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REVIEW

The poly(C)-binding proteins: A multiplicity of functions and a search for mechanisms

ALEKSANDR V. MAKEYEV and STEPHEN A. LIEBHABER

Departments of Genetics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

ABSTRACT

The poly(C) binding proteins (PCBPs) are encoded at five dispersed loci in the mouse and human genomes. These proteins, which can be divided into two groups, hnRNPs K/J and the αCPs (αCP1-4), are linked by a common evolutionary history, a shared triple KH domain configuration, and by their poly(C) binding specificity. Given these conserved characteristics it is remarkable to find a substantial diversity in PCBP functions. The roles of these proteins in mRNA stabilization, translational activation, and translational silencing suggest a complex and diverse set of post-transcriptional control pathways. Their additional putative functions in transcriptional control and as structural components of important DNA-protein complexes further support their remarkable structural and functional versatility. Clearly the identification of additional binding targets and delineation of corresponding control mechanisms and effector pathways will establish highly informative models for further exploration.

Keywords: αCP; hnRNP K; mRNA stability; mRNA translation; poly(C) binding proteins; post-transcriptional controls

PERSPECTIVE

It has long been noted that certain RNA-binding proteins can be characterized, grouped, and purified on the basis of their binding to nucleic acid homopolymers (Swanson & Dreyfuss, 1988). A defined set of RNA-binding proteins is characterized by high affinity and sequence-specific interaction with poly(C). These poly(C)-binding proteins (PCBPs) comprise two subsets in mammalian cells; hnRNPs K/J (Matunis et al., 1992) and the αCP proteins (α-complex proteins). The exact structural and genetic relationship of hnRNP K and J remains to be fully defined. The αCPs are encoded at four dispersed loci, with additional isoforms generated via alternative splicing (Makeyev & Liebhaber, 2000). The PCBPs studied in greatest detail are hnRNP K, αCP-1, and αCP-2. The latter two proteins are alternatively referred to as PCBP1 and PCBP2 or hnRNP-E1 and hnRNP-E2 (Kiledjian et al., 1995; Leffers et al., 1995). Recent studies, summarized in this review, reveal that the PCBPs are involved in a remarkable array of biological processes. Members of this protein family are linked to mRNA stabilization (Weiss & Liebhaber, 1994, 1995; Holcik & Liebhaber, 1997), translational silencing (Ostareck et al., 1997; Collier et al., 1998), and translational enhancement (Blyn et al., 1997; Gamarnik & Andino, 1997). These proteins also appear to be involved as determinants of transcriptional controls (Michelotti et al., 1996; Tomonaga & Levens, 1996) and apoptotic pathways (Charroux et al., 1999; Zhu & Chen, 2000) and constitute candidate structural components of recombination complexes within retrotransposon long terminal repeats (Goller et al., 1994) and in telomere complexes (Lacroix et al., 2000). The common denominator of PCBP activities appears to reflect binding to C-rich single-strand motifs. Determining how such interactions might factor into this broad array of diverse biologic functions constitutes a major challenge to current research efforts.

Initial studies of PCBPs have been previously reviewed (Bomsztyk et al., 1997; Ostareck-Lederer et al., 1998). This article will focus with particular attention on three areas of recent interest: (1) identification and characterization of novel PCBP isoforms and posttranslational modifications that may underlie their functional diversity; (2) new insights into the evolutionary history

Reprint requests to: Dr. Stephen A. Liebhaber, University of Pennsylvania School of Medicine, Department of Genetics, CRB—Room 428, 415 Curie Blvd., Philadelphia, Pennsylvania 19104-6148, USA; e-mail: liebhabe@mail.med.upenn.edu.

of the PCBPs that may shed light on their conserved structure-function relationships, and (3) the expanding spectrum of PCBP functions in transcriptional and post-transcriptional controls.

hnRNP K AND THE αCPS REPRESENT DISTINCT PCBP SUBSETS WITH COMMON EVOLUTIONARY ORIGINS

Shared structures and origins of the PCBPs

PCBPs belong to the KH domain superfamily of nucleic acid-binding proteins. The KH (hnRNP K homology) domain was initially identified in hnRNP K and has subsequently been noted in a wide range of RNA-binding proteins in organisms extending throughout the procaryotic and eukaryotic evolutionary spectrum (for a recent review, see Adinolfi et al., 1999). The KH domain can occur in a protein as a single unit or in multiple copies. Isolated KH domains can act as independent nucleic acid-binding units and can dictate, either independently or in concert, a wide spectrum of nucleic acid-binding specificities.

The PCBPs share an overall anatomy consisting of two KH domains grouped near the N-terminus and a third KH domain located at the C-terminus (Fig. 1). The region of greatest structural divergence among family members occurs between the second and third KH domains (Gibson et al., 1993; Siomi et al., 1993). It is important to note that the triplicated KH domain structure common to all of the PCBPs does not dictate their poly(C)-binding specificity. For example, the Nova-1 and Nova-2 proteins share the triple KH domain structure and yet have a distinct specificity (Buckanovich & Darnell, 1997; Yang et al., 1998; Thisted et al., 2001). Thus, the PCBPs are defined both by their triple KH structure and by their poly(C)-binding specificity.

There are five PCBP loci: *HNRNPK* and *Pcbp1*, *2*, *3*, and *4*. These loci are dispersed to separate chromosomes within both the mouse and human genomes (Tommerup & Leffers, 1996; Makeyev & Liebhaber, 2000). Comparative analyses of PCBP protein and gene structures establish a clear evolutionary relationship reflecting a history of gene duplications as well as an unusual retrotransposition event (see below; Makeyev & Liebhaber, 2000). Alignment of the four mammalian αCPs reveals that the greatest sequence conservation is maintained between corresponding KH domains (Fig. 2). However sequence conservation extends into the inter-KH regions as well. The limitation of this extended sequence conservation to the αCP subset sup-

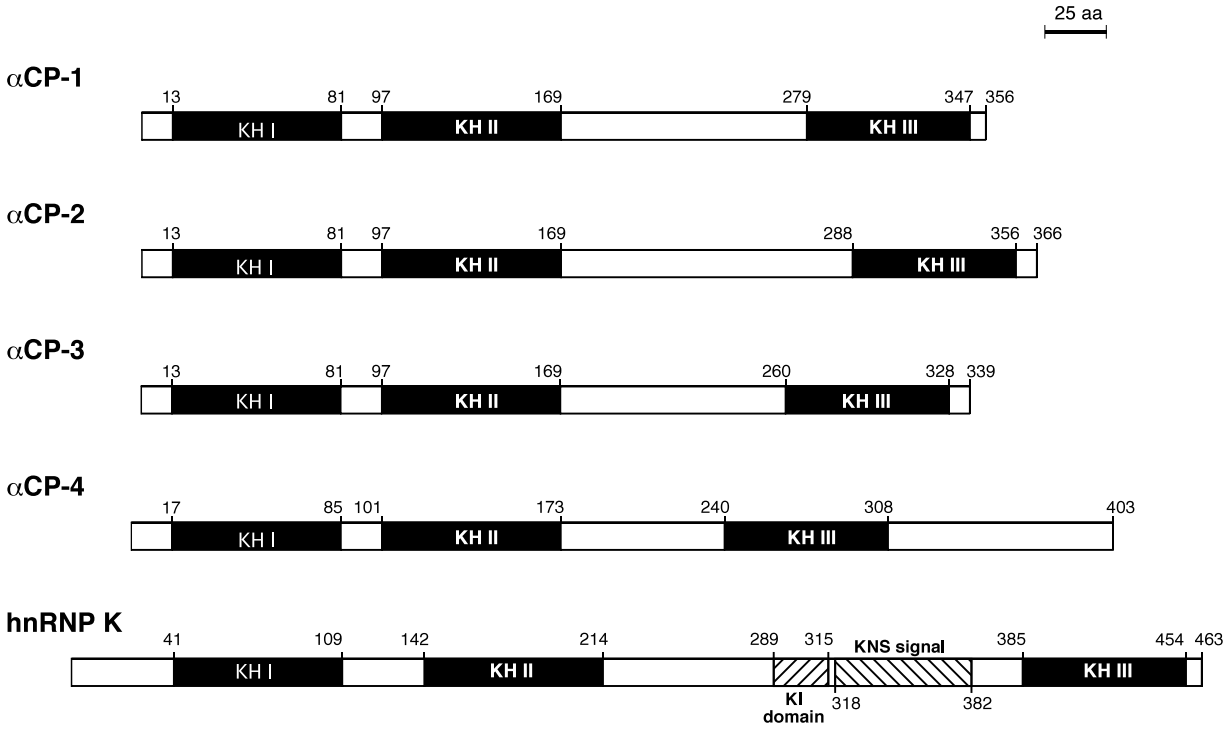

FIGURE 1. Multidomain structure of poly(C)-binding proteins. The five major members of the PCBP family are shown. Numbers of amino acid residues are indicated for each respective human sequence. The conserved KH domains (I, II, and III) are shaded. Specific domains of hnRNP K are labeled and discussed in the text. The sequences between KH II and KH III are the most variable in length and primary sequence.

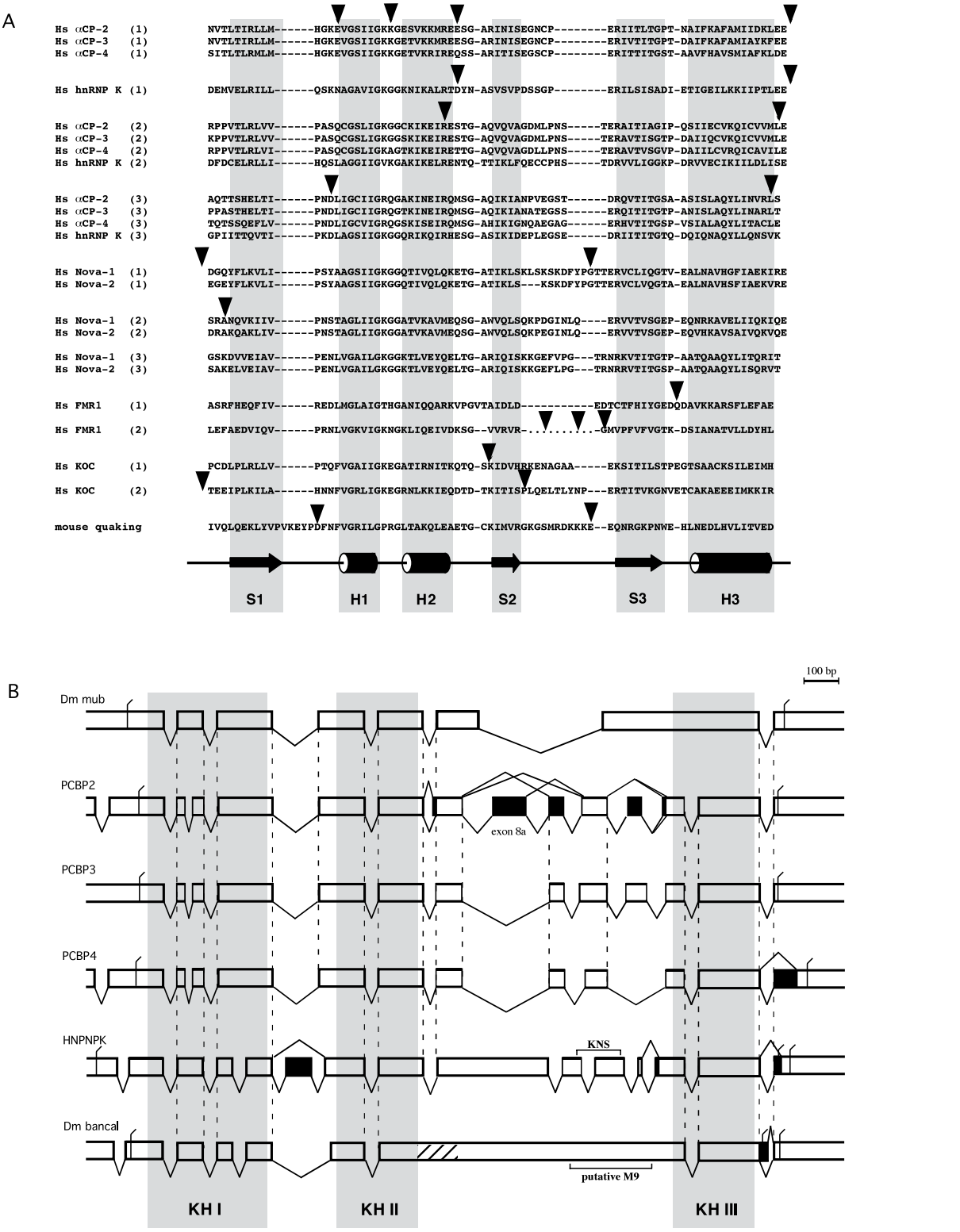

FIGURE 2. Comparative analysis of PCBP protein and gene structures. **A:** Alignment and exon–intron organization of KH domains from various mammalian KH-containing proteins. Numbers in parentheses refer to the number of the corresponding KD domain. Secondary structural elements, β-sheets (S) and α-helices (H), are indicated below the alignment, and are as previously defined (Lewis et al., 1999). Arrowheads indicate positions of introns in the corresponding gene sequences. **B:** Comparative genomic anatomy of mammalian and *Drosophila* (Dm) poly(C)-binding proteins. Exons are shown as boxes; the black exons or exon segments represent alternatively spliced sequences of the transcript. Regular splice junctions are indicated below the transcript and alternative splicing pathways are indicated above the transcript. The regions of the transcript encoding the KH domains are indicated in the shaded overlay. The black exon in hnRNP K between the first and second KH domain is hypothesized to be selectively deleted in hnRNP J. The exons are drawn to scale, and the introns are represented in a uniform manner.

ports their common origins and their remote bifurcation from the hnRNP K/J precursor (Fig. 3; Makeyev & Liebhaber, 2000).

The common origin of the αCP-2, -3, and -4 genes is supported by shared gene structure. The number and size of the exons are generally conserved among these three genes as is the positioning of the introns vis-à-vis the protein structure (Fig. 2). With this in mind, it was remarkable to find that the αCP-1 gene is intronless. Detailed analysis supported the conclusion that the αCP-1 locus was generated by retrotransposition of a minor αCP-2 splicing product. This event apparently occurred prior to mammalian radiation as the intronless αCP-1 gene has been identified in both human and mouse (Makeyev et al., 1999). Although resembling a processed pseudogene, the αCP-1 gene is, in fact, transcriptionally active, has the same wide tissue distribution as the originating αCP-2 gene, and encodes a functional protein. Thus, this retrotransposition event resulted in fixation of a minor αCP-2 isoform in mammalian genome. Conservation of αCP-1 during the mammalian radiation suggests that it serves a useful and nonredundant role.

A number of PCBP homologs have been identified in lower organisms (Table 1). Two of the nine KH-containing proteins in the *Saccharomyces cerevisiae* ORF database (YBD2 and YB83) contain three KH domains. These two proteins, whose functions remain to be determined, display equivalent structural similarity to the mammalian αCPs and hnRNP K (Currie & Brown, 1999). Two PCBP homologs have been identified in *Xenopus laevis*: one resembles hnRNP K and the other resembles αCP-2. αCP and hnRNP K homologs have also been identified in *Drosophila melanogaster*: the product of *mub* gene (Grams & Korge, 1998) and Hrb57A, the product of *bancal* gene (Hovemann et al.,

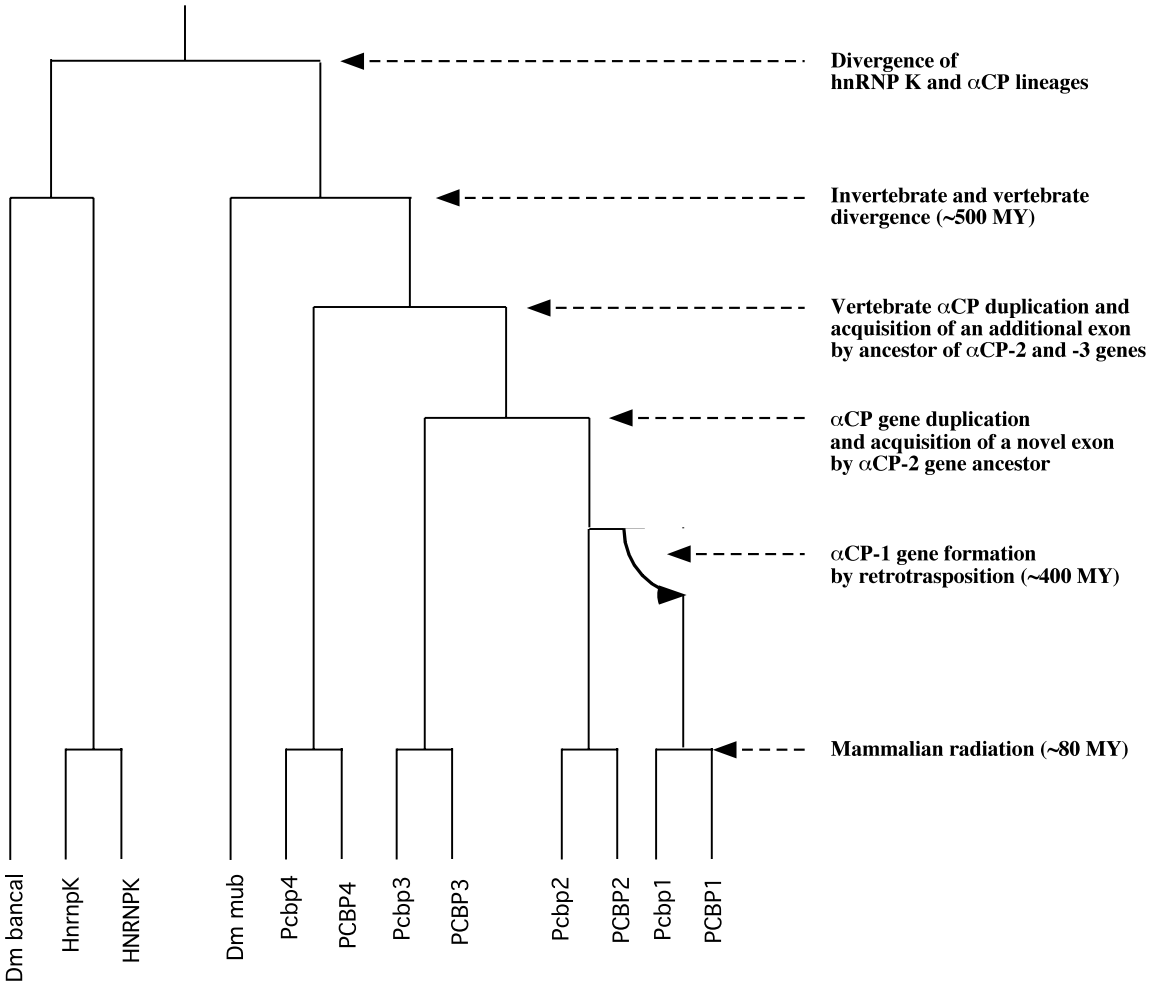

FIGURE 3. Phylogenic tree of PCBP genes. This tree traces the evolution of the PCBP gene family from a single originating gene to five separate and dispersed mammalian loci. The two *D. melanogaster* loci corresponding to the mammalian hnRNP K and αCP loci are indicated as well (*Dm bancal* and *Dm mub*, respectively). A clocklike maximum-likelihood rooted phylogenic tree was calculated with the use of Puzzle software (Strimmer & von Haeseler, 1996). Because there are no ambiguously aligned regions in PCBP alignment, the entire amino acid sequences were used for the phylogenetic analysis. The evolutionary distances among sequences are not drawn to scale. Gene duplication events are indicated by the bifurcations, and the retrotransposition event that generated αCP-1 is indicated by a curved arrow.

TABLE 1. Identities/similarities (%) between human PCBPs and their putative homologs from other organisms.

Protein	Accession number	αCP-1	αCP-2	αCP-3	αCP-4	hnRNP K
Xenopus laevis (Michael et al., 1997)						
hnRNP E2	CAB50743	76/84	86/88	73/80	47/61	25/40
hnRNP K	S41224	25/42	28/45	29/45	20/37	77/80
Drosophila melanogaster (Grams & Korge, 1998; Hovemann et al., 2000)						
mub gene product	CAA67719	43/59	45/59	48/62	43/59	23/38
Hrb57A	CAB64936	20/34	21/35	21/34	16/30	37/48
Caenorhabditis elegans (The *C. elegans* Sequencing Consortium, 1998)						
F26B1.2	T30168	26/42	26/45	27/45	21/39	35/48
Saccharomyces cerevisiae (Currie & Brown, 1999)						
YBD2	S45766	13/29	20/33	16/33	12/24	11/22
YB83/Pbp2p	S46109	22/42	22/42	24/41	22/39	17/33

2000). Both of these *Drosophila* proteins demonstrate a binding preference for poly(C). Remarkably, the human hnRNP K and *Drosophila* Hrb57A proteins demonstrate functional equivalence (see below). Only a single PCBP homolog, most closely resembling hnRNP K, has been predicted from the *Caenorhabditis elegans* genome database (hypothetical protein F26B1.2; The *C. elegans* Sequencing Consortium, 1998). These evolutionary comparisons indicate that the PCBP family is ancient and is likely to subserve fundamental functions (Fig. 3).

Diversity of PCBP isoforms

The structural diversity of PCBPs is likely to contribute to the multiple functions of these proteins. Although the intronless αCP-1 gene encodes a single protein, a systematic investigation of αCP-2 mRNAs by RT-PCR and dbEST searches (Funke et al., 1996; Makeyev & Liebhaber, 2000) revealed alternative splicing of multiple alternative exons and the utilization of competing splice acceptors, resulting in a wide variety of processed αCP-2 mRNAs (Fig. 2B). The major αCP-2 isoform, αCP-2KL, lacks a 31-amino-acid segment encoded by a single exon (exon 8a; Fig. 2B). At least four additional αCP-2 mRNAs are expressed at reasonable abundance (Funke et al., 1996; Makeyev & Liebhaber, 2000). We have found no evidence for alternative splicing of αCP-3 by analysis of mRNAs from a variety of cell types or by EST searches. The full coding sequence for αCP-4 can be deduced by a comparison with EST clones (UniGene cluster Hs.20930) and with other αCP homologs. Two major alternatively spliced forms of αCP-4 transcript have been detected in mouse and human tissues. These two forms differ in the length and primary sequence at their respective C-termini (Makeyev & Liebhaber, 2000; Fig. 2B). An additional 5′-truncated form of αCP-4 has also been described as MCG10 protein (accession nos. AF257770 and AF257771) and has been implicated in an apoptotic pathway (see below; Zhu & Chen, 2000). It was surprising to note that this 5′-truncated form of αCP-4 was absent in dbEST and it contains an unusually long 5′ UTR with predicted exon and intron sequences. These observations suggest that this reported αCP-4 cDNA may represent an incompletely spliced αCP-4 transcript.

Four alternatively spliced hnRNP K transcripts have been identified (Dejgaard et al., 1994) reflecting the use of alternative splice acceptors and inclusion of alternative C-terminal exons (Fig. 2B). hnRNP J was initially proposed to represent an isoform of hnRNP K based on immunologic cross-reactivity (Dejgaard et al., 1994). We have recently identified a novel splice variant of hnRNP K that is abundant in the current dbEST and has a calculated mass and pI similar to that determined for hnRNP J (our unpubl. data). The cassette exon that is absent in this putative hnRNP J mRNA is located between KH domains I and II of the hnRNP K transcript (Fig. 2B). At this point, none of the PCBP isoforms has been assigned to a particular, isoform-specific function.

A consistent observation in the analyses of αCP and hnRNP K splice variants is that all insertions/deletions are confined to regions between KH domains (Fig. 2B). Some of the optionally spliced exons in these regions of the mammalian PCBP genes are absent in the *Drosophila* and *Xenopus* homologs. This suggests that the mammalian-specific exons represent recent insertions that encode gained functions.

Posttranslational modifications

Posttranslational modifications further increase the diversity of PCBP structure and their potential functions. Phosphorylation of αCP-1 and αCP-2 results in a marked decrease in poly(rC)-binding activity (Leffers et al., 1995). In vivo phosphorylation of hnRNP K has been noted in response to growth factors, acute phase

reactants, oxidative stress, and other changes in the extracellular environment (Ostrowski et al., 1991; Van Seuningen et al., 1995; Schullery et al., 1999). Ser^{302} in the middle of the KI domain of hnRNP K is subject to phosphorylation by protein kinase Cδ (Schullery et al., 1999), and this modification may stimulate subsequent phosphorylation of neighboring tyrosines (Ostrowski et al., 2000). These structural modifications increase association of hnRNP K with the proto-oncogene Vav, the transcriptional repressors Eed, and protein kinases Src, Lck, and Cδ, while at the same time dramatically decreasing RNA-binding activity in vitro (Schullery et al., 1999; Ostrowski et al., 2000). Treatment of hepatocytes with insulin or administration of insulin to mice increases tyrosine phosphorylation within hnRNP K and alters hnRNP K interactions with RNA and DNA (Ostrowski et al., 2001). These modifications of hnRNP K, with the consequent alterations in binding properties, may serve to link signal transduction pathways to nucleic acid-dependent processes such as transcription, translation, and RNA processing.

SUBCELLULAR LOCALIZATION OF THE PCBPs

The diverse functions of the PCBPs suggest that they act both in the nucleus and in the cytoplasm. Indeed, it has been found that hnRNP K (Michael et al., 1997) as well as αCP-1, αCP-2, and αCP-4 (our unpubl. data) all shuttle between nucleus and cytoplasm. Mammalian hnRNP K contains a classical, bipartite basic NLS at its N-terminus and a specific KNS (hnRNP K nuclear shuttling) shuttling domain located between KH domains II and III that promotes bidirectional transport through the nuclear pore complex (Michael et al., 1997; Figs. 1 and 2B). At steady-state level, immunofluorescence staining of hnRNP K demonstrates a dotted, nucleoplasmic staining that is excluded from the nucleolus. Phosphorylation of hnRNP K by mitogen-activated protein kinase/extracellular-signal-regulated kinase (MARK/ERK) stimulates cytoplasmic accumulation of hnRNP K under physiological growth conditions (Habelhah et al., 2001). A shift of hnRNP K from nucleus to cytoplasm prestages its function as a silencer of LOX mRNA translation (see below; Ostareck et al., 1997).

Hrb57A, the *Drosophila* ortholog of hnRNP K, contains bipartite basic NLS but the KNS domain found in the mammalian homolog is substituted by a distinct shuttling domain (Fig. 2B). This shuttling motif, M9 motif, was first identified in mammalian hnRNP A1 (Charroux et al., 1999). The distribution of Hrb57A in *Drosophila* nuclei is similar to that of its mammalian counterpart and the dotted patterning becomes more pronounced after heat shock (Buchenau et al., 1997). This dotted pattern coincides with ω-speckles associated with nuclear noncoding hsr-ω transcripts (Prasanth et al., 2000). αCP-1 and αCP-2 also display both dotted and nucleoplasmic distributions in mammalian nuclei (our unpubl. data). However, only αCP-1, but not αCP-2, has been found in biochemically purified interchromatin granules (Mintz & Spector, 2000).

THE MULTIPLE KH DOMAINS OF THE PCBPS: COMPLEX CORRELATIONS TO BINDING ACTIVITY AND THE NEED FOR FURTHER STRUCTURAL ANALYSIS

The KH domain has been identified in a wide spectrum of RNA-binding proteins (Gibson et al., 1993; Siomi et al., 1993). Although this domain was initially identified as a repeated 45-amino-acid motif in hnRNP K (Siomi et al., 1993), subsequent alignments and structural studies have defined a more extensive 68–72 residue KH "maxi domain" (Musco et al., 1996). NMR spectroscopic and X-ray crystallographic analyses of a number of KH domains (Musco et al., 1996, 1997; Baber et al., 1999; Lewis et al., 1999; Wimberly et al., 2000) have revealed that the initially described 45-residue core is configured as a $\beta\alpha\alpha\beta$ unit. The data further reveal two KH domain subtypes based on the more extensive structure: The type I KH domain (e.g., KH-3 of hnRNP K) includes a C-terminal $\beta\alpha$ extension and the type II KH domain (e.g., ribosomal protein S3) contains an N-terminal $\alpha\beta$ extension (Grishin, 2001). All three KH repeats within the PCBPs belong to the type I KH domain comprising a three-stranded antiparallel β-sheet packed against three α-helices ($\beta\alpha\alpha\beta\beta\alpha$; Fig. 2A). Two unstructured surface loops extend from this structure, one containing an invariant GXXG and a second of variable length and sequence. These loops may be of central importance in determining nucleic acid binding specificity (Adinolfi et al., 1999).

An extended survey of PCBP gene organization including the inferred intron–exon organization of the genes encoding human FMR-1 (Eichler et al., 1993), mouse *quaking* (Kondo et al., 1999), Nova-1 and Nova-2, and KOC (known also as IGF-II mRNA-binding protein 3; Fig. 2A) reveals that introns are restricted to positions between secondary structural segments of the KH domain. This positioning suggests that the present-day KH domain may have arisen from a combination and assortment of mini-exons encoding individual structural elements. This model would suggest that prokaryotic and eukaryotic KH domains might have evolved by convergent evolution of nucleic acid-binding modules (Graumann & Marahiel, 1996).

The presence of multiple KH domains in each of the PCBPs brings up the question of which domain, or combination of domains, dictates RNA-binding specificity and affinity. At present, there is no simple answer to this question. Although individual KH domains from PCBPs can bind single-stranded nucleic acids (Dejgaard & Leffers, 1996), it is clear that the binding affinity and specificity of the intact protein reflects a

complex sum of its parts. For example, the high affinity poly(C) binding by hnRNP K can be abolished or significantly reduced by modifying or deleting any of the three individual KH domains (Siomi et al., 1994). The first and/or the second KH domains of hnRNP K exhibit minimal binding activity towards poly(rC) on their own (Dejgaard & Leffers, 1996) and the isolated third KH domain of hnRNP K exhibits a much lower affinity to CT-rich DNA sequence than does the protein as a whole (Baber et al., 2000). In the case of αCP-1 and αCP-2, the first and second KH domains can independently bind poly(rC) with a high affinity and specificity whereas the third KH domain has no such activity (Dejgaard & Leffers, 1996). However, when a more complex native target of αCP-2 (poliovirus 5′-UTR; see below) was studied, only the first KH domain demonstrated the target RNA-binding activity (Silvera et al., 1999). Such observations lead to the conclusion that the binding specificity of each protein can reflect collaboration of the multiple KH repeats and/or may reflect contributions from the inter-KH regions as well (Dejgaard & Leffers, 1996). Structural studies of the intact PCBP proteins with their RNA targets will be needed to gain further insight into this problem.

The presence of multiple KH repeats in each of PCBPs, each of which may theoretically bind independently to RNA, and the ability of PCBPs to directly interact with themselves and with other proteins (see below) suggests that RNP complexes involving PCBPs may differ significantly in their stoichiometry. To address this issue, the stoichiometry of several of the PCBP-containing RNP complexes has been explored. NMR measurements demonstrate a 1:1 interaction between the isolated third KH domain of hnRNP K and its targets (Baber et al., 2000). Analysis of the complex formed at the C-rich segment of the human α-globin mRNA revealed that this "α-complex" is comprised of a single molecule of αCP bound to the mRNA target site (Chkheidze et al., 1999). This binary interaction of the αCP with the α-globin 3′ UTR (3′ untranslated region) target could reflect interaction between the RNA target and a single KH domain of αCP or by interactions between each of the three KH domains and the target sequence. In this regard, comparison of optimized RNA targets generated by in vitro SELEX revealed that αCP-2 and hnRNP K have distinct requirements for high-affinity RNA binding: The optimal target sequence for hnRNP K was found to be a single short "C-patch," whereas that of αCP-2 encompassed three such short C-patches within a highly exposed single-stranded conformation (Thisted et al., 2001). These data suggest that whereas a single C-patch may mediate a high affinity interaction with a single KH domain in hnRNP K, a tandem array of three C-patches maximizes αCP-2 binding to its RNA target. Thus, the binding of αCP to its optimized target might reflect individual interactions by each of the three KH domains. The interesting questions of how these multiple contacts are organized and why the closely related αCP and hnRNP K differ in this seemingly fundamental aspect of their biology must await structural analyses of the respective RNA–protein complexes.

PROTEIN–PROTEIN INTERACTIONS: MULTIPLE PARTNERS IN SEARCH OF VERIFIED FUNCTIONS

The structural variability of the PCBP isoforms may contribute to the multiplicity of reported protein–protein interactions. αCP-2 can form homodimers (Gamarnik & Andino, 1997; Kim et al., 2000) and, in the yeast two-hybrid system, it has been reported to interact with hnRNP L (Funke et al., 1996; Kim et al., 2000), hnRNP K and I (Kim et al., 2000), Y-box-binding protein, splicing factor 9G8, and filamin (Funke et al., 1996). It has been shown that the N-terminal half of αCP-2 including two exons after the second KH domain is required for both homodimerization and interaction with hnRNP I, K, and L (Kim et al., 2000). In vivo confirmation of most of these interactions and their corresponding functional implications awaits further studies.

hnRNP K can dimerize and oligomerize with multiple proteins (Denisenko & Bomsztyk, 1997; Kim et al., 2000). Initial studies demonstrated that hnRNP K interacts with Src family tyrosine kinases (Taylor & Shalloway, 1994; Weng et al., 1994), the proto-oncogene Vav (Bustelo et al., 1995; Van Seuningen et al., 1995), and with protein kinase C (Schullery et al., 1999). It has been proposed that these interactions reflect a role of hnRNP K in signal transduction (Bomsztyk et al., 1997). In vitro and in vivo interaction with TATA-binding protein (Michelotti et al., 1996) and with transcriptional repressors Eed, Zik-1, Kid-1, and MZF-1 (Denisenko et al., 1996; Denisenko & Bomsztyk, 1997; Bomsztyk et al., 1997) suggest a role for hnRNP K in transcriptional control. Remarkably, hnRNP K and αCP-2 can interact with one another (Kim et al., 2000) and have a subset of common protein partners including Y-box-binding protein, splicing factor 9G8, and hnRNP L (Shnyreva et al., 2000). Thus, the two subsets of PCBPs may intersect in their protein–protein interaction networks.

The structural basis for the interactions of the PCBPs with various partners appears to be complex. Interaction of hnRNP K with SH3 domains from Src, Fyn, Lyn, and Vav as well as transcriptional repressors and Y-box-binding protein is mediated by the centrally located proline-rich domain, denoted KI (Fig. 1). In contrast, the TATA-binding protein associates with hnRNP K via a site adjacent to the KI domain (Denisenko & Bomsztyk, 1997; Shnyreva et al., 2000) whereas homodimer formation and interactions with αCP-2, hnRNP L, hnRNP I, and the transcriptional factor CCAAT/enