An RNA Binding Motif in the Cbp2 Protein Required for Protein-stimulated RNA Catalysis*

(Received for publication, March 1, 1999, and in revised form, July 26, 1999)

Hymavathi K. Tirupati, Lynn C. Shaw, and Alfred S. Lewin‡
From the Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida 32605

The fifth and terminal intron of yeast cytochrome \( b \) pre-mRNA (a group I intron) requires a protein encoded by the nuclear gene \( CBP2 \) for splicing. Because catalysis is intrinsic to the RNA, the protein is believed to promote formation of secondary and tertiary structure of the RNA, resulting in a catalytically competent intron. \( \text{In vitro} \), this mitochondrial intron can be made to self-splice or undergo protein-facilitated splicing by varying the \( \text{Mg}^{2+} \) and monovalent salt concentrations. This two-component system, therefore, provides a good model for understanding the role of proteins in RNA folding. A UV cross-linking experiment was initiated to identify RNA binding sites on Cbp2 and gain insights into Cbp2-intron interactions. A 12-amino acid region containing a presumptive contact site near the amino terminus was targeted for mutagenesis, and mutant proteins were characterized for RNA binding and stimulation of splicing. Mutations in this region resulted in partial or complete loss of function, demonstrating the importance of this determinant for stimulation of RNA splicing. Several of the mutations that severely reduced splicing did not significantly shift the overall binding isotherm of Cbp2 for the precursor RNA, suggesting that contacts critical for activity are not necessarily reflected in the dissociation constant. This analysis has identified a unique RNA binding motif of alternating basic and aromatic residues that is essential for protein facilitated splicing.

The terminal intron (intron 5) of the yeast gene encoding cytochrome \( b \) (COB) can self-splice \( \text{in vitro} \) at high concentrations of \( \text{Mg}^{2+} \) (1, 2), but the protein encoded by the nuclear \( CBP2 \) gene is essential for splicing at physiological concentrations of \( \text{Mg}^{2+} \) (3). Although this group IA intron possesses the conserved secondary and tertiary structures found in all group I introns, it varies in important ways from the prototype \( Tetranychus \) rRNA intron (4). In particular, the internal guide sequence that establishes the substrate specificity starts 220 nucleotides downstream of the 5′ splice junction, rather than the usual 14–20 nucleotides found in most other group I introns. The intron is AU-rich, and requires higher levels of \( \text{Mg}^{2+} \) for stabilization of the active structure than the GC-rich \( Tetranychus \) group I intron. Like other group IA introns, the fifth intron of \( COB \) pre-mRNA (bI5) is also devoid of the peripheral RNA element, P5abc, that is important for stabilizing the catalytic core of the \( Tetranychus \) rRNA intron (5). It is therefore conceivable that Cbp2 compensates for this RNA structure and stabilizes its RNA partner by contributing substantial binding energy in a manner similar to the CYT-18 protein of \( Neurospora \) (6, 7). UV cross-linking, chemical, and enzymatic modification studies indicate that Cbp2 contacts bI5 at multiple sites in the catalytic core and peripheral RNA elements and stimulates the formation of the catalytically active structure (8, 9). Based on these and kinetic studies, Weeks and Cech (10) proposed that Cbp2 serves as a tertiary structure capture protein. However, Cbp2 also induces the formation of RNA secondary structure, in addition to the stabilization of tertiary structure (8, 11). Chemical modification studies (1) show that Cbp2 binds to intron 5 RNA even in the absence of \( \text{Mg}^{2+} \) and nucleates the formation of the catalytic core by stabilizing the P4/P6 domain. Thus, Cbp2 appears to be involved in a dynamic process of stabilizing RNA structure both at the secondary and tertiary structure levels, stimulating the formation of the catalytically active RNA structure.

Weeks and Cech (9, 12) provided a kinetic framework for both Cbp2-mediated and self-splicing reactions of intron 5 RNA. At low \( \text{Mg}^{2+} \) levels (5 mM), the self-splicing reaction is estimated to be 1,000-fold slower than the protein-facilitated reaction. At near saturating concentrations of \( \text{Mg}^{2+} \) (40 mM), the protein-independent reaction is still 8-fold slower, indicating that high levels of the cation cannot completely compensate for Cbp2 function. The self-splicing reaction has to proceed through two additional transitions compared with protein-stimulated splicing. The first step involves a transition in secondary structure to an intermediate state that is efficiently promoted by \( \text{Mg}^{2+} \). However, self-splicing must still overcome a second barrier, which is the transition from the intermediate to an active enzyme state that finally gives rise to products. The kinetics of Cbp2-mediated splicing, on the other hand, include two significant steps, namely, guanosine binding to the Cbp2-active intron 5 RNA complex followed by efficient conversion of this ternary complex to products. Studies on phosphate substitution at the 5′ splice site and pH profiles indicate that at physiological pH the self-splicing reaction is limited by chemistry, whereas the Cbp2-facilitated reaction is limited by a conformational step (12). These studies indicate that Cbp2 binding compensates for at least two structural defects while increasing the rate of chemistry.

The availability of a two-component \( \text{in vitro} \) system to study autocatalytic and protein-facilitated splicing offers the advantage of studying RNA catalysis in isolation or in combination with the RNA-binding protein simply by varying the \( \text{Mg}^{2+} \) and monovalent salt concentrations. Studies so far have focused on mapping the Cbp2 contact sites on bI5 RNA and the kinetics of splicing in the presence and absence of the protein. Little is

---

* This work was supported by a grant from the College Incentive Fund of the University of Florida College of Medicine. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Molecular Genetics and Microbiology, University of Florida, Box 100266 Gainesville, FL 32605. Tel.: 352-392-0676; Fax: 352-392-3133; E-mail: lewin@ufl.edu.

