Vertebrates control intracellular iron concentration principally through the interaction of iron regulatory proteins with mRNAs that contain an iron responsive element, a small hairpin with a bulged C. The hairpin loop and bulged C have previously been assumed to be critical for binding and have been proposed to make direct contact with the iron regulatory proteins. However, we show here that a U or G can be substituted for the bulged C provided that specific nucleotides are also present within internal loops. The Kd, IC50 and chemical modifications of the iron responsive element variants are similar to the wild-type. Results are more consistent with a role in which the C-bulge functions to orient the hairpin for optimal protein binding rather than to directly contact the protein. Characterization of these novel iron responsive element variants may facilitate the identification of additional mRNAs whose expression is controlled by iron regulatory proteins, as well as provide insight into the nature of a critical RNA-protein interaction.

A decrease in cytosolic iron concentration can stimulate the RNA binding activity of two different iron regulatory proteins (IRP-1 and IRP-2). IRP-1 and cytoplasmic aconitase are the same protein, but the RNA binding and enzymatic activities are mutually exclusive (1–4). When the intracellular iron concentration decreases, an iron-sulfur cluster necessary for aconitase activity is disassembled, which then activates the RNA binding site. IRP-2 has 57% overall amino acid identity to IRP-1 but contains an additional 73-amino acid insertion and is not converted to an inactive form under iron-replete conditions (12). IRP-1 but contains an additional 73-amino acid insertion and is not converted to an inactive form under iron-replete conditions, IRP-2 has 57% overall amino acid identity to IRP-1 and contains five IREs within its 3′-untranslated region. IRP binding inhibits translation by preventing recruitment of the small ribosomal subunit to these mRNAs, which leads to decreased iron storage (16). As a result, IRP binding to the transferrin and ferritin mRNAs can function synergistically to elevate the cytosolic iron concentration. The expression of the citric acid cycle enzyme mitochondrial aconitase and the heme biosynthetic enzyme erythroid δ-aminolevulinate synthase are also regulated through IRE-IRP interactions (17, 18). In addition, putative IREs have also been identified on the mRNAs encoding the iron transporters DMT1 and IREG1 (19, 20). Characterization of the IRE-IRP interaction, therefore, is of high biological significance.

Mutagenesis (21–24), phylogenetic analysis (25), in vitro selection (26, 27), chemical probing (28, 29), and NMR (30–32) have defined structural features of the IRE, and there is significant overlap in the structures recognized by both IRPs. The natural IREs all contain the hairpin loop sequence CAGWGH (where W is A or U and H is C, A, or U; Fig. 1A) that is necessary for high affinity binding to both IRPs. NMR and in vitro selection results indicate that the first and fifth positions of the hairpin loop form a Watson-Crick pair. A disordered C nucleotide is present in all identified natural IREs five base pairs from the hairpin loop (Fig. 1A, C–12) and has been assumed to directly contact the IRPs. In most IREs, the C is a single bulge in the helix of the IRE stem (32), but it is also found as part of a larger dynamic internal loop (31). This internal loop increases binding affinity for the IRPs and has been proposed to be part of a Mg2+ binding site that could affect the conformation of the hairpin (33). Our results indicate that a U or G can substitute for the C with little effect on binding to IRP-1 provided that additional nucleotides are also present within the bulged loops. Results are more consistent with a role for the IRE bulge/bulge loop in positioning the hairpin for optimal IRP-1 binding rather than in directly contacting the protein.

EXPERIMENTAL PROCEDURES

Protein Preparation—Human IRP-1 with an N-terminal His6 tag was expressed in E. coli as previously described (34) and purified by Q-Sepharose and nickel-chelate chromatography followed by dialysis against 25 mM Hepes, pH 7.6, 150 mM KOAc, and 1.5 mM MgCl2 to remove the imidazole. The protein was 98% pure as estimated from silver staining. Glycerol was added to a final concentration of 5% prior
to freezing.

RNA Preparation—The starting random RNA population was synthesized by T7 transcription from the double stranded DNA template: CCGAAGCCTTCTGCTAATGCAATGGA(N)50CACGUTTATAGTCUCCTCCCTATA GTGAGTCGTATTA, where N in theory is an equal representation of each of the four standard nucleotides. For the chemical modification studies, the selected U-bulge RNA (Fig. 1B) was synthesized by T7 transcription from a DNA template that added a 3’ extension (AUAAUACAGGAUAGGUGUAGG). This extension has less than a 2-fold effect on binding affinity (data not shown) and was used as a primer binding site for the detection of the modifications. All other RNAs were synthesized by T7 transcription from the corresponding oligodeoxynucleotide template.

