at 150% polyacrylamide gels, and unlabeled transcripts were purified as described previously (Henderson et al., 1993).

RNA Band-shift Assay—RNA-protein complexes were resolved by band-shift assay. Gel-purified DNA-labeled RNAs were incubated for 10 min at 25°C with cytoplasmic extract, enriched IRP fractions, or recombinant human IRP-1 expressed as fusion proteins with glutathione S-transferase (Draper & Hentze, 1993) or a (His)₅ tag (Gray et al., 1993), and resolved on 6% non-denaturing polyacrylamide gels at 4°C as described previously (Müller et al., 1989). Unless otherwise stated, the binding reactions (20 μl final volume) were performed in the presence of 5 μg/ml heparin to displace nonspecific RNA-protein interactions, and with 2% 2-ME to activate IRPs in vitro.

More specific conditions were required to detect individual human IRPs using selected IRP-specific probes. Importantly, human IRP-2 appears to be more susceptible to oxidation than rodent IRP-2, and thus extracts were prepared fresh and processed quickly for experiments to be performed in the absence of reducing agent. Up to 4 μg of human cytoplasmic extract was preincubated with 300 ng of yeast tRNA, and then mixed with 5 × 10⁴ cpm of IRE probe (mutant GG1 or CG125) for 10 min in the presence or absence of 1% 2-ME (higher concentrations of 2-ME can decrease human IRP-2 activity), and in the presence of 5 μg/ml heparin, each selected RNA was 32P-labeled and gel-purified, incubated for 15 min at 25°C in the presence of 2% 2-ME, and IRP-I/IRE complexes detected by band-shift assay. IRP in the cell extract used was pre-determined by titration curves to bind [32P]IRE at a 1:1 molar ratio. Competition curves were plotted for each RNA, and the ratio of the D₅₋ VALUES of wild-type and mutant IRE sequences yielded K₁ₒ, the relative binding efficiency of each RNA (further details given by Henderson et al., 1993).

A further addition to competition for sequences that bind poorly to the IRPs, and are less likely to reach saturable binding, we adopted two approaches to measure relative binding affinities. The first approach was a competition assay to determine the D₅₋ VALUES of each IRP-binding sequence selected, where D₅₋ is the concentration of competitor required to reduce IRP binding by a 10-fold decrease (see Dingwall et al., 1991). The IRE probe (done 42 RNA; 10⁴ cpm; 0.093 μM) was added at first to a 1–1000-fold excess of unlabeled competitor RNA to IRP-enriched B16 fractions, incubated for 15 min at 25°C in the presence of 2% 2-ME, and IRE-IRP complexes detected by band-shift assay. IRP in the cell extract used was pre-determined by titration curves to bind [32P]IRE at a 1:1 molar ratio. Competition curves were plotted for each RNA, and the ratio of the D₅₋ VALUES of wild-type and mutant IRE sequences yielded K₁ₒ, the relative binding efficiency of each RNA (further details given by Henderson et al., 1993).

The second approach was to determine the dissociation constant for all the selected mutant IREs, which would prove difficult for sequences that bind poorly to the IRPs and are less likely to reach saturable binding, we adopted two approaches to measure relative binding affinities. The first approach was a competition assay to determine the D₅₋ VALUES of each IRP-binding sequence selected, where D₅₋ is the concentration of competitor required to reduce IRP binding by a 10-fold decrease (see Dingwall et al., 1991). The IRE probe (done 42 RNA; 10⁴ cpm; 0.093 μM) was added at first to a 1–1000-fold excess of unlabeled competitor RNA to IRP-enriched B16 fractions, incubated for 15 min at 25°C in the presence of 2% 2-ME, and IRE-IRP complexes detected by band-shift assay. IRP in the cell extract used was pre-determined by titration curves to bind [32P]IRE at a 1:1 molar ratio. Competition curves were plotted for each RNA, and the ratio of the D₅₋ VALUES of wild-type and mutant IRE sequences yielded K₁ₒ, the relative binding efficiency of each RNA (further details given by Henderson et al., 1993).