‡ L. C. Shaw and A. S. Lewin, submitted for publication.
known about the structure of Cbp2 protein or its interaction with b15 from the protein point of view. To understand the role of Cbp2 in stimulation of splicing, we have attempted to identify potential RNA binding regions of Cbp2 using the technique of UV cross-linking and label transfer. Following the mapping of major contact sites, site-directed mutagenesis was employed to confirm the importance of various amino acid residues in these sites for interaction with intron 5 RNA and facilitation of splicing. UV cross-linking identified two major RNA contact sites in Cbp2, with the NH2-terminal site occurring within the first 37 residues of the protein. Mutagenesis of this region identified an RNA binding domain essential for protein-stimulated RNA catalysis.

MATERIALS AND METHODS

Preparation of native and His-tagged Cbp2—Plasmid pET15b-CBP2 was constructed by cloning the NdeI-ClaI fragment carrying the CBP2 gene from pET3a-CBP2, downstream of the 6x histidine tag in the T7 expression vector, pET15 (Novagen). *Escherichia coli* strain JM109(DE3), which carries the T7 RNA polymerase gene on a lambda lysogen, was used for the expression of His-tagged Cbp2. Native Cbp2 protein was expressed from pET3a-CBP2 plasmid in BL21(DE3), another *E. coli* strain carrying the vector. The plasmid was transcribed by introducing an NdeI site at the start codon of CBP2 gene by polymerase chain reaction mutagenesis and cloning the NdeI-SmaI fragment between the Ndel-BamHI sites of pET3a expression vector (13). The Cbp2 protein expressed from this construct is 20 amino acid residues shorter than the His-tagged version.

An overnight culture of bacteria carrying the Cbp2 expression plasmid was used to inoculate 2 liters of LBampicillin medium at 1:100 dilution. The cultures were grown at 37 °C until they reached an *A*_opt of 0.35, and the growth of Cbp2 was induced with 0.4–1 mM isopropyl-1-thio-β-D-galactopyranoside for 1–3 h. Cells were pelleted after addition of 17 μg/ml promylthethylsulfonyl fluoride, washed with 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, snap frozen in a dry ice/ethanol bath, and stored at −70 °C until purification.

The His-tagged protein was purified on nickel-nitritotriacetic acid superflow (Qiagen) column. The bacterial pellet was resuspended in 10 ml of column buffer (50 mM HEPES, pH 7.6, 700 mM NaCl, 1 mM imidazole, 17.5 μg/ml promylthethylsulfonyl fluoride) and lysed by two passages through a French pressure cell at 14,000 p.s.i. The lysate was cleared by centrifugation at 35,000 rpm for 30 min in a Beckman Ti 42.1 rotor, and the supernatant was loaded on a 1-ml nickel-nitritotriacetic acid superfllow column pre-equilibrated with 10 volumes of column buffer. The column was washed with 10 volumes of column buffer (1 mM imidazole) followed by 7.5 volumes of wash buffer (20 mM imidazole). Cbp2 protein was then eluted with 7.5 volumes each of buffers containing 80 and 200 mM imidazole. The fractions containing Cbp2 (detected by SDS-polyacrylamide gel electrophoresis) were pooled and dialyzed twice against 1 liter each of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20% glycerol, and once with a liter of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50% glycerol, and stored at −70 °C after rapid freezing in a dry ice/ethanol bath. Native Cbp2 protein was isolated according to the purification protocol described by Shaw and Lewin (8).

In Vitro Transcription—Plasmid pSF15 DNA was purified by CsCl gradients and linearized with *SmaI* and used for *in vitro* transcription with T7 RNA polymerase (2). The transcripts contain the entire intron 5 RNA sequence and the flanking exon sequences. The transcripts were internally labeled using [α-32P]UTP and/or [α-35S]ATP (ICN).

UV Cross-linking—Cbp2-RNA complexes were generated according to the UV cross-linking technique of Zamore and Green (14). 5 pmol of [32P]-labeled b15 transcripts were incubated at room temperature or 37 °C for 30 min with an excess of His-tagged Cbp2 or native Cbp2 in a low salt buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 50 mM NH4Cl), containing excess tRNA as a nonspecific competitor. Each sample was split into several 10-μl aliquots in a 96-well microtiter plate (Falcon) placed on ice and exposed to 600 μl of UV radiation in a Stratalinker (Stratagene). The aliquots of each sample were pooled in a 1.5-ml Eppendorf tube and incubated with 0.32 μg/ml pronase E (Calbiochem) and 100 μg/ml of RNase T1 (Roche Molecular Biochemicals) at 37 °C for 2 h to remove uncleaved RNA. The samples were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, and the band containing the Cbp2 excised after Coomassie Blue staining. The Cbp2 thus purified contained both the cross-linked and uncleaved-forms of the protein.

Generation of Peptides—The gel pieces were washed four times with distilled water over a period of 20 min, placed into appropriate cleavage buffer and thoroughly macerated with a Kontes Eppendorf pestle. The slurry was completely covered with the cleavage buffer and incubated overnight at appropriate temperature.

The asparaginyl side chain has a tendency to form a cyclic imide that is susceptible to nucleophilic attack by hydroxamine (15). The cyclization is more favored in the minor UV cross-linked form of Cbp2 resulting in increased susceptibility of Asn-Gly bonds. Chemical cleavage of proteins with hydroxylamine generates relatively large peptides due to the infrequency of Asn-Gly bonds. Hydroxylamine (NH2OH) cleavage of Cbp2 was performed by overnight incubation of Cbp2 containing gel pieces in 2.4 M guanidine-HCl, 2 mM hydroxylamine buffer, pH 9, at room temperature, as described above. NH2OH was used to neutralize the guanidine-HCl and hydroxylamine-HCl.