Selections—The initial cycle of selection contained 200 pmol of the 50N random RNA. Assuming no other biases, this would have resulted in a 99% probability of having representation of any combination of 22 contiguous nucleotides (35). The RNA was incubated with 20 pmol of purified IRP-1 in 100 µl of 1× native buffer (10 mM Hepes, pH 7.5, 5 mM MgCl2, 40 mM KCl, 50 ng/µl bovine serum albumin, and 1% 2-mercaptoethanol). The binding reaction for the first 9 cycles was done at 22 °C. This was increased to 37 °C for the last four cycles to remove less stable structures. For cycles 1 to 7 and 10 to 13, the bound RNA was partitioned from the bulk population by filtration through nitrocellulose. The bound complex was partitioned on a native polyacrylamide gel for cycles 8 and 9 to decrease the likelihood that RNAs would be selected for an unintended criterion. In addition, a negative selection was exploited before cycles 2 through 6 so as to remove RNAs within the population with affinity to nitrocellulose or minor contaminants of the IRP-1 preparation. A 100-fold molar excess of the ferritin IRE (Fig. 1A) was included in the binding reaction of the 9th cycle of selection to compete away lower affinity RNAs. The selected U-bulge RNA was randomized at eight positions and subjected to three rounds of re-selection. The IRP-RNA complex was partitioned by filtration through nitrocellulose for the first 2 cycles and by native gel electrophoresis for the third. A 100-fold molar excess of the ferritin IRE was also included in the binding reaction of the third cycle of re-selection.

RESULTS

A Bulged C Is Not Essential—We initially exploited in vitro selection (36, 37) to generate novel high affinity IRP-1 binding RNAs from a starting population that had 50 random positions (50N). Unlike previous selections (26, 27), the random region was large enough to permit the selection of alternative structures requiring more sequence space, and in addition was designed to select for the highest affinity interactions (38). RNAs were cloned and sequenced after both the 9th and 13th cycles of enrichment. The sequence phylogeny and IRP-1 binding affinities are similar for both RNA populations (data not shown). All of the 27 sequenced clones contain the IRE hairpin loop (CAG-WGH) confirming the importance of this sequence. However, two of the selected high affinity RNAs have a U or a G at the bulge position rather than a C.

Binding affinity of the IRE variants for IRP-1 was determined by two methods. First, binding curves were generated by incubating the radiolabeled RNA with increasing quantities of purified IRP-1 and partitioning the bound complexes from the free RNA by filtration through nitrocellulose. The Kd was determined from an analysis of the binding curve that did not require an assumption that [IRP]free — [IRP]total (39). The measured value of 40 ± 30 pm (n = 3) for the affinity of the ferritin IRE is in agreement with previously published values (24, 40). The second method determined the Kd relative to that of a human ferritin IRE using a competition assay (Fig. 1C). Radiolabeled ferritin IRE RNA was incubated with IRP-1 in the presence of increasing quantities of unlabeled RNA competitor. A value for Kn, is defined as the IC50 of the unlabeled competitor RNA normalized to the concentration of the radiolabeled ferritin IRE. All data points for the binding curves were done in triplicate and the R2 for the curve fits is >0.9 for those variants that have a Kd or Kn, value within a factor of 20 of the wild-type ferritin IRE and >0.8 for the other variants. The Kd or Kn, values for some RNAs were obtained from several inde-

![Fig. 1](http://www.jbc.org/)
folding of the ferritin IRE, for which there is a larger body of supporting data. An RNA selected after 9 cycles of enrichment has a U at the bulge position and also differs significantly from the wild-type ferritin IRE in that there are two extra nucleotides at the 3'-end of the hairpin loop (CA), one additional C nucleotide within the top bulge and two within the bottom bulge (Fig. 1B). The PCR primer binding sequences were deleted from the selected RNA, and the lower stem was stabilized with two additional G–C pairs. The affinity of IRP-1 for this RNA, measured both directly or by competition ($K_{rel}$), is ~50% that of the wild-type ferritin IRE. In the absence of the additional CA, the selected U-bulge motif has binding affinity equal to that of the wild-type ferritin sequence ($K_{rel} = 0.97 \pm 0.02$, $n = 2$) suggesting that the CA not only does not contribute binding energy but is detrimental. This is supported by the observation that the addition of the extra CA to the hairpin loop of the ferritin IRE also decreased binding affinity by 2-fold (Fig. 1A).

