Determinants of RNA Binding and Translational Repression by the Bicaudal-C Regulatory Protein*

Yan Zhang1, Sookhee Park, Susanne Blaser, and Michael D. Sheets2
From the Department of Biomolecular Chemistry, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, Wisconsin 53706

Background: Bicaudal-C RNA binding proteins are important translational repressors in many different biological contexts.

Results: A minimal site for RNA binding and translation repression by Bicaudal-C was identified.

Conclusion: The RNA binding site for Bicaudal-C forms a stem-loop structure important for binding and translational repression.

Significance: This will facilitate the identification of Bicaudal-C binding sites in other mRNAs.

Bicaudal-C (Bic-C) RNA binding proteins function as important translational repressors in multiple biological contexts within metazoans. However, their RNA binding sites are unknown. We recently demonstrated that Bic-C functions in spatially regulated translational repression of the xCR1 mRNA during Xenopus development. This repression contributes to normal development by confining the xCR1 protein, a regulator of key signaling pathways, to specific cells of the embryo. In this report, we combined biochemical approaches with in vivo mRNA reporter assays to define the minimal Bic-C target site within the xCR1 mRNA. This 32-nucleotide Bic-C target site is predicted to fold into a stem-loop secondary structure. Mutational analyses provided evidence that this stem-loop structure is important for Bic-C binding. The Bic-C target site was sufficient for Bic-C mediated repression in vivo. Thus, we describe the first RNA binding site for a Bic-C protein. This identification provides an important step toward understanding the mechanisms by which evolutionarily conserved Bic-C proteins control cellular function in metazoans.

Recently, we identified the Xenopus Cripto-1 mRNA (referred to as xCR1) as a direct Bic-C target (7). Translational repression of xCR1 mRNA by Bic-C in vegetal cells of the embryo restricts the xCR1 protein, a ligand that regulates multiple developmental signaling pathways, to cells of the animal hemisphere (8). We identified a region of the xCR1 mRNA 3’-UTR that was necessary and sufficient for Bic-C binding to RNA as well as Bic-C-dependent translational repression. We called this region the translational control element (TCE) and proposed that it contained one or more specific Bic-C binding sites (8).

The N-terminal half of Bic-C proteins contain three predicted hnRNP-K-homolog (KH) domains and two predicted KH-like domains (1, 9). Although KH domains are known to bind RNA, there is only limited information about the general molecular rules that govern formation of KH-domain-RNA interfaces (10, 11). In our previous study identifying the xCR1 mRNA as a direct Bic-C target, we showed that the N-terminal half of Bic-C that contains the KH and KH-like domains was sufficient for specific binding to the xCR1 3’-UTR in vivo and in vitro. Studies in Drosophila (2) and human cells (12) have also identified mRNAs regulated by Bic-C; however, the mechanistic basis of Bic-C binding to specific RNAs is unknown.

Here, we used EMSA, RNase protection, and RNA footprinting assays to define a 32-nucleotide region within the xCR1 TCE that functions as a minimal Bic-C binding site. This site is predicted to form a stem-loop secondary structure, and mutational analyses supported the importance of this secondary structure for Bic-C-RNA interactions. Finally, the results of translational reporter assays in living embryos demonstrated that this minimal Bic-C binding site was also sufficient for Bic-C-mediated translational repression. Thus, we have defined the first Bic-C target site that is functional for both specific Bic-C binding in vitro and Bic-C-mediated translational repression in vivo.

EXPERIMENTAL PROCEDURES

Luciferase Reporter mRNA Plasmids and in Vitro mRNA Synthesis—Different regions of the xCR1 3’-UTR were generated from the xCR1 cDNA by PCR. DNA fragments were cloned into the BglIII and BamHI sites of the pT7LucBglIII plasmid.
Defining a Bicaudal-C Minimal RNA Binding Site

mid to generate luciferase reporter plasmids (14). The luciferase reporter plasmid containing the Xenopus cyclin B1 3′-UTR has been described previously (14). Plasmids containing three tandem copies of the Bic-C binding site and its derivatives were generated by cloning synthetic DNA fragments (Gene Blocks, Integrated DNA Technology) into the pT7LucBglII plasmid. All plasmids were linearized with appropriate restriction enzymes and used to generate capped mRNAs as described (7, 8, 13, 14) with reagents from New England Biolabs.

mRNA Injections and Luciferase Assays—Each reporter mRNA was diluted to a concentration of 2.5 nM, and 5 nanoliters (12.5 amol) were injected into embryonic cells of Xenopus embryos. For animal cell assays of Bic-C repression, reporter mRNAs were injected alone or together with 500 pg of mRNA encoding Xenopus Bic-C into one animal cell of eight-cell embryos. For assays of vegetal cell repression, a single animal cell of one set of embryos and a single vegetal cell of a separate set of eight-cell embryos were injected with reporter mRNAs. Injected embryos were cultured until stage 7, extracts were prepared from samples of 10 embryos and analyzed for luciferase activity as described previously (7, 8, 13, 14).

EMSA—Recombinant GST-Bic-C N-terminal fusion protein (residues 1–506) was expressed and purified as described (15, 16). The various RNA substrates were derived from the xCR1 3′-UTR and uniformly radiolabeled by in vitro transcription reactions that included P32UTP (8). The negative control RNA (negative control, 261 nucleotides) was generated from the poly linker of the pSTBlue-1 plasmid (Novagen/EMD Biosciences). Binding reactions (20 µl) contained GST-Bic-C-NT protein (either 0, 50, 200 nM), 10 mM Hepes, pH 8.0, 1 mM EDTA, 50 mM KCl, 0.02% Tween 20, 0.2 mg/ml yeast tRNA, 100 µg/ml BSA, 2 mM DTT, and 0.5 mM RNA. Reaction products were analyzed on 4% (1 × TBE) native polyacrylamide gels (7, 8).

Bic-C Protection Assays—Uniformly radiolabeled RNAs were combined with recombinant Bic-C N-terminal fusion protein (200 nM) using conditions identical to those used for electrophoretic mobility assays with the addition of RNase T1 (1000 units) or RNaseOne (10 units, Promega) to some of the reactions. The reaction products were analyzed by denaturing 20% polyacrylamide gel electrophoresis.

RNase Footprinting—The 287–464 xCR1 RNA substrate was radiolabeled at its 5′ end using polynucleotide kinase and γ32P-labeled ATP. Footprinting reactions (20 µl) contained 10 mM Hepes, pH 8.0, 1 mM EDTA, 50 mM KCl, 0.02% Tween 20, 0.2 mg/ml yeast tRNA, 100 µg/ml BSA, 2 mM DTT, and 0.5 mM RNA. Reactions also contained various amounts of GST-Bic-C N-terminal protein or Bic-C N-terminal protein. The Bic-C N-terminal protein was generated from a recombinant SUMO-Bic-C-N-terminal protein using SUMO protease to cleave off the SUMO tag (17). Reactions were incubated 10 min at 20 °C. RNaseA (10 pg) was added, and incubation was continued for 2 min. The reaction products were analyzed by denaturing 15% polyacrylamide gel electrophoresis and compared with RNA molecular weight markers generated by 5′-radiolabeling RNAs of the low range ssRNA ladder (New England Biolabs). RNA markers to delineate the boundaries of the 32-nucleotide Bic-C binding site were generated by treating the 5′ end-labeled 287–464 RNA with oligonucleotides and RNase H. Mk1

FIGURE 1. Defining a minimal Bic-C binding site within the 3′-UTR of the xCR1 mRNA. A, diagram depicting the regions of the xCR1 3′-UTR analyzed for Bic-C binding using native gel electrophoresis. The full-length xCR1 3′-UTR is 963 nucleotides long, and the numbers indicate the nucleotide position with the 3′-UTR. The gray vertical bar highlights the position of the 32-nucleotide minimal Bic-C binding site in the RNA substrates analyzed. B, protein gel stained of GST Bic-C N-terminal protein stained with Coomassie Blue. The position of molecular mass markers (in kDa) (MW) is indicated. The expected molecular mass of the protein was 82 kDa. C and D, radiolabeled RNAs were mixed with 0, 10, 50, and 200 nM GST Bic-C N-terminal protein (C, lanes 1–12) or 0, 50, and 200 nM GST Bic-C N-terminal protein (D, lanes 1–18), and binding analyzed by native gel electrophoresis.

was created with oligonucleotide ctttgtcaattaataaaaaatttaaaagtttgtgaa and Mk2 was created with oligonucleotide gctgttaatgtaacggtcttaaag.

RESULTS

Defining a Minimal Bic-C Binding Site within the 3′-UTR of the xCR1 mRNA—To biochemically analyze the interaction between Bic-C and the xCR1 3′-UTR, three independent RNA-protein interaction assays were used. First, we used EMSAs to analyze RNA binding by Bic-C (Fig. 1). We previously demonstrated that a GST fusion protein containing the N-terminal half of Bic-C, including the KH and KH-like domains (Bic-C N-terminal; amino acids 1–506 of Bic-C) binds the xCR1 TCE but not other similarly sized RNAs (7, 8). To define a region of

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 289 • NUMBER 11 • MARCH 14, 2014

7498
the TCE sufficient for Bic-C binding, radiolabeled RNAs corresponding to different regions of the TCE were mixed with purified Bic-C N-terminal protein, and the reactions were analyzed by EMSA. Bic-C N-terminal efficiently bound the intact TCE RNA (nts 286–637) and the RNA containing the 5'-half of the TCE (nts 286–464) (Fig. 1, A and C, lanes 1–8). In contrast, Bic-C bound the RNA containing the 3'-half of the TCE (nts 465–637) less efficiently (Fig. 1C, lanes 9–12). Further analysis of smaller RNAs derived from the 5'-half of the TCE (nts 286–464) (Fig. 1D, lanes 1–18) identified a 32-nucleotide RNA (nts 358–389 of the TCE) that was sufficient for Bic-C binding (Fig. 1D, lanes 13–15).

As a second approach, Bic-C binding to the xCR1 TCE RNA was analyzed with an RNase protection assay (Fig. 2). In these assays, uniformly 32P-labeled xCR1 TCE RNA (nts 286–637 of the xCR1 3'-UTR), was incubated with Bic-C N-terminal protein and the binding reactions were treated with RNase T1 that cleaves RNA only after guanine residues. Bound Bic-C N-terminal protein should protect G residues within the Bic-C binding sites from cleavage by the RNase. RNase T1 treatment of the TCE RNA in the absence of Bic-C protein produced the predicted RNA oligonucleotides (Fig. 2, A–C, lane 2). When Bic-C N-terminal protein was present in the reaction, a new protected RNA product 86 nucleotides in length was observed (Fig. 2C, lanes 3–5). This protected RNA fragment was gel-purified and treated with RNase T1, and the products were analyzed by denaturing gel electrophoresis (lane 2) in comparison with RNase T1 cleavage products of the untreated radiolabeled 286–637 RNA (lane 1). Bic-C protects a region of the xCR1 3'-UTR from RNase T1 cleavage in the presence of increasing amounts of GST Bic-C N-terminal protein (lanes 1–5) were analyzed by denaturing polyacrylamide gel electrophoresis. The 86-nucleotide region protected by Bic-C from RNase T1 cleavage is highlighted in gray. The thick black line indicates the 32-nucleotide region identified by native gel electrophoresis. Bic-C specifically protects a region of the xCR1 3'-UTR from RNase T1 cleavage that encompasses the minimal 32 nucleotide Bic-C binding site. Untreated 32P-labeled 286–637 RNA (lane 1), RNase T1-treated labeled 286–637 RNA (lane 2), and labeled 286–637 RNA treated with RNase T1 in the presence of increasing amounts of GST Bic-C N-terminal protein (lanes 3–5) were analyzed by denaturing polyacrylamide gel electrophoresis. Further analysis of the Bic-C RNaseOne protected RNA fragments. Bic-C RNaseOne protected RNA fragments were purified from polyacrylamide gels and reanalyzed by denaturing polyacrylamide gel electrophoresis after no treatment (lane 2) or treatment with RNase T1 (lane 3). Lane 1 contains the untreated radiolabeled 286–637 RNA cleaved with RNase T1 (lane 1) to serve as RNA size markers.
Defining a Bicaudal-C Minimal RNA Binding Site

RNA oligonucleotides (Figs. 2D, lane 2). The oligonucleotides from the Bic-C-protected fragment were a subset of oligonucleotides generated from RNase T1 treatment of the TCE RNA (Figs. 2, A, B, and D, lane 2). These results indicate that the Bic-C protected RNA fragment includes the same 32-nucleotide region of the TCE sufficient for Bic-C binding as measured in EMSAs (Fig. 1).

The specificity of RNase T1 (i.e. RNase T1 cleaves RNA only after G residues) (18) limits the resolution of the protection assays performed with this enzyme. To address this bias, protection experiments were also performed with RNaseOne, an enzyme with no nucleotide specificity for cleavage (19, 20). Uniformly radiolabeled TCE RNA (nts 286–464 of the xCR1 3′-UTR) treated with RNaseOne was cleaved into short RNA oligonucleotides <11 nucleotides in length (Fig. 2E, lane 3). The addition of Bic-C N-terminal protein to the reaction produced Bic-C protected RNA products 31 to 34 nucleotides in length (Fig. 2E, lane 5, and F, lane 2). The RNase One protected fragments were purified, cleaved with RNase T1, and the products were analyzed by polyacrylamide gel electrophoresis. The resulting RNA oligonucleotides indicated that the RNaseOne protected RNAs were composed of a distinctive set of T1 RNA oligonucleotides characteristic of the 32-nucleotide region sufficient for Bic-C binding (Fig. 2, F, lane 3, and see Fig. 2, A and B, fragments indicated by blue line).

RNase footprinting was used as third approach to analyze Bic-C-TCE interactions. RNA consisting of the 5′-half of the TCE (nts 286–464 of the xCR1 3′-UTR, Fig. 3A) was radiolabeled at its 5′ end. The labeled RNA was treated with limiting amounts of RNase A in the presence or absence of the Bic-N-terminal protein (Fig. 3C), and the products were analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 3B). The Bic-C N-terminal protein protected a region of the TCE RNA from RNase A cleavage in a concentration-dependent manner (Fig. 3B, lanes 5–12). Comparison of the reaction products to specific RNA markers (Fig. 3, A and B, Mk1 and Mk2) demonstrates that the Bic-C N-terminal protected region coincided with the same 32-nucleotide minimal Bic-C binding site defined independently by EMSAs and RNase protection (Figs. 1 and 2). Thus, three independent and complementary biochemical approaches for examining RNA-protein interactions support the conclusion that a single 32-nucleotide RNA region within the xCR1 mRNA 3′-UTR constitutes a minimal Bic-C binding site.

Characterization of the Bic-C RNA Binding Site—Inspection of the Bic-C binding site (Figs. 1–3) using mFOLD (21) indicated that this 32-nucleotide RNA could potentially form a secondary structure that consisted of a five nucleotide stem with a six-nucleotide loop (Fig. 4A). To test the importance of this predicted structure for Bic-C binding, RNA variants containing specific nucleotide base changes were analyzed for Bic-C binding by EMSAs (see Fig. 1). RNAs containing three nucleotide changes of one or the other strand of the predicted stem impaired Bic-C binding (Fig. 4B, Stem1 and Stem2 RNAs, and Fig. 4C, lanes 5–8). However, efficient binding could be restored using another RNA variant that contained three additional nucleotide changes predicted to restore complementary base pairing between strands of the putative stem (Fig. 4B, Stem1/Stem2 RNA, and Fig. 4C, lanes 9 and 10). In addition, Bic-C efficiently bound RNAs that contained either two additional GC base pairs added to the base of the stem (Fig. 4B, Stem+2GC RNA, and Fig. 4D, lanes 3 and 4) or 10 nucleotide changes of the putative stem predicted to maintain base pairing between strands (Fig. 4B, Stem10 RNA, and Fig. 4D, lanes 5 and 6). These results support a model in which a stem structure rather than sequence of the stem is an important characteristic of the Bic-C binding site present in the xCR1 3′-UTR.

Although the stem was important for binding, it was not sufficient. Bic-C did not bind efficiently to an RNA substrate
that contains the stem but lacks the region preceding the stem (RNA 368–389, Fig. 4, lanes 11 and 12). Also, the importance of putative loop for Bic-C binding was analyzed with an additional RNA variant in which the nucleotide sequence of the loop was changed (Fig. 4B, Loop). Bic-C failed to bind this RNA variant (Fig. 4C, lanes 3 and 4). Taken together, these results provide evidence that the RNA binding site for Bic-C within the xCR1 mRNA TCE formed a stem-loop structure in which both the stem and the sequence of the loop are important determinants for recognition by Bic-C.

The Bic-C Binding Site Is Sufficient for Translational Repression—The central region of the xCR1 mRNA 3′-UTR, termed the TCE (translational control region; nts 286–637) is sufficient for Bic-C-mediated translational repression (7, 8). The mRNA that encodes the Bic-C protein is localized to vegetal cells of Xenopus embryos. Consequently, animal cells of the embryo are normally deficient for Bic-C (22), and as a result of this deficiency, the xCR1 mRNA is translated in animal cells. Thus animal cells of Xenopus embryos provide a context to assay for Bic-C-mediated repression (7). Specifically, injection of animal cells with a luciferase reporter mRNA containing the xCR1 3′-UTR with an mRNA that expresses full length Bic-C protein leads to specific repression of the reporter mRNA in Xenopus embryos (Fig. 5A) (7).

This assay was used to determine the region of the xCR1 3′-UTR sufficient for Bic-C-mediated repression by examining reporter mRNAs bearing sub-segments of the TCE (Fig. 5B). Whereas removal of 100 or 174 nucleotides from the 3′ end of the TCE had small affects on Bic-C repression (Fig. 5, B and C, reporters 286–554 and 286–464), the removal of an additional 106 nucleotides that included the 32-nucleotide Bic-C binding site eliminated repression (Fig. 5, B and C, reporter 286–358). Thus, sequences critical for Bic-C mediated repression reside in the region of the xCR1 3′-UTR between nucleotides 358 and 464 and included the Bic-C binding site.

To determine whether the minimal Bic-C binding site identified biochemically was sufficient for translational repression, luciferase reporter mRNAs containing one or three copies of
the binding site were analyzed in the animal cell assay. A single binding site was sufficient to elicit a low level of repression (Fig. 5D, xCR1 3'-UTR nts 358–389 × 1). However, three copies of the minimal Bic-C binding site elicited repression almost as efficiently as the TCE of xCR1 mRNA 3'-UTR (Fig. 5D, xCR1 3'-UTR nts 358–389 × 3). Thus, the Bic-C binding site defined biochemically provides sufficient information to direct robust translational repression.

Competition experiments were used as a second approach to analyze the relationship between the Bic-C binding site and translational repression. We reasoned that if Bic-C was essential for translational repression of xCR1 mRNA, then the presence of excess Bic-C binding sites would disrupt binding to the TCE of the xCR1 3'-UTR and disrupt repression of TCE-containing reporter mRNAs. Reporter assays were performed as described above with the inclusion of a 1000-fold excess of different potential RNA competitors (Fig. 5E). Translational repression of the TCE-containing luciferase reporter mRNA (nts 286–637 of the xCR1 3'-UTR) was attenuated by the presence of RNAs containing Bic-C binding sites (Fig. 5E, either the TCE (nts 286–637) or the 358–389 × 3 RNA). In contrast, repression was unaffected by the presence of an RNA that lacked Bic-C binding sites; (Fig. 5E, xCR1 3'-UTR nucleotides 1–308).

To investigate the structural requirements of the Bic-C binding site for translational repression, different luciferase reporter mRNAs containing three copies of different variants of the Bic-C binding site were analyzed in the animal cell assay (Fig. 5A). Reporter mRNA where the Bic-C binding site contained five nucleotide changes on one strand of the predicted stem impaired Bic-C repression (Fig. 6A, Stem5 reporter mRNA). However, efficient repression of the reporter mRNA could be restored by using a variant that contained the five additional nucleotide changes predicted to restore complementary base pairing between strands of the putative stem (Fig. 6A, Stem10 reporter mRNA). In addition, the reporter mRNA in which the nucleotides of the putative loop in the Bic-C binding site were changed was inefficiently repressed by Bic-C (Fig. 6A, loop reporter mRNA).

The normal mechanism of xCR1 translational repression is vegetal cell-specific, consistent with the localization of Bic-C protein to these cells. Thus, another prediction was that reporter mRNAs containing the minimal Bic-C binding site would be translationally repressed in vegetal cells. We tested this prediction by injecting reporter mRNAs into vegetal cells and comparing their translation to reporter mRNAs injected into animal cells, cells in which the xCR1 mRNA is normally translated (Fig. 6B). The reporter mRNA containing three copies of the Bic-C binding sites was repressed in vegetal cells and repression was comparable with a reporter containing the TCE of the xCR1 3'-UTR (Fig. 6C). Reporter mRNAs were inefficiently repressed by vegetal cells when they contained either the
Defining a Bicaudal-C Minimal RNA Binding Site

Bic-C binding sites with nucleotide changes to one strand of the potential stem (Fig. 6C, stem 5) or nucleotide changes to the potential loop (Fig. 6C, loop). In contrast, the reporter containing Bic-C binding sites with nucleotide changes predicted to restore base pairing and restore formation of the potential stem was repressed as efficiently as the reporter containing the wild type binding site (Fig. 6C, stem 10). Thus, repression in vegetal cells exhibited the same requirements for the Bic-C binding site as observed in biochemical experiments of Bic-C binding (Fig. 4) and repression in animal cells expressing Bic-C (Fig. 6A).

DISCUSSION

Bic-C proteins are expressed in embryos and specific adult tissues of invertebrate and vertebrate organisms where they regulate diverse biological processes (1). All evidence to date indicates that Bic-C in all metazoans functions primarily by repressing the translation of a select subset of mRNAs; yet, there is a paucity of information about the molecular determinants that specify RNAs for Bic-C binding. In Xenopus, Bic-C is suggested to be important for establishing the fundamental differences between animal and vegetal cells of the embryo (22). In particular, during early embryonic cell divisions, Bic-C protein accumulates only within the vegetal cells of the embryo because the mRNA encoding Bic-C is specifically localized to the vegetal cortex of developing oocytes. We recently identified the xCR1 mRNA as a bona fide target of Bic-C-mediated translation repression in vegetal cells (7). We hypothesize that Bic-C repression of this mRNA in vegetal cells allows for the accumulation of xCR1 protein specifically within the animal cells of the embryo, where it can regulate multiple developmentally important signaling pathways (8, 23).

Here, we substantially extended these findings by using several independent and complementary biochemical approaches to define a minimal Bic-C target site within the xCR1 mRNA 3’-UTR. Our data provide evidence that the Bic-C target site consists of both structural and sequence elements, suggesting a level of complexity that must be considered for developing computational tools to predict additional Bic-C target RNAs. Although recent studies have identified mRNAs potentially regulated by Bic-C (2, 12), the data presented here represent the first description of an RNA binding site for any Bic-C protein.

The evolutionarily conserved KH domains are found in a variety of different proteins that regulate splicing, translation, and other events of RNA metabolism (10, 11, 24). Many of these proteins employ multiple KH domains to direct specific RNA binding and provide substrate specificity. Often in such proteins the multiple KH domains function together to recognize either short sequence elements or structural features of their RNA substrates. For example, the KH domains of the Nova-1 protein prefer to bind RNA substrates that contain short sequence motifs within stem-loop structures. The sequence of the stem is less important than its structure, but the sequence of the loop is critical (25, 26). In contrast, the KH domains of the FMRP (Fragile-X mental retardation protein), mediate the recognition of RNAs that contain a specific tertiary structure (27, 28). Our results indicate that the KH domains of Bic-C mediate its binding to the 3’-UTR of the xCR1 mRNA and that secondary structure is an important feature of this binding. Additional experiments are required to define the exact RNA structure optimal for Bic-C binding and to determine what other sequence features are important for binding as well.
Defining a Bicaudal-C Minimal RNA Binding Site

The Bic-C binding site from the xCR1 3′-UTR was sufficient for translational repression. Although a single Bic-C binding site was able to direct a low, but detectable amount of repression, three sites used in combination repressed translation nearly as efficiently as the intact TCE region of the xCR1 3′-UTR. These results suggest that Bic-C binding to a single target site within the xCR1 3′-UTR may be facilitated by additional factors that interact with both the RNA substrate and Bic-C. For example, we previously demonstrated that the TCE region of the xCR1 3′-UTR contains binding sites for the pumilio and CELF1 (also called CUGBP1) proteins (8). In the context of the intact xCR1 3′-UTR with a single efficient Bic-C binding site these proteins may function together to facilitate efficient Bic-C binding. Indeed, stabilizing Bic-C binding to the xCR1 3′-UTR could be their primary function in xCR1 mRNA translational regulation. The role of these factors may be circumvented by the presence of multiple binding sites that increase the probability that Bic-C will bind the 3′-UTR.

Although our results provide evidence that a Bic-C-RNA element interaction is sufficient for efficient translational repression in Xenopus embryos, recent studies in kidney cells suggest that Bic-C represses target mRNAs in collaboration with specific miRNAs (4,12). Our results suggest that Bic-C can function autonomously in binding an mRNA target and repressing translation, but efficient Bic-C-dependent translational regulation in adult cell types may depend upon additional components and mechanisms. Defining how Bic-C binds RNA is a critical step to understanding how this conserved cellular regulatory protein functions in vivo.

Acknowledgments—We thank Catherine Fox for insightful and useful comments. We thank Elsebet Lund for technical guidance and comments on the manuscript and Laura Vanderploeg for preparing figures.

REFERENCES

1. Gamberi, C., and Lasko, P. (2012) The bic-C family of developmental translational regulators. Comp. Funct. Genomics 10.1155/2012/141386
2. Chicoine, J., Benoit, P., Gamberi, C., Paloureas, M., Simonelig, M., and Lasko, P. (2007) Bicaudal-C recruits CCR4-NOT deadenylase to target mRNAs and regulates oogenesis, cytoskeletal organization, and its own expression. Dev. Cell 13, 691–704
3. Saffman, E. E., Styhler, S., Rother, K., Li, W., Richard, S., and Lasko, P. (1998) Premature translation of oskar in oocytes lacking the RNA-binding protein bicaudal-C. Mol. Cell. Biol. 18, 4855–4862
4. Tran, U., Zakin, L., Schweickert, A., Agrawal, R., Däger, R., Blum, M., De Robertis, E. M., and Wessely, O. (2010) The RNA-binding protein bicaudal-C regulates polycystin 2 in the kidney by antagonizing miR-17 activity. Development 137, 1107–1116
5. Maisonneuve, C., Guillerot, L., Vick, P., Weber, T., Andre, P., Beyer, T., Blum, M., and Constan, D. B. (2009) Bicaudal C, a novel regulator of Dvl signaling abutting RNA-processing bodies, controls cilia orientation and leftward flow. Development 136, 3019–3030
6. Kraus, M. R., Clain, S., Pfister, Y., Di Maio, M., Ulinski, T., Constan, D., Bellanné-Chantelot, C., and Grapin-Botton, A. (2012) Two mutations in human BICCl resulting in Wnt pathway hyperactivity associated with cystic renal dysplasia. Hum. Mutat. 33, 86–90
7. Zhang, Y., Cooke, A., Park, S., Dewey, C.N., Wickens, M., and Sheets, M.D. (2013) Bicaudal-C spatially controls translation of vertebrate maternal mRNAs. RNA 11, 1575–1582
8. Zhang, Y., Forinash, K. D., McGivern, J., Fritz, B., Dorey, K., and Sheets, M. D. (2009) Spatially restricted translation of the xCR1 mRNA in Xenopus embryos. Mol. Cell. Biol. 29, 3791–3802
9. Nakel, K., Hartung, S. A., Bonnefo, F., Eckmann, C. R., and Conni, F. (2010) Four KH domains of the C. elegans Bicaudal-C ortholog GLD-3 form a globular structural platform. RNA 16, 2058–2067
10. Valverde, R., Edwards, L., and Regan, L. (2008) Structure and function of KH domains. FEBS J. 275, 2712–2726
11. Ankö, M. L., and Neugebauer, K. M. (2012) RNA-protein interactions in vivo: global gets specific. Trends Biochem. Sci. 37, 255–262
12. Piazzon, N., Maisonneuve, C., Guillerot, I., Rotman, S., and Constan, D. B. (2012) BicC1 links the regulation of CAMP signaling in polycystic kidneys to microRNA-induced gene silencing. J. Mol. Cell. Biol. 4, 398–408
13. Fritz, B. R., and Sheets, M. D. (2001) Regulation of the mRNAs encoding proteins of the BMP signaling pathway during the maternal stages of Xenopus development. Dev. Biol. 236, 230–243
14. Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., and Wickens, M. (1994) The 3′-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. Genes Dev. 8, 926–938
15. Hou, Z., Danzer, J. R., Mendoza, L., Bose, M. E., Müller, U., Williams, B., and Fox, C. A. (2009) Phyleogenetic conservation and homology modeling help reveal a novel domain within the budding yeast heterochromatin protein Sir1. Mol. Cell. Biol. 29, 687–702
16. Hou, Z., Bernstein, D. A., Fox, C. A., and Keck, J. L. (2005) Structural basis of the Sir1-origin recognition complex interaction in transcriptional silencing. Proc. Natl. Acad. Sci. U.S.A. 102, 8489–8494
17. Malakhov, M. P., Mattern, M. R., Malakhova, O. A., Drinker, M., Weeks, S. D., and Butt, T. R. (2004) SUMO fusions and SUMO-specific protease for precise translation and purification of proteins. J. Struct. Funct. Genomics 5, 75–86
18. Takahashi, K., Uchida, T., and Egami, F. (1970) Ribonuclease TI, structure and function. Adv. Exp. Biol. 1, 53–98
19. Meador, J., 3rd, Cannon, B., Cannistraro, V. J., and Kennell, D. (1990) Purification and characterization of Escherichia coli RNase I. Comparisons with RNase M. Eur. J. Biochem. 187, 549–553
20. Meador, J., 3rd, and Kennell, D. (1990) Cloning and sequencing the gene encoding Escherichia coli ribonuclease I: exact physical mapping using the genome library. Gene 95, 1–27
21. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415
22. Wessely, O., and De Robertis, E. M. (2000) The Xenopus homologue of Bicaudal-C is a localized maternal mRNA that can induce endoderm formation. Development 127, 2053–2062
23. Dorey, K., and Hill, C. S. (2006) A novel Cripto-related protein reveals an essential role for EGF-CFCs in Nodal signalling in Xenopus embryos. Dev. Biol. 292, 303–316
24. Ray, D., Kazan, H., Cook, K. B., Weirchau, M. T., Najafabadi, S. H., Li, X., Gueroussou, S., Albu, M., Zheng, H., Yang, A., Na, H., Irimia, M., Matzat, L. H., Dale, R. K., Smith, S. A., Yarosh, C. A., Kelly, S. M., Nabet, B., Mecenas, D., Li, W., Laishram, R. S., Qiao, M., Lipshitz, H. D., Piano, F., Corbett, A. H., Carlsten, R. P., Frey, B. J., Anderson, R. A., Lynch, K. W., Penalva, L. O., Lei, E. P., Fraser, A. G., Blencowe, B. J., Morris, Q. D., and Hughes, T. R. (2013) A compendium of RNA-binding motifs for decoding gene regulation. Nature 499, 172–177
25. Lewis, H. A., Musunuru, K., Jensen, K. B., Edo, C., Chen, H., Darnell, R. B., and Burkeley, S. K. (2000) Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. Cell 100, 323–332
26. Jensen, K. B., Musunuru, K., Lewis, H. A., Burkle, S. L., and Darnell, R. B. (2000) The tetranucleotide UCAY directs the specific recognition of RNA by the Nova K-homology 3 domain. Proc. Natl. Acad. Sci. U.S.A. 97, 5740–5745
27. Darnell, J. C., Warren, S. T., and Darnell, R. B. (2004) The fragile X mental retardation protein, FMRP, recognizes G-quartet mRNAs important for neuronal function. Cell 107, 489–499

7504 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 289 • NUMBER 11 • MARCH 14, 2014