Cleaveage with 2-nitro-5-thiocyanobenzoate (NTCB) was a two-step process. First, the thiol groups on cysteine residues of denatured proteins were modified to SCN groups by NTCB (16, 17), followed by cleavage at the amino group of the modified cysteine by exposure to alkaline pH conditions. Gel-purified Cbp2 protein was incubated in 2.4 M guanidine-HCl, 5 mM dithiothreitol, 1 mM EDTA, 0.2 mM Tris acetate, pH 8, buffer at 37 °C for 2 h to denature the protein and reduce the disulfide bonds to SH groups. A 10-fold excess of NTCB (50 mM) over the total thiol was added to the gel slurry, and the incubation was continued for 0.5 h at the same temperature to effect modification of the SH groups to SCN groups. The slurry was filtered through a 0.22-mm low protein binding filter (Corning-Costar), and washed once with distilled water. The slurry was later transferred to a 1.5-ml Eppendorf tube and incubated overnight in 2.4 M guanidine-HCl, pH 9, cleavage buffer at 37 °C.

Extraction of Peptides—After cleavage, the slurry was filtered through a Costar filter, washed once with distilled water, and incubated overnight at 37 °C in the extraction buffer (0.1% SDS, 50 mM Tris-HCl, pH 8.8, 0.1 mM EDTA, and 0.2 mM ammonium bicarbonate). On the third day, the gel slurry was heated at 85 °C for 5 min and rapidly filtered through a Costar filter to recover soluble peptides. The slurry was further incubated with 0.5% SDS, 10 mM Tris-HCl, pH 8, for 20 min at room temperature and filtered to extract the residual peptides in the gel.

**Protein-stimulated RNA Catalysis**—The filtrates containing the peptides were pooled, dried in a Speed-Vac (Savant), resuspended in water, and precipitated overnight at −20 °C with 9 volumes of acidified acetone. Peptides were pelleted at 12,000 rpm in a microcentrifuge for 20 min and resuspended in 15 μl of SDS gel-loading buffer. The samples were dried in a Speed-Vac to remove the residual acetone, brought to a final volume of 40 μl with water, resolubilized on 15% (for hydroxylamine) or 75% (for NTCB) TCA-Tricine gels (18), and analyzed by 15% SDS-polyacrylamide gel, low molecular weight peptide markers (Amersham Pharmacia Biotech), and exposed to x-ray film.

Site-Directed Mutagenesis—The NH2-terminal RNA contact site on Cbp2 (identified by the UV cross-linking strategy) was subjected to site-directed mutagenesis to identify key residues for Cbp2 function. Mutations were designed to either delete the region of interest (residues 17–25) or make point mutations within that region. The Xhol-BamHI fragment encoding the first 97 codons of CBP2 from pET15b-CBP2 was subcloned into the M13mp19 vector and used as the template for oligonucleotide-directed mutagenesis, performed according to the double primer method of Zoller and Smith (19) using the mutagenic oligonucleotides listed in Table 1. The resulting mutants were plaque purified, and the single-stranded DNA sequenced. The CBP2 segment carrying the mutation of interest was then re-cloned in pET15b-CBP2 expression vector and was sequenced using Sequenase 2.0 kits (Amersham Pharmacia Biotech).

**In Vitro Splicing Assay**—The activity of various mutant Cbp2 proteins was determined by an *in vitro* splicing assay (2). 32P-labeled precursor RNA (1 pmol) containing 615 was incubated with wild-type or mutant Cbp2 proteins in a 100-μl reaction including low salt buffer (see above), in the presence of 5 mM dithiothreitol and 2 units of RNasin RNase inhibitor (Promega), at 37 °C for 10 min. Splicing was initiated with 0.2 μM GTP (Amersham Pharmacia Biotech), and the reactions allowed to continue for varying lengths of time. Reactions were terminated by the addition of equal volumes of 90% formamide, 25 mM EDTA, and resolved on 4% polyacrylamide 8 M urea gels and exposed to x-ray film or a PhosphorImager screen (Molecular Dynamics). Initial rates of

---

2 The abbreviations used are: NTCB, 2-nitro-5-thiocyanobenzoate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; aa, amino acid; NH2OH, hydroxylamine; ds, double-stranded; dsRBM, dsRNA binding motif.
splicing were determined by the fraction of exon products (ligated exons and 5′ exon) expressed as a fraction of the exon sequences present in the precursor.

**Splicing Competition Assays**—These assays were done essentially as described above but in the presence of a constant amount of the wild-type protein and increasing concentrations of mutant Cbp2 proteins. As a control, the concentration of wild-type Cbp2 was increased to the same level of total Cbp2 protein (wild-type and mutant) used in the above reactions but in the absence of mutant proteins. Spliced products were resolved on denaturing gels and quantitated using PhosphorImager (Molecular Dynamics).

**Partial Proteolysis of Cbp2**—The conformation of mutant Cbp2 proteins was determined by comparing the partial proteolytic profiles of wild-type and mutant Cbp2. Partial proteolysis was done by incubating 0.5–1 μg of the wild-type (native or heat-denatured) or mutant Cbp2 protein with trypsin, at protease:Cbp2 ratios of 1:50 and 1:100 (w/w), for 1 h at room temperature, and the peptides were resolved on 12% SDS-polyacrylamide gel electrophoresis gels. The peptide profiles were detected by Western blotting performed according to Tobin et al. (20), with a Cbp2-specific polyclonal antibody (a generous gift of Dr. Alexander Tzagoloff).