Mutation of the bulged C in the wild-type ferritin IRE to a U decreased binding affinity by almost an order of magnitude, in agreement with published results (Fig. 1A; Refs. 23, 27). To determine why a U at the bulge position of the selected RNA does not likewise inhibit binding, a series of mutations were made. Mutations to the top stem of the selected RNA (nt 11–15 and 24–28 in Fig. 1B) had no effect on binding affinity provided that Watson-Crick pairing was maintained, which was demonstrated for the ferritin IRE (21). Deletion of the additional C nucleotides from the bulge region of the selected RNA (nt 7 or 29 and 30) dramatically inhibited IRP-1 binding. The importance of the conformation of this region is emphasized by the significant inhibition caused by the addition of an A between nucleotides 31 and 32 ($K_{rel} >40$, $n = 3$). The corresponding

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Fig. 2. Chemical probing of the selected U-bulge RNA. RT terminations that are independent of modifying reagent are indicated with (-). Terminations resulting from base modification or phosphodiester bond cleavage are one nucleotide shorter than those resulting from dideoxynucleotide incorporation during the sequencing reactions. The gels are each representative of four independent sets of reactions. A, DMS and CMCT modifications. B, OP-Cu cleavage in the presence of increasing Mg$^{2+}$ concentrations. C, localization of the modifications and cleavages.
addition to the ferritin IRE also inhibited IRP binding, although not as dramatically (Fig. 1A). For both RNAs, the added nucleotide potentially stabilizes the lower helix. In the context of the ferritin IRE, the inserted A could completely pair the lower stem as is the case for the transferrin receptor type IRE, which also has a lower affinity for the IRPs (41).

The U–A and C–G pairs of the lower ferritin stem (bp 7–32 and 8–31 in Fig. 1A) and the U–G and G–C pairs adjacent to the bulged C (bp 10–30 and 11–29) can also form in the selected U-bulge RNA. The significance of this sequence to the binding affinity of the ferritin IRE has not been fully appreciated. However, its presence in the selected U-bulge RNA emphasizes the contribution of this region. The U–A and C–G pairs in the lower stem of the selected RNA contribute base-specific binding affinity as indicated by the substitutions that maintain Watson-Crick pairing and cause a 2-fold increase in $K_{\text{av}}$ (bp 4–34 and 5–33 in Fig. 1B). The corresponding nucleotides in the ferritin IRE have previously been suggested to be part of a Mg$^{2+}$-binding site that optimizes IRP binding (31). The mutagenesis indicates, therefore, that the additional bulged nucleotides and the presumed Mg$^{2+}$ binding site of the selected U-bulge RNA are necessary to compensate for the lack of a C at the bulge position.

**Chemical Probing**—To obtain support for the proposed structure of the selected U-bulge RNA, the molecule was probed with dimethylsulfate (DMS) and 1-cyclohexyl-3-(2-morpholinooethyl) carbodiimide metho-p-toluene sulfonate (CMCT) under denaturing and native conditions (Ref. 42; Fig. 2A). Primer extension analysis was used to detect DMS modifications at N1-A and N3-C and the CMCT modifications at N3-U and N1-G (Fig. 2A). These functional groups would be protected from modification by secondary or tertiary interactions and by protein contacts. The detected modifications were consistent with the proposed structure with two exceptions (Fig. 2C).

First, no evidence was obtained for the intraoop base pair ($^{16}$C–G$^{20}$), however the dynamic nature of a single base pair may not be sufficient to provide protection. Second, C-7 is predicted to be bulged yet is protected from modification under native conditions. This protection could have resulted either from a tertiary interaction or alternatively the model of the secondary structure of the bulge region may not be completely correct (Fig. 1B). In the NMR structure of the frog ferritin IRE, the G corresponding to G-32 of the selected U-bulge RNA is base-paired in alternative conformations to either a U corresponding to U-8 or a U that is at the position corresponding to C-7 of the selected U-bulge RNA (31). The chemical probing is more consistent with this dynamic model. Structural information for C-5 and C-6 could not be obtained as neither base is reactive under denaturing conditions, and the evaluation of the reactivity of A-14, C-15, C-16, C-35, and C-36 is not possible because of modification-independent RT terminations.

The hairpin loop nucleotides A-17, G-18, U-19, G-20, and U-21 are protected from modification by IRP-1 consistent with the protection of the transferrin receptor IREs (Fig. 2A, *Janes 8* and 12; Ref. 28). The entire ferritin IRE has previously been reported to be protected by IRPs when probed with enzymes and metal coordination complexes (43, 44). However, this discrepancy probably reflects the properties of the different probes and as a result, the two sets of protections are not necessarily inconsistent. IRP-1 also protects U-8 and U-10 from CMCT modification. These protections could have resulted either from a direct contact with the protein or alternatively because the protein induces a conformational change within the RNA. The extra loop nucleotides C-22 and A-23 are not protected, suggesting that they are not making direct contact with the protein, in agreement with the mutagenesis.