UV Cross-linking of RNA-Protein Complexes—For UV cross-linking, 20 μg of gel-purified RNA was incubated with an excess of IRP-containing cytoplasmic extract, in the presence of 15% 2-ME, for 5 min at room temperature. The specificity of the reaction was enhanced by the addition of 100 ng of yeast tRNA and 200 ng of a random stem-loop RNA (Henderson et al., 1993). Unbound probe was degraded by addition of 0.1 unit of RNase T1 (Calbiochem) for 5 min, after which heparin was added to 5 mg/ml for 10 min. The reaction mixtures were then subjected to a UV-C cross-link was performed on ice. After incubation at 4°C, a 15-watt UV lamp for 25 min, followed by an additional 5-min incubation at 25°C with 1 unit of RNase T1. Samples were then denatured in SDS buffer and separated on a denaturing 8% SDS-polyacrylamide gel.
FIG. 1. Detection of IRE-binding proteins in mouse extract. Mouse B16 melanoma cytoplasmic extract was fractionated by linear gradient over a Mono-Q column, and 1 μl of selected fractions analyzed by band-shift assay. Samples were probed with 5 × 10^6 cpm of 32P-labeled IRE, and IRE-protein complexes corresponding to IRP-1 and IRP-2 are clearly indicated. A third IRE-binding protein was detected in fractions 16–19; however, this RNA-binding protein (RBP) was not specific for IRE stem-loops. The gel was scanned with a Molecular Dynamics PhosphorImager, and relative signal strength is plotted in the lower panel. The graph does not accurately portray the total relative amounts of IRP, as while each fraction volume was 1 ml, most IRP-1 eluted in the flow-through (24 ml volume). Cross-contamination of IRP fractions was minimal; < 0.3% of total IRP-2 activity eluted in the first wash, and in the major IRP-2 peak fraction (175 mM KCl), IRP-1 comprised <0.2% of total IRE binding activity.

RESULTS

Sequence-specific Binding of Two Distinct Proteins to Iron-responsive Elements—The objective of this study was to define and compare the RNA-binding specificities of IRP-1 and IRP-2. Since the cellular forms of each IRP can undergo modification (Eisenstein et al., 1993; Henderson and Kühn, 1995), we began with a systematic approach to enrich for all IRE-binding proteins within a single cell line, and then screened for IRE-binding sequences. Cytoplasmic extract from B16.F1 mouse melanoma cells (treated with desferrioxamine for 20 h to ensure IRP activation) was fractionated over a Mono-Q anion exchange column, and proteins eluted by linear gradient from 5 to 600 mM KCl. IRP-1 and IRP-2 activities were identified by RNA band-shift assay (see Fig. 1), and their specificity confirmed by UV cross-linking and RNA competition experiments (data not shown). Immunoblot analysis revealed a close correlation between IRP RNA binding activities and protein levels (see "Materials and Methods"). While most IRP-1 was recovered in the flow-through as previously observed (Barton et al., 1990; Henderson et al., 1993), a minor peak was eluted with 150–160 mM KCl (Fig. 1). This small peak of IRP-1 may represent modified protein; however, it did not appear to differ in RNA-binding specificity compared to the major form of IRP-1 (data not shown) and awaits more detailed characterization. Recovery of IRP-2 peaked at 175 mM, then trailed off, with some IRP-2 still detectable in 400 mM salt fractions. Interestingly, a third RNA-binding protein complexed with the wild-type ferritin IRE probe (see fractions 16–19, Fig. 1); however, this protein bound equally as well to other RNA stem-loops of different structure (e.g. yeast tRNA; data not shown) and therefore was not studied further.

As expected, the above analysis identified IRP-1 and IRP-2 as the only sequence-specific IRE-binding proteins in B16 cells. To test whether IRP-related proteins may exist that bind RNA hairpins of similar structure but different sequence to the IRE, we probed B16 fractions with a 32P-labeled ferritin IRE probe made degenerate at the 7 conserved loop and bulge nucleotide positions (see Henderson et al. (1994) and Fig. 2A), and detected several additional RNA-protein complexes (data not shown). In order to assess the specificity of the extra band-shift complexes, a set of four less degenerate RNA mixtures were prepared, in which bases at the bulge and the first loop position were fixed (see Series 1 RNA pools in Fig. 2A). Band-shift analysis of the mouse B16 fractions revealed binding of IRP-1 and IRP-2 to certain [32P]RNA pools (e.g. C1N5 and G1N5 probes in Fig. 2B); however, following detailed examination and a series of competition assays, we identified no other proteins with specificity for IRE-like sequences. We therefore have no evidence to suggest the existence of other IRPs.

RNA Recognition by IRP-1 and IRP-2 Requires a Structured IRE Loop—Previously, we reported binding of IRP-1 to IREs containing C1G5 or U1A5 loops (base pairing between positions 1 and 5), but not to G1C5-type IREs (Henderson et al., 1994). Since the G1C5 IREs tested could alternatively fold as tetraloops, which showed reduced binding to rodent IRP-1 and in particular to IRP-2 (Henderson et al., 1994), we have re-examined the IRP requirement for intra-loop base pairing using paired RNA mixtures incapable of tetraloop formation. A second series of IRE-like hairpin mixtures was prepared, less degenerate than the Series 1 RNAs (see Fig. 2A). The Series 2 RNA pools differed from the first series only in that the fifth base of the loop was constrained to pair with the first. This single change increased IRP-binding of certain IRE-like RNA pools by up to 4-fold (see band-shift in Fig. 2B). Quantitation of paired RNA probes (Fig. 2A) confirmed the preference of IRP-1 for C1G5 and U1A5 IREs, and in addition revealed strong binding of IRP-1 to G1C5 IREs (a 2-fold increase was expected, as IRP-1 also binds well to G1G5 IREs) (Henderson et al., 1994). Surprisingly, IRE binding by IRP-2 demonstrated a similar requirement for 1:5 loop pairing; however, IRP-2 did not recognize U1A5 IREs, and neither IRP bound detectably to IREs with an A1U5 loop (summarized in Fig. 2A).

Relative Binding Affinities of Selected IRP-1 and IRP-2 Binding Sequences—We predicted that 1:5 base pairing might position the IRE loop nucleotides 2, 3, and 4 for direct protein contact (see Henderson et al. (1994)). These bases may in part determine the binding specificity of IRP-1 and IRP-2. Working on this premise, we deduced degenerate DNA fragments corresponding to the CNNNCG and GNNNCN loop-containing IRE probes (described in Fig. 2) into pGEM-32f(–), and screened about 300 clones from each mixture for binding to IRP-1 or IRP-2 (procedure outlined in Fig. 3A). Individual positive clones were sequenced, and the binding affinities of in vitro transcripts then tested by competition assay and band-shift assay as described (see Henderson et al. (1994) and "Materials and Methods"). We identified 11 different positive C1G5-type
RNAs and 7 different G1C5-type hairpins. Selected 32P-labeled transcripts were gel-purified and compared for binding to mouse IRP-1 and IRP-2 relative to a wild-type ferritin IRE (see Fig. 3B). The 5'-GAGUCG-3' loop IRE (mutant GC6), which is wild type at loop positions 2-4, bound both IRP-1 and IRP-2 almost as well as the C1G5 wild-type sequence. The binding ratios (quantitation of band-shift complex formed relative to ferritin IRE) for individual sequences are summarized in Fig. 4 and are presented with relative affinity values (K_rel) calculated from competition assays. The two approaches indicated a similar trend in the binding affinities of each RNA, although the competition assays were performed in the absence of heparin and generally (with a few exceptions) scored higher values than the binding ratios obtained in the presence of 5 mg/ml heparin (see Fig. 4 and Henderson et al. (1994)).

Identification of IRP-2-specific RNA Target Sequences—
Some of the C1G5 sequences tested (CG mutants 305, 153, and 218) had been selected previously using a PCR selection/amplification approach (Henderson et al., 1994), and similar binding affinity values were obtained. Several sequences from each group bound well to both IRP-1 and IRP-2; however, we selected no sequence with preference for mouse IRP-1. By contrast, we identified four different suboptimal sequences, which bound preferentially to IRP-2 (binding 12 to 140 times stronger than to IRP-1; see Table I). Each of the IRP-2-specific RNAs maintain a G at loop position 3, but differ from the wild-type sequence at positions 2 and 4. Two of these RNAs share the same central loop sequence CGC, but differ in their type of intraloop base pair (compare mutants GC147 and GC157). The loop nucleotide G3 is clearly important in this context, as its replacement by U3 abolishes binding to both IRPs (seen negative G1C5 mutant GC-1, Figs. 3B and 4).

The IRE mutant CG125 (loop: 5'-CCGAGC-3') showed the greatest specificity for IRP-2, as judged by comparison of $K_{rel}$ values and binding ratios (Fig. 4, Table I). We tested further the specificity of this IRE variant by band-shift analysis (Fig. 5). Binding of the mutant CG125 probe to mouse IRP-1 and IRP-2 was compared to a wild-type IRE, in the presence or absence of heparin. Remarkably, in the absence of heparin, the CG125 probe bound mouse IRP-2 50% as well as the wild-type IRE, but it showed no detectable binding to IRP-1 (see Fig. 5).
IRP-1 and IRP-2 Differ in RNA-binding Specificity

The values shown are the binding ratios (taken from Fig. 4) and represent relative binding of RNAs to IRP-1 or IRP-2 in the presence of 5 mM heparin. Bold type indicates those bases with the potential to pair; underlined bases differ from wild type.

**Table 1**

| Clone | Loop sequence position | Relative binding ratio |
|-------|------------------------|-----------------------|
|       |                        | 1 | 2 | 3 | 4 | 5 | 6 | IRP-1 | IRP-2 |
| Wild-type | C | A | G | U | G | C | 1.0 | 1.0 |
| CG125 | C | G | C | G | C | C | 0.01 | 0.14 |
| CG284 | C | U | G | C | C | C | 0.01 | 0.15 |
| CG147 | C | G | G | C | C | C | 0.01 | 0.12 |
| GC157 | G | G | C | G | C | C | 0 | 0.10 |

**Fig. 4.** Sequence and binding affinities of selected RNAs. The loop sequence of selected RNAs is shown relative to the wild-type sequence (clone 42: 5'-GAGAGU-3'). Bases capable of pairing (positions 1 and 5) are in large type, and non-wild-type nucleotides are underlined. The relative binding affinities of each RNA were determined by competition assay (K\textsubscript{rel} values), or by \( ^{32}P\)-labeling each RNA and comparing IRP binding of each sequence with a wild-type IRE probe (see "Binding Ratios"; see Fig. 3B for an example). Both approaches utilized the band-shift assay (see "Materials and Methods" for details), and all binding affinities are relative to the wild-type IRE, set at a value of 1. Binding assays were performed in the presence of 2% 2-ME. ND, not determined. Mutant GC1-loop (loop: 5'-GCUCCG-3') is a negative control.

Increasing the stringency of the reaction by adding heparin, reduced binding of the CG125 probe to IRP-2 as expected (binding decreased to about 12% of wild type). This experiment confirms mutant CG125 as an IRP-2-specific RNA target.

**Conserved Specificity of IRE Variant Probes for Human IRP-1 and IRP-2**—The range of RNA sequences targeted by IRP-1 and IRP-2 overlap, but are clearly different. If these differences are of functional importance, then they should likewise be observed in human cells. Currently, there is no means to distinguish the RNA binding activities of human IRP-1 and IRP-2, as the human IRP-IRE complexes co-migrate on band-shift gels (Henderson et al., 1993). We therefore investigated whether RNA probes specific for mouse IRP-1 or IRP-2 might show similar preference for the human IRPs. In addition to demonstrating conservation of the binding specificity, such probes would provide a valuable tool to distinguish IRP-1 from IRP-2 in different human cell types. Toward this goal, we tested two partially characterized variant IREs, one specific to mouse IRP-2 (mutant CG125; this study), and a previously selected IRP-1-specific RNA, mutant GG1 (loop sequence, 5'-GAGAGU-3'; Henderson et al. (1994)). The variant IREs bind to rodent IRP-1 or IRP-2 with affinities ~50% and ~14%, respectively, that of a wild-type IRE in the presence of 5 mM heparin (see Fig. 4 and Henderson et al. (1994)).
in Different Human Cell Lines—Cytoplasmic extracts from different human cell types were treated in vitro with 2-ME to activate IRP RNA binding activity and analyzed by band-shift assay (Fig. 7A). The migration of human IRE-protein complexes was compared to that of mouse IRP-1 and IRP-2 fractions, and in human Molt-4 cytoplasmic extract (see "Materials and Methods"). Cross-linked RNA-protein complexes were separated on a denaturing 8% SDS-polyacrylamide gel. The probes covalently bound to ∼97-kDa proteins corresponding to mouse IRP-1 and/or IRP-2 as expected. All probes, including mutant CG125, cross-linked to a similar sized ∼97-kDa protein in human Molt-4 extracts, in addition to some smaller sized proteins that may include IRP degradation products. The autoradiographs shown represent 4-day exposures of x-ray film to the gels.

IRP-1 and IRP-2 Differ in RNA-binding Specificity

The IRP-1-specific probe generated a single human band-shift complex with a pattern very similar to that of the wild-type ferritin IRE probe; IRP-1 activity was lowest in human TK6 lymphoblasts and HL60 promyelocytic leukemia cells. By contrast, the IRP-2-specific probe formed a band-shift complex migrating at the same position as mouse IRP-2 (mIRP-2); however, IRP-1 and IRP-2-specific probes generated different patterns, corresponding to variations in expression of the human IRPs (hIRPs). The autoradiographs shown represent different exposures (e.g. ratio of exposure times was 1:2:9 for wild-type, mutant GG1, and mutant CG125 probes, respectively) to compensate for differences in binding affinities of the probes. This scaling was made according to the relative affinities of these probes for the mouse IRPs, as evident by comparison of the mouse IRP signals. B, gels were quantitated and the relative signal intensities plotted as shown, after normalizing the values for IRP-1 (mutant GG1; ×2) and IRP-2 (mutant CG125; ×9) probes against the wild-type IRE. To aid comparison, the IRP-2 activity profile is shown superimposed over the wild-type expression profile.

Regulation of Human IRP-1 and IRP-2 Activity by Iron and Reducing Agents—We next tested whether the activity of IRP-1 and IRP-2 is iron regulated in human cells. As illustrated in Fig. 8, IRP-1 was strongly induced by 20 h of desferrioxamine treatment and repressed by the re-addition of iron salts for 4 h in HL60 and HeLa cells, but not in Molt-4 cells. IRP-1 activities were mostly normalized following in vitro reduction with 2-ME.
log-phase or treated for 20 h with 100 μM Fe/C. The extract was prepared from Molt-4, HL60, and HeLa cells, untreated to account for differences in binding affinity. This experiment was tested with 10 μg of each extract was mixed with 6 x 10^7 cpm of gel-purified mutant GG1 (IRP-1-specific) and mutant CG125 (IRP-2-specific) RNA probes in the presence of 300 ng yeast tRNA, 5 μg/ml heparin, and the presence (+) or absence (−) of 1% 2-ME, and complexes analyzed by band-shift. Autoradiographs were exposed for 24 h (IRP-1 gel) or 48 h (IRP-2 gel). Gel bands were quantitated and the relative intensities plotted in the lower panel, after normalizing values to account for differences in binding affinity. This experiment was performed twice with similar results.

Interestingly, IRP-2 responded in much the same way as IRP-1 to changes in iron levels; however, its activity could not be recovered by 2-ME in extracts from iron-treated cells (Fig. 8). This finding is in accord with the distinction between IRP-1 and IRP-2 in rodent cells (Henderson et al., 1993). Our data suggest that iron decreases IRP-2 protein levels in HL60 and HeLa cells, which is supported by recent Western blot analysis of IRP-2 protein in HeLa cells (Guo et al., 1995b). IRP-2 was more active in untreated Molt-4 cells and HeLa cells than in HL60 cells. IRP-2 activity, like that of IRP-1, was also refractory to iron modulation in Molt-4 cells.

This experiment reveals that endogenous human IRP-1 and IRP-2 activities are iron-regulated. In addition, we observed that pretreatment of HeLa cells with cycloheximide blocked the inactivation of IRP-2 by iron, but not the inactivation of IRP-1 (see Fig. 8, lower panel, for quantitation). The same result was observed with the translation inhibitor anisomycin (data not shown) and correlates perfectly with recent data on rodent IRP-2 (Henderson and Kühn, 1995). When considered together, our findings strongly suggest that our probes do indeed detect and discriminate between the two human IRPs. Furthermore, the features that distinguish binding activity of IRP-1 from IRP-2 are well conserved between human and rodent cells.

**DISCUSSION**

The IRP-IRE interaction represents a unique cellular mechanism underlying the post-transcriptional regulation of several genes. Recent evidence supports the view that IRP-1 and IRP-2 each function as mRNA trans-regulators involved in maintaining intracellular iron homeostasis, as both proteins (i) are regulated by iron levels (Henderson et al., 1993), (ii) inhibit IRE-containing mRNA translation in vitro (Guo et al. 1994; Kim et al., 1995), and (iii) bind ferritin and transferrin receptor mRNA IREs with equally high affinity (Henderson et al., 1993; Guo et al., 1994). In this study, we further show that the two IRPs have conserved the ability to individually recognize exclusive and distinct sets of RNAs. This novel finding suggests the possibility that the IRPs may regulate additional mRNA targets. We also describe features of the IRE hairpin that permit binding by either or both IRPs and have used this information to establish an assay for the detection of individual IRP RNA binding activities in human cells.

IRPs Differ in RNA-binding Specificity—A two-step screening protocol was designed to define the RNA-binding specificities of mouse IRP-1 and IRP-2 (Figs. 2 and 3). The first stage revealed that IRE-binding by both IRPs required a (1:5) base pair interaction between positions 1 and 5 of the 6-base IRE loop. This was previously discovered as a critical feature of RNA-binding by IRP-1 (Henderson et al., 1994). The fact that optimal binding by IRP-2 also favored a defined loop structure may implicate some similarity in the RNA-binding domains of the two proteins. The type of loop (1:5) base pair influenced IRP binding efficacy, and, perhaps in turn, the stability of different 5 loop base pairs was affected by IRP contact. In the final selection, we identified C1G5 and C1C5 base pairs as the only ferritin IRE loop interactions tolerated by both IRPs.

IRP-1 was generally more tolerant of IRE base changes than was IRP-2, particularly of specific base alterations in the bulge and loop (1:5) base pairs. Only IRP-1 recognized IRE loops containing a U3A3 pair, and some less stable non-Watson-Crick base pairs (Henderson et al., 1994). While none of the 600 randomly selected C1G5 or C1C5-loop RNAs were specific for IRP-1, we identified four such sequences that preferentially bound to IRP-2. Each contained a double mutation of two loop bases (positions 2 and 4) predicted to be unpaired and accessible for protein contact. These findings are not restricted to the ferritin IRE, as fusion of an IRP-2-specific loop (mutant CG125: 5'-CCGAGC-3') to the erythroid 5-aminolevulinic acid synthase IRE stem resulted in comparable selectivity for IRP-2, although the binding affinity was greatly reduced (data not shown). We have thus dissected the wild-type IRE loop and identified specific changes in paired or unpaired nucleotides that are tolerated by only IRP-1 or IRP-2. These findings reveal that the IRPs bind overlapping, but quite distinct, sets of RNA targets.

The list of regulatory RNA-binding proteins is growing rapidly, and several of these bind with specificity to defined RNA hairpin structures (reviewed in Varani and Pardi (1994), Burd and Dreyfuss (1994), and McCarthy and Kolmus (1995)). Aside from the IRPs and tRNA synthetases, there are relatively few sets of related proteins known to discriminate between similar RNA hairpin targets via differences in loop sequence. The eukaryotic splicing snRNPs proteins U1A and U2B+ are one example; they share strong sequence similarity and bind closely related U1/U2 small nuclear RNA stem-loops (reviewed by Nagai and Mattaj (1994)). Furthermore, the bacteriophage MS2 and GA coat proteins are 62% identical in sequence and...
as translation repressors that recognize similar RNA hairpins differing primarily in the 4-base loop sequence (see Lim et al. 1994). Discrimination of the different RNA loop sequences by MS2 and LA coat proteins was attributed to a single amino acid residue (Lim et al., 1994). Thus, while the relatedness of IRP-1 and IRP-2 (Rouault et al., 1992) may account for their shared capacity to bind a wild-type IRE, it should prove at least equally as interesting to learn the amino acid differences which decide the individual specificities of the two IRPs.

In our view, intra-loop base pairing induces a conformational change within the IRE loop, such that the intervening three bases (positions 2, 3, and 4; see Fig. 2A for numbering) are made accessible for contact with IRP-1 and IRP-2 (model outlined by Henderson et al. 1994). We propose that the unpaired loop bases splay outward to form hydrogen bonds with specific IRP amino acids, as was shown previously for other RNA-protein interactions including that between the tRNA^Gin and tRNA^Asp anticodon loops and their respective glutaminyl- and aspartyl-tRNA synthetases (Rould et al., 1991; Cavarello et al., 1993), and for the U1 small nuclear RNA stem-loop and U1A spliceosomal protein (Oubridge et al., 1994). The distinction in binding by the IRPs arises following base substitution at IRE loop positions 2 and 4, which is clearly far better tolerated by IRP-2. It is interesting that IRP-1 is more tolerant of different 1:5 base pair combinations in the IRE loop. Perhaps after initial binding, IRP-1 is better able to fold the IRE into a conformational loop interaction, thereby enabling base pairs with lower energy to form. This explains the ability of IRP-1, but not IRP-2, to bind IRE-like hairpins with less stable non-Watson-Crick base pairs previously observed in other RNAs (Gutell, 1993; Henderson et al., 1994). Similar reasoning predicts RNA hairpins with C1G5 and G1C5-type loops to be the most stable, possibly even forming spontaneously in solution, and thus facilitating easier recognition by IRP-2. Indeed, the wild-type IRE loop may form a C1G5-type base pair in solution (Sierzputowska-Gracz et al., 1994), and it will be interesting to learn if alternate loop base pairs also form prior to RNA interaction with the IRPs. Ultimately, refined structural analyses of the IRP-RNA complexes are required to confirm these proposals.

Coordinate Iron Regulation of IRP-1 and IRP-2 in Human Cells—A novel and practical application was found for the selected RNA sequences, which were used as 32P-labeled probes to distinguish human IRP-1 from IRP-2. Unlike the rodent IRPs, human IRP-1 and IRP-2 are of similar charge, and their RNA binding activities cannot be distinguished by bandshift assay using conventional IRE probes. This has precluded any detailed analysis of individual endogenous human IRP activities; as a consequence, earlier studies that examined human IRE binding activity actually detected a combination of the two IRPs. Recent investigations have managed either to detect recombinant human IRP-2 expressed following transfection into human cells (Samaniego et al., 1994) or to supershift human IRP-2:IRE complexes with specific antibodies (Guo et al., 1995a).

In this study, we demonstrated that IRE-like hairpins specific for mouse IRP-1 or IRP-2 showed similar selectivity for the human IRPs. Despite an increase in background caused by a lower binding affinity of the IRP-2-specific probes, the availability of different IRP-2-specific and non-binding sequences enables a controlled assay for human IRP-2 activity. A survey of human cell lines revealed different IRP expression patterns, and as in most rodent cell lines (Henderson and Kühn, 1995), IRP-1 activity was always highest. In fact, the human IRPs displayed most of the previously defined characteristics that distinguish rodent IRP-1 and IRP-2. Both of the human proteins were regulated by iron, but only IRP-1 activity was recovered in extracts from iron-treated cells by in vitro reduction with 2-ME. We recently reported that iron-mediated inactivation of mouse IRP-2, but not IRP-1, was translation-dependent (Henderson and Kühn, 1995). This difference in the iron regulatory pathways of the two IRPs also applies in human cells. These findings establish several aspects of IRP regulation that are well conserved and describe a general assay to detect individual human IRP RNA binding activities.

Growing Complexity in IRP Regulation and Function—IRP-1 and IRP-2 are related proteins that share several common features; they each bind strongly to "wild-type" IREs conserved phylogenetically in all known IRE-containing mRNAs (Thel, 1994), their RNA binding activities are coordinately modulated by iron levels (Henderson et al., 1993; this study), and they inhibit translation of IRE-containing transcripts in vitro (Guo et al., 1994; Kim et al., 1995). Recent work, however, has uncovered some interesting differences between the two proteins. For instance, while iron inactivates both proteins, only IRP-2 is selectively degraded (Guo et al., 1994; Samaniego et al., 1994), and by a translation-dependent mechanism (Henderson and Kühn, 1995), suggesting that IRP-2 levels are independently controlled by another protein. Moreover, IRP-2 responds preferentially to certain stimuli, and in rat liver cells IRP-2 activity increases during tissue regeneration (Cairo and Pietrangelo, 1994), but decreases following oxidative stress in vivo (Cairo et al., 1994). These differences in IRP gene or protein regulation may reflect in the localization of these proteins. Expression patterns of IRP mRNA (Samaniego et al., 1994), protein (Guo et al., 1995a) and RNA binding activities (Henderson et al., 1993) vary among different tissues. The cell type-specific expression reported in this study further implies that the IRPs might function at different cellular locations or stages of development. Our current findings add to this complexity, by showing that each IRP has a distinct RNA-binding specificity, which is conserved between species. This implies that each IRP may regulate its own distinct set of mRNA targets in vivo. We are currently searching the nucleic acid data bases to identify such mRNA target sequences, in the hope that this might further elucidate the function of these proteins, and potentially link iron metabolism with other cellular processes.

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