**Equilibrium Binding Analysis**—The affinity of wild-type and mutant Cbp2 proteins for b15 RNA was determined by the double-filter binding assay (21), with the exception that a charged nylon membrane (Hybond N+ from Amersham Pharmacia Biotech) was used in place of DEAE. Protein-RNA complexes were generated by incubating a low concentration (16 pm) of 32P-labeled intron 5 RNA with increasing concentrations of mutant Cbp2 proteins. As a control, the concentration of wild-type Cbp2 protein was increased to the same level of total Cbp2 protein (wild-type and mutant) used in the above reactions but in the absence of mutant proteins. Spliced products were resolved on denaturing gels and quantitated using PhosphorImager (Molecular Dynamics).

**RESULTS**

**Identification of Cbp2 Peptides That Contact Intron 5 RNA**—UV photolysis provides a useful approach to determine the contact points between nucleic acid and protein, because it produces direct cross-links in contrast to chemical cross-linking agents. A free radical mechanism has been proposed to explain the process of UV cross-linking of amino acids to nucleic acid bases (22). The extent of photo-cross-linking also depends on the intrinsic structure of the nucleic acid or protein. Photo-chemical cross-linking has been adapted to detect protein bound to specific sites on double-stranded DNA using 32P-labeled site-specific probes (23). This method permits transfer of 32P from specific phosphodiester bonds to amino acid residues at the interface upon photo-cross-linking (24). We have employed a similar method to detect intron 5 RNA binding sites on Cbp2 protein (25). We synthesized transcripts that b15 (internally labeled with [α-32P]UTP), UV cross-linked them to purified Cbp2 under conditions that favor specific complex formation. We generated peptides from these complexes by chemical cleavage and detected the cross-linked peptides by label transfer using gel electrophoresis and autoradiography.

To optimize the yields of cross-linked species, we varied the dosage of ultraviolet radiation used. In these experiments, 32P-labeled transcripts were incubated with native Cbp2 under low salt conditions without GTP. Cbp2 binds to intron 5 RNA under these conditions and induces formation of the catalytic RNA conformation (8). The Cbp2-RNA complexes generated were UV cross-linked in the presence of excess tRNA (added as a nonspecific competitor) (14), as described under “Materials and Methods.” The samples were irradiated at an increasing UV dosage ranging from 100 to 950 mJ. As a control, intron 5 RNA was irradiated with noncoaginate proteins, bovine serum albumin and the RNA-binding protein PUB1 (a gift from Dr. Maurice Swanson) (25). All samples were extensively treated with RNase A and RNase T1 to remove uncross-linked RNA, and the protein-RNA complexes were resolved on SDS-polyacrylamide gels (data not shown). No cross-linked complex was observed with bovine serum albumin or PUB1 even at high doses of UV radiation. The extent of cross-linking of intron 5 RNA to Cbp2 was 2-fold higher at a UV dosage of 600 mJ than at 100 mJ and remained relatively constant at higher doses. Thus a UV dosage of 600 mJ was chosen as the lowest UV dosage that yielded optimal amounts of ribonucleoprotein complex.

The Cbp2-RNA complexes thus generated were purified on SDS-polyacrylamide gels. The gel fragments were then soaked in different chemical cleavage reagents like NH2OH and NTCB to generate peptides. The peptides were separated on high percentage gels (18), and the cross-linked peptides that retained the label were identified by autoradiography (Fig. 1). NH2OH cleaves proteins at asparaginyl-glycyl peptide bonds (15) and would yield three large peptides (15.1, 21.6, and 37.1 kDa) in a complete digestion of Cbp2. Upon cleavage of the cross-linked Cbp2-RNA complex (Fig. 1A), the 21.6-kDa amino-terminal and the 15.2-kDa carboxy-terminal fragments of Cbp2 strongly cross-linked with intron 5 RNA. In addition to native Cbp2, we analyzed the cross-linking of a histidine-tagged version of the protein that stimulated splicing as well as untagged wild-type Cbp2 (Fig. 1C). The histidine tag added an additional 20 amino acid residues (2.3 kDa) to the NH2 terminus of the Cbp2 protein. Therefore, the indirectly labeled NH2-terminal peptide and the partials obtained with this tagged Cbp2 would migrate slower in the gels than their nontagged counterparts. This difference in electrophoretic mobility was used as an analytical tool to confirm the assignment of the cross-link site to the NH2-terminal fragment. As expected, the NH2-terminal peptide of the His-tagged protein obtained by NH2OH cleavage migrated at apparent molecular mass of 24 kDa, whereas the nontagged peptide (Fig. 1A) migrated faster (at 21.6 kDa). Migration of the 15.2-kDa COOH-terminal peptides derived from both versions of Cbp2 was unaffected. The cleavage reagent NTCB is specific to amino groups of cysteines (16, 17). NTCB cleavage of Cbp2 would produce 9 peptides ranging from 0.17 to 29.5 kDa if the reaction proceeded to completion. However, only a partial digestion of the protein could be achieved despite our efforts to optimize the
conditions. Incomplete cleavage often results from β-elimination and/or incomplete modification due to the reversible nature of the cyanlation reaction (17). Failure to completely denature the protein may also result in poor cleavage at internal sites. Cbp2, a relatively large protein (630 residues), appears to be somewhat refractile even to the strong denaturation conditions used in these cleavage reactions. Cleavage of cross-linked Cbp2-RNA complexes with NTCB (Fig. 1B) generated several peptides that retained the label. Among the various indirectly labeled peptides, the 4.6-kDa NH2-terminal peptide, which is the size expected of the NH2-terminal peptide, and fragments of apparent molecular mass 6.3 and 16.9 kDa (indicated by asterisks) could be readily identified. All three fragments were shifted upwards in the histidine-tagged version of Cbp2 suggesting that the 6.3- and 16.9-kDa bands were partial digestion fragments including the NH2 terminus. These results suggest that the NH2-terminal RNA contact site is located close to the His-tag (within the first 37 residues of untagged Cbp2).

To demonstrate that the conditions employed for UV cross-linking promote the formation of active Cbp2-intron complexes, portions of the reactions were removed before UV irradiation, incubated with 0.2 mM GTP at 37 °C for 30 min, and the products resolved on denaturing gels. (Fig. 1C). Reaction mixes containing either native (lane 2) or His-tagged Cbp2 (lane 3) clearly demonstrated splicing, whereas the RNA alone (lane 1) could not splice under similar low salt conditions.

The results of these and similar experiments are represented in Fig. 1D. The map shows the two strong RNA binding regions (filled boxes), one site within the first 37 residues and the other located near the COOH terminus (aa 502–aa 582). The theoretical cleavage sites of NH2OH and NTCB on Cbp2 are also indicated. Precise identification of the residues that participate in photo cross-linking by amino acid analysis requires cross-linking efficiencies greater than those obtained under the conditions employed (usually less than 10%). Therefore, we focused on the amino-terminal cross-linking site and attempted to identify essential residues by mutagenesis.

**Mutagenesis of a Presumptive RNA Binding Site**—The putative NH2-terminal RNA contact site on Cbp2 (spanning 37 residues) contains a 12-residue sequence, \^{1}\text{SSSSR}YRYK-FNME\^{28}, with characteristics similar to the double-stranded RNA binding motif, which is rich in hydrophobic and basic residues (26). Although the charges may promote ionic interactions between protein and RNA, the aromatic residues could engage in stacking interactions. The stretch of serines might participate in hydrogen bonding interactions. A series of mutations was introduced into this sequence by oligonucleotide-directed mutagenesis (Table I and Fig. 1D). All mutant His-tagged Cbp2 proteins were purified by metal affinity chromatography as described under “Materials and Methods.” The serine deletion mutant could not be successfully purified. The His-tagged protein eluted in the wash fractions with other *E. coli* proteins. The serine deletion (ΔI17–19) appears to have altered the global conformation of Cbp2 (perhaps the topology of the histidine tag), resulting in poor binding to the nickel column. The Y23L mutant co-purified with an RNA nuclease activity despite several independent attempts to purify the Y23L protein with fresh reagents and columns.
raises the possibility that this mutation has conferred a nuclease function to Cbp2. This problem was not encountered in parallel preparations of any of the other mutant proteins. We have verified that the nuclease activity is dependent on magnesium and is not specific to the intron sequence, because an antisense b15 substrate was degraded at the same rate as the splicing substrate. Degradation was sufficiently slow for us to determine that the Y23L mutant was unable to stimulate splicing of b15 (see below). The mutant Cbp2 proteins purified from E. coli were first analyzed by Western blotting with a Cbp2-specific polyclonal antibody to check for production of the full-length protein. Mutant proteins were separated by gel electrophoresis and analyzed by immunoblotting (20). All mutant proteins except the triple aromatic to leucine mutant exhibited electrophoretic mobility similar to that of wild-type His-tagged Cbp2 (data not shown). This mutant appeared to migrate more slowly than the wild-type protein on some gels. The deletion mutant (Δaa 17–aa28) was shorter by about 1.3 kDa, as expected.

To test the effects of these mutations on splicing function, in vitro splicing assays were carried out. 32P-labeled precursor transcripts containing b15 were incubated with increasing concentrations of wild-type or mutant Cbp2 proteins under low salt splicing conditions. The reaction products were separated on 4% polyacrylamide-8 M urea gels and visualized by autoradiography (Fig. 2). Increasing concentrations of deletion mutant (Δaa 17–aa28) and triple aromatic mutant failed to stimulate splicing of b15, whereas wild-type Cbp2 spliced normally at both concentrations tested. These results suggest that the NH2-terminal residues (aa 17–aa28) may comprise a domain essential for Cbp2 function. Functional analysis of the remaining mutations is reported below.

**NH2-terminal Mutations Do Not Alter the Global Conformation of Cbp2**—One explanation of the loss of function of Cbp2 mutants is structural destabilization caused by the mutations. Partial proteolysis is a useful technique to analyze the conformational states of proteins (27–30). To determine whether the mutations have caused major conformational changes, wild-type and mutant proteins were incubated separately with trypsin under conditions that favored partial proteolysis. Digestion of heat-denatured wild-type Cbp2 was performed for comparison.
tively, in comparison with wild-type Cbp2. Thus, tyrosine residues at position 21 and particularly at position 23 are critical for activity, whereas a phenylalanine at position 25 is dispensable. The charged residues (arginine and lysine) at positions 20, 22, and 24 are also important for Cbp2 function.

Binding of Cbp2 Mutants with bI5 RNA—To determine whether lower splicing activity of the Cbp2 mutants corresponded to a reduction in overall affinity for intron 5 RNA, equilibrium binding assays were performed. 32P-labeled bI5 RNA was incubated with Cbp2 variants under low salt splicing conditions at a ratio of 7 pmol of protein to 1 pmol of RNA in a 100-µl reaction. Reactions were terminated at the times indicated, and samples were resolved on 4% polyacrylamide–8 M urea gels. The figures are from autoradiographs of those gels. Symbols in the left margin indicate the identity of the radioactive bands: boxes represent exon RNA and lines indicate intron sequences. The circle represents the circular form of the intron. A, wild-type Cbp2; B, F25L; C, triple charged mutant; D, Y21L.

**TABLE II**

| Cbp2 allele | RNA fraction spliceda | Initial rateb | kd c (pM) |
|-------------|-----------------------|---------------|-----------|
| Wild-type   | 0.94 ± 0.22           | 0.189         | 0.189 ± 0.12 |
| A17–28      | ND                    | ND            | 58 ± 3    |
| LRLRKL      | ND                    | ND            | 247 ± 14  |
| LYLRYF      | 0.11 ± 0.01           | 0.474 × 10⁻²  | 543 ± 6   |
| Y21L        | 0.05 ± 0.005          | 0.417 × 10⁻²  | 1595 ± 58 |
| Y23L        | ND                    | ND            | 61 ± 9    |
| F25L        | 0.68 ± 0.09           | 0.162         | 171 ± 12  |
| Y21L        |                       |               |           |

a Fraction 5' exon cleaved or cleaved and ligated in a 60-min reaction.

b Fraction of RNA processed per min during the period of linear increase with time (within first 5 min).

c kd values of wild-type protein and F25L mutant were not significantly different (Table II).

d ND, not detected above background.

quets were filtered through a double filter as described under “Materials and Methods.” Each filter binding experiment (in duplicate) was repeated at least two times, and the RNA binding data (Fig. 6) used to calculate dissociation constants (kd) (Table II).

The F25L mutant (filled triangles), which showed wild-type-like splicing activity, demonstrated RNA binding levels comparable to that of wild-type (open circles). The kd values of wild-type protein and F25L mutant were not significantly different (Table II). The triple charged (open triangles) and the Y21L mutants (filled squares) that displayed partial splicing activity showed reduced binding. These two mutants showed 3.2- and 9.4-fold increase in kd values, respectively, relative to wild-type Cbp2. The deletion mutant (open squares) and Y23L showed tighter binding (kd of 58 and 61 pM, respectively) than wild-

**FIG. 4**. Splicing time course for bI5 in the presence of wild-type and mutant Cbp2. Radiolabeled precursor RNA was incubated with Cbp2 variants under low salt splicing conditions at a ratio of 7 pmol of protein to 1 pmol of RNA in a 100-µl reaction. Reactions were terminated at the times indicated, and samples were resolved on 4% polyacrylamide–8 M urea gels. The figures are from autoradiographs of those gels. Symbols in the left margin indicate the identity of the radioactive bands: boxes represent exon RNA and lines indicate intron sequences. The circle represents the circular form of the intron. A, wild-type Cbp2; B, F25L; C, triple charged mutant; D, Y21L.

**FIG. 5**. Aromatic mutants dramatically decrease splicing enhancement by Cbp2. Gels such as those shown in Fig. 4 were analyzed using a PhosphorImager, and the fraction of RNA spliced was determined as described under “Materials and Methods.”filled boxes, wild-type Cbp2; open boxes, F25L; filled circles, triple charged mutant; open circles, Y21L; open diamonds, Y23L. 

**Protein-stimulated RNA Catalysis**
of Cbp2:intron RNA and not on the absolute concentration of Cbp2. Therefore, protein aggregation did not seem to be a contributing factor to loss of activity.

To determine whether the cross-linking site mutations behaved in a similar manner, we added increasing amounts of mutant Cbp2 to a reaction containing a 7:1 molar ratio of wild-type Cbp2 to intron 5 RNA and increasing amounts of the residue 17–28 deletion mutant (B) or the triple aromatic mutant (C) were added to parallel samples. The products were analyzed by electrophoresis and PhosphorImager as described above. The fraction spliced is presented as a function of the mutant to wild-type Cbp2 ratio.

**FIG. 7.** A, increasing the ratio of Cbp2 to intron 5 RNA decreases the rate of splicing. Splicing reactions were conducted for 10 min at 37°C and separated on 4% polyacrylamide–8 M urea gels. The fraction of RNA spliced was determined as described in the legend to Fig. 6, and the highest value (74%) at a 7:1 ratio of Cbp2:RNA was set as 100. B and C, mutant Cbp2 proteins inhibit splicing in the presence of wild-type Cbp2. A normal splicing reaction was set up with a 7:1 molar ratio of Cbp2 to bI5 RNA and increasing amounts of the residue 17–28 deletion mutant (B) or the triple aromatic mutant (C) were added to parallel samples. The products were analyzed by electrophoresis and PhosphorImager as described above. The fraction spliced is presented as a function of the mutant to wild-type Cbp2 ratio.
This ratio corresponds to a total Cbp2:RNA ratio of 196:1. This result is typical of competition experiments using the other Cbp2 mutants. For example, increasing the level of the A17–28 protein led to 95% inhibition of splicing at a ratio of 6:1 mutant to wild-type (42:1 protein to RNA) (Fig. 7C). If the total protein to RNA ratio was kept below 7:1, then increasing the ratio of mutant to wild-type protein did not inhibit splicing (data not shown). Our interpretation is that inhibition by the mutant proteins is analogous to the inhibition by high levels of wild-type Cbp2. Consequently, the nonspecific binding of b15 by the binding motif mutants is probably similar to that of the wild-type protein.

**DISCUSSION**

UV cross-linking identified a 37-amino acid region of Cbp2 that is likely to contain a close contact site in its RNA partner, intron 5 of COB. Altering the surface properties of a 6-residue sequence (RYRYKF) within this region by mutagenesis impaired the ability of Cbp2 to stimulate splicing. It was important to determine, however, that these changes did not alter the conformation and stability of mutant proteins. Unfortunately, Cbp2 has no known function other than facilitating the processing of intron 5 (31) and the ω intron (32), unlike the CYT-18 protein of *Neurospora crassa*. CYT-18, which facilitates the splicing of several mitochondrial group I introns, is a tyrosyl RNA synthetase (33). This bifunctionality of CYT-18 was exploited to demonstrate the native conformation of splicing-defective mutants by assays for synthetase function (34). Because our system did not afford this luxury, the conformational integrity of the Cbp2 mutants was addressed using partial proteolysis and equilibrium RNA binding assays.