The selected U-bulge RNA was also probed with a 1,10-phenanthroline-copper complex (OP-Cu), which cleaves single-stranded regions of RNA (45). Sites of cleavage were mapped by primer extension (Fig. 2B). There are three principal cleaved regions: the bottom and top bulges as well as the 3’-end of the hairpin loop (Fig. 2C). These regions of the ferritin IRE were previously shown to be cleaved by OP-Cu, and the cleavage was sensitive to the Mg$^{2+}$ concentration (29). Titration of Mg$^{2+}$ also inhibits the OP-Cu-dependent cleavage of the U-bulge RNA. In both the ferritin and the U-bulge RNAs, the Mg$^{2+}$ could be competing for the same binding sites as the OP-Cu complex. Because the effect of the Mg$^{2+}$ titration is similar for all three regions, it is possible that the bulge loops and hairpin loop could be forming a single Mg$^{2+}$/OP-Cu binding site that functions to optimize IRP-1 binding. Alternatively, there could be a binding site for Mg$^{2+}$, which when occupied dramatically alters the conformation of the IRE, rendering all of the sites inaccessible to OP-Cu cleavage.

**Internal Loop Sequence Requirement**—Because the mutagenesis clearly indicates that the internal loops of the selected U-bulge RNA are critical for IRP binding, we were interested in a more detailed analysis of the sequence requirements of this region. Only one RNA was initially selected with a U at the bulge position, possibly because the requirement of the internal loop nucleotides resulted in less representation within the starting random RNA population. To generate a phylogeny with which to obtain additional structural information, three nucleotides in the top bulge loop (nt 7–9 in Fig. 3A) and three in the bottom (nt 29–31) were made random and were reselected for high affinity binding to IRP-1. The additional CA (nt 22–23) of the hairpin loop was also randomized for the reselection. The measured values for the $K_{\text{av}}$ of the reselected clones are within a factor of three of the parental RNA (Fig. 3B). The parental top and bottom loop sequences were reselected (Fig. 3B, *clone 12*), and no RNA was selected with an A at position 7, 8, or 31 or a G at position 30, emphasizing a sequence preference in the internal loops. All five of the clones that were selected with a G at position 8 also have a C at position 30. Furthermore, two additional clones with a U at position 8 also have an A at position 30. This co-variation would suggest that these two positions interact. However, the remaining seven reselected clones and the parental sequence would either have a U–C or C–U non-Watson-Crick pair at these positions, suggesting either that these particular mismatches were tolerated or that the RNAs form an alternative structure in this region more consistent with that in Fig. 1B. A significant degree of variability is tolerated at positions 22 and 23 of the hairpin loop, but only one RNA was selected with a G at either position possibly because of the potential to compete with G-20 for the formation of the intra-loop base pair with C-16 (Fig. 3A).

An IRE variant was also obtained from the 13th cycle of the original 50N selection with a G at the bulge position (Fig. 3C). The PCR primer binding sequences were deleted from the RNA, and the lower stem was stabilized with two additional G–C base pairs. The $K_{\text{av}}$ and $K_{\text{op}}$ for the binding of this RNA to IRP-1 indicates the affinity for IRP-1 is approximately the same as the wild-type ferritin IRE, yet substitution of the C with a G at the bulge position of the ferritin IRE results in a 6-fold decrease in affinity, consistent with previously published results (Fig. 1A; Refs. 23, 27). The G-bulge RNA, like the U-bulge, contains additional internal loop nucleotides. Clone 11 (Fig. 3B) from the reselection of the U-bulge RNA has the same internal loop sequence as that found in the G-bulge RNA, suggesting that these nucleotides perform the same function in both RNAs.
The identification of the novel high affinity IRE variants lacking a bulged C enabled expanded searches of DNA data banks for additional mRNAs whose expression could be regulated by IRPs. A human EST data bank was searched using an algorithm that exploits RNA secondary structure constraints (46).\(^2\) ESTs were identified that have the potential to form the CAGWGH hairpin loop five base pairs removed from a C, G, or U that is present as part of a bulge or internal loop. Because an IRE needs to be within 100 nucleotides of the mRNA 5' -end to regulate translation (16), only the identified ESTs encoding mRNAs with a mapped 5'-end could be evaluated for biological relevancy. We initially identified 696 unique ESTs that have the potential to form an IRE structure within 100 nucleotides of the EST 5'-end.\(^3\) Of these, 105 encode an annotated mRNA with a mapped 5'-end, and seven of the mRNAs potentially can form an IRE-like structure within 100 nucleotides of its 5'-end. However, the bulged loop sequences of the seven putative IREs are not identical to those described here (Fig. 3B), and the measured \(K_d\) of these RNAs for IRP-1 is higher than that expected for biological relevancy. The remaining 591 non-annotated ESTs could not be readily evaluated for biological relevancy, but as the genome continues to be characterized, the novel U and G bulge variants may facilitate the identification of additional IRP-regulated mRNAs.

**DISCUSSION**

The absolute conservation of the CAGWGH hairpin loop within the RNAs selected from the starting 50N RNA population emphasizes the importance of this sequence, and it is remarkable that more highly divergent RNAs were not obtained given the available sequence space. However, the selection was set-up to isolate the highest affinity IRP-1 binders, and as a result RNAs with dramatically different conformations and lower binding affinities, analogous to those that interact with the \(E. coli\) aconitase (47), would not have been selected. In addition, high affinity binding RNAs requiring significantly more sequence space than the IRE may have been underrepresented within the starting RNA pool. Other unintended biases such as the impact of the flanking PCR primer sequences also have to be considered, and as a result the possibility of additional high affinity IRP-binding RNA motifs cannot be excluded. Alternatively, the binding affinity of the natural IRE to an IRP is one of the strongest known RNA-protein interactions, and the CAGWGH hairpin loop may simply be an integral part of the best possible IRP binding RNAs.

The \textit{in vitro} selections and mutagenesis indicated that the addition of a CA to the 3'-end of the hairpin loop only results in a 2-fold decrease in binding of the ferritin IRE and selected U-bulge RNA (Fig. 1). Previous insertions to the hairpin loop had a significantly greater detrimental effect (23, 48). However, these RNAs had nucleotides added to both the 5'- and 3'-sides of the hairpin loop, and it is likely that the location and size of the insertion as well as the sequence bias against G (Fig. 3B) affects the relative impact of the insertions. This is supported by the absence of insertions at other locations within the hairpin loop of the RNAs obtained from the initial 50N selection.

On the basis of limited \textit{in vitro} selection and mutagenesis, it had previously been assumed that a bulged C was essential for high affinity binding of the IRE to the IRPs. The initial direction of this study used IRP-1 to test the hypothesis that there are additional high affinity variants of the IRE that could not have been detected by the previous studies. The internal bulg-
es/loops that can compensate for a U or G at the bulge position make this point. Putative IREs with a G or U at the bulge position have also been identified within the crayfish ferritin mRNA (49) and the trout ferritin mRNA (50) respectively. Both putative IREs also have the potential to form internal loops although not with the same sequence as that described here (Fig. 3B), and their physiological relevance has not yet been demonstrated. IRP-2 has previously been shown to be more sensitive to the presence of the internal loops than IRP-1 (41). As a result, even though IRP-1 and IRP-2 are highly similar proteins with overlapping and similar binding affinities, it is possible that IRP-2 may not interact with the selected RNAs in the same manner as IRP-1.

There are at least two mechanisms through which the internal loops could be facilitating the binding of the IRE variants containing a U or G substitution at the bulge position. First, the selected loops could provide binding energy by making direct contact with IRP-1, in effect compensating for the loss of IRP-2. Alternatively, if the role of the ferritin IRE bulged C is to position the phylogenetically conserved hairpin loop for optimal binding, the selected nucleotides may provide another means of obtaining this conformation when there is a U or G at the bulge position. This is consistent with NMR results suggesting the internal loop influences the conformation of the hairpin loop (33, 41). However, because the loss of one or more potential hydrogen bond contacts may only have a slight impact on $K_d$, the possibility that the bulged C of the ferritin IRE also makes some minor contacts with the protein cannot be completely excluded. Acomitate purified from Bacillus subtilis binds to a $B. subtilis$ mRNA that has the potential to form an IRE-like structure containing the CAGWGH loop sequence but missing the canonical bulged C (51). This further suggests that the hairpin loop sequence is the central feature of the IRE-IRP interaction and implies that the internal bulge could have subsequently evolved to optimize the interaction.

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The Hairpin Loop but Not the Bulged C of the Iron Responsive Element Is Essential for High Affinity Binding to Iron Regulatory Protein-1
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