Partial proteolysis is a simple and powerful tool to analyze the conformational states and domain structure of proteins. It is based on the principle that protease-susceptible sites often occur between independently folded domains. The potential cleavage sites within these domains are protected from proteases in the native state and exposed to various degrees in the denatured or partly unfolded states (35, 36). Partial proteolysis has been successfully used to study conformational changes in proteins induced by DNA binding (27–29) or binding to a specific ligand (37–39). This approach was also used to compare the conformations of wild-type and mutant proteins in mutagenesis experiments to detect structural perturbations caused by the mutations. For instance, Liu et al. (30) analyzed the folding patterns of several rhodopsin point mutants by partial tryptic digestions and CD spectroscopy obtaining analogous conclusions from each approach. The partial proteolytic profiles obtained with mutant Cbp2 proteins were similar to that of the native but not the heat-denatured Cbp2 (Fig. 3). These results suggest that the mutants possess a conformation similar to that of wild-type protein. The binding isotherms of the mutant proteins also suggest that these variants retain a native conformation, because the nonspecific binding of RNA by Cbp2 demonstrates a sigmoidal profile (11). Changes within the potential RNA binding site had varying effects depending on whether aromatic or charged residues were altered. The triple charged mutant (Arg20, Arg24, Lys24 to Leu) yielded a protein with partial activity in splicing assays (Fig. 5), in contrast to the triple aromatic mutant (Tyr21, Tyr22, and Phe25 to Leu), which was completely defective in splicing (Fig. 2). Therefore, the charged residues at positions 20, 22 and 24 are important for Cbp2 function, though not as critical as the aromatic residues at positions 21, 23, and 25.

Single mutations affecting the aromatic residues severely impaired splicing of b15. The Y23L mutation led to complete loss of splicing enhancement and the acquisition of Mg-dependent nuclease activity by Cbp2. We believe that the nuclease is an intrinsic property of Y23L Cbp2 because it consistently co-purifies with the protein, but we cannot exclude a contaminant that did not occur in the preparation of any of the other variants. The Y21L mutant gave very low partial activity, with initial rates of splicing lowered by 45-fold compared with wild-type (Table II), whereas the F25L mutant behaved almost like wild-type Cbp2 in the splicing time course experiments (Fig. 5). In addition, the Y21L and triple charged mutations appear to have affected the first step of splicing, because the splicing intermediates (5′ exon and intron 3′ exon) are markedly diminished compared with the spliced products (ligated exons and free intron).

The importance of the Tyr21 residue is also highlighted by the fact that the tyrosine to leucine change at this position alone made a significant impact on the $k_d$. A 9-fold increase in $k_d$ was measured for a single amino acid change in a protein of 630 amino acids. The partial activity of Y21L could be due to the presence of the other important residues in the aa 17–aa 28 region, partial compensation by the COOH-terminal RNA binding domain, or both.

Cbp2 is reminiscent of proteins containing the double-stranded RNA binding motif (DSRM or daRBM). The double-stranded RNA binding motif is a conserved 65–68 amino acid region with basic and hydrophobic residues scattered throughout the motif (40–42). 44 daRNA binding motif sequences have been identified from 27 functionally diverse proteins (26). Mutational analysis indicates that nearly all of the conserved residues are important for double-stranded RNA binding (43, 44). The daRBMs occur in single or multiple copies and specifically bind double-stranded RNA in a sequence-independent manner (45). Studies on the human dsRNA-dependent protein kinase, PKR, indicate that the specificity for double-stranded RNA is largely due to nonelectrostatic interactions with a network of 2′-OH groups on both strands of RNA (46). Hydroxyl radical footprinting experiments with PKR indicate that the daRBM interacts directly with the minor groove of dsRNA. Cbp2 has hydrophobic and basic residues scattered throughout the length of the protein. Hence, it is likely that different regions of this protein may participate in sequence-independent recognition of the conserved intron structure, in addition to engaging in specific interactions with the bases.

Experiments with the triple charged mutant of Cbp2 indicate that the charged residues are important but not absolutely essential for activity, suggesting that these residues may not form specific ion pairs with the phosphates of RNA. It is possible that Cbp2 might be involved in a variety of nonspecific interactions with the sugar-phosphate backbone of intron 5 RNA. These might include nonelectrostatic interactions with 2′-OH groups of sugar residues or functional groups of bases. The tyrosine residues at positions 21 and 23 appear to be essential for activity, suggesting that these aromatic side chains (and that of Phe25) may participate in stacking interactions with bases in the intron RNA. It is also possible that the hydroxyl groups on these tyrosine residues are involved in polar interactions with the RNA. These possibilities can be dissected by additional mutagenesis experiments, namely, changing Tyr21 and Tyr22 to phenylalanine or to serine.

We found that Cbp2-facilitated splicing of b15 is inhibited by increasing the molar ratio of protein to RNA above 7:1. This result is reminiscent of the chaperone activity of the nucleocapsid protein of human immunodeficiency virus, type 1, a nonspecific RNA-binding protein (47). Similarly to Cbp2, this protein enhances the activity of a hammerhead ribozyme in vitro only in a narrow range of protein concentrations. It therefore appears that nonspecific interactions may precede the formation of specific contacts between Cbp2 and intron 5 RNA.
that lead to catalysis. At high protein:RNA ratios, however, non-specific interactions may predominate and preclude the formation of specific contacts that are essential for promoting the catalytic conformation of RNA. It can be speculated that the charged residues on Cbp2 may, in part, be important for these initial nonspecific encounters, whereas aromatic residues (such as Tyr-21 and Tyr-23) may be involved in making specific contacts with intron 5 RNA.

Acknowledgments—We thank Bert Flanagan, Henry Baker, Phil Laipis, and Mark Caprara for reading and criticism of early versions of this manuscript and Alan Lambowitz and Nancy Denslow for advice on experimental design.

REFERENCES

1. Gampel, A., and Tzagoloff, A. (1987) Mol. Cell. Biol. 7, 2545–2551
2. Partono, S., and Lewin, A. S. (1988) Mol. Cell. Biol. 8, 2562–2571
3. Gampel, A., Nishikimi, M., and Tzagoloff, A. (1989) Mol. Cell. Biol. 9, 5424–5433
4. Michel, F., and Westhof, E. (1990) J. Mol. Biol. 216, 585–610
5. Cate, J. H., Gooding, A. R., Podell, E., Zhou, K., Golden, B. L., Kundrot, C. E., Cech, T. R., and Doudna, J. A. (1996) Science 273, 1678–1685
6. Weeks, K. M., and Cech, T. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 5423–5427
7. Weeks, K. M., and Cech, T. R. (1996) Science 270, 512–513
8. Shaw, L. C., and Lewin, A. S. (1995) J. Biol. Chem. 270, 21552–21562
9. Weeks, K. M., and Cech, T. R. (1995) Cell 82, 221–230
10. Weeks, K. M., and Cech, T. R. (1996) Science 271, 345–348
11. Shaw, L. C., Thomas, J., and Lewin, A. S. (1996) Nucleic Acids Res. 24, 3415–3424
12. Weeks, K. M., and Cech, T. R. (1995) Biochemistry 34, 7728–7738
13. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
14. Zamore, P. D., and Green, M. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9242–9247
15. Bohnet, P., and Balian, G. (1977) Methods Enzymol. 48, 3235–3238
16. Jacobson, G. R., Schaffer, M. H., Stark, G. R., and Vanaman, T. C. (1973) J. Biol. Chem. 248, 6583–6591
17. Degani, Y., and Patchornik, A. (1974) Biochemistry 13, 1–11
18. Schagger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
19. Zoller, M. J., and Smith, M. (1984) DNA 3, 479–488
20. Tobin, H., Staelhelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. 76, 4350–4354
21. Wong, I., and Lohman, T. M. (1993) Proc. Natl. Acad. Sci. 90, 5428–5432
22. Sheltair, M. (1990) in Photochemical and Photobiological Reviews (Smith, K., ed) pp. 105–107, Plenum Press, New York
23. Safer, B., Cohen, R. B., Garfinkel, S., and Thompson, J. A. (1988) Mol. Cell. Biol. 8, 105–113
24. Williams, R. K., and Konigsberg, W. H. (1991) Methods Enzymol. 208, 516–539, 516–539
25. Anderson, J. T., Paddy, M. R., and Swanson, M. S. (1993) Mol. Cell. Biol. 13, 6102–6113
26. Kharrat, A., Macias, M. J., Gibson, T. J., Nilges, M., and Pastore, A. (1995) EMBO J. 14, 3572–3584
27. Chang, B. Y., and Doi, R. H. (1993) Biochem. J. 294, 43–47
28. Hay, R. T., and Nicholson, J. (1993) Nucleic Acids Res. 21, 4592–4598
29. Ikeda, M., Wilcox, E. C., and Chin, W. W. (1996) Nucleic Acids Res. 24, 221–230
30. Liu, X., Garriga, P., and Khorana, H. G. (1996) Proc. Natl. Acad. Sci. 93, 4554–4559
31. Hill, J., McGraw, P., and Tzagoloff, A. (1985) J. Biol. Chem. 260, 3235–3238
32. Shaw, L. C., and Lewin, A. S. (1997) Nucleic Acids Res. 25, 1587–1604
33. Akins, R. A., and Lambowitz, A. M. (1987) Cell 50, 331–345
34. Cherniack, A. D., Garriga, G., Kittle, J. D., Jr., Akins, R. A., and Lambowitz, A. M. (1990) Cell 62, 745–755
35. Cleghorn, V., and Klessig, D. F. (1992) J. Biol. Chem. 267, 17872–17881
36. Parker, E. J., Botting, C. H., Webster, A., and Hay, R. T. (1998) Nucleic Acids Res. 26, 1240–1247
37. Vaisanen, S., Junutunen, K., Ikonen, A., Vihko, P., and Maenpaa, P. H. (1997) Eur. J. Biochem. 248, 156–162
38. Kobrak, K. J., Opolko, J., Xu, M., Sarlis, N. J., and Simons, S. S., Jr. (1997) J. Biol. Chem. 272, 23886–23894
39. Chu, D. M., Corbin, J. D., Grimes, K. A., and Francis, S. H. (1997) J. Biol. Chem. 272, 31922–31928
40. St. Johnston, D., Brown, N. H., Gall, J. G., and Jantsch, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10979–10983
41. Green, S. R., and Mathews, M. B. (1992) Genes Dev. 6, 2478–2490
42. McCormack, S. J., Thomis, D. C., and Samuel, C. E. (1992) Virology 188, 47–56
43. Gatignol, A., Bucker-White, A., Berkhout, B., and Jeang, K. T. (1991) Science 251, 1597–1600
44. Gatignol, A., Bucker, C., and Jeang, K. T. (1993) Mol. Cell. Biol. 13, 2193–2202
45. Hunter, T., Hunt, T., Jackson, R. J., and Robertson, H. D. (1975) J. Biol. Chem. 250, 409–417
46. Bevilacqua, P. C., and Cech, T. R. (1996) Biochemistry 35, 9983–9994
47. Herschlag, D., Khoura, M., Tsukihashi, Z., and Karpel, R. L. (1994) EMBO J. 13, 2913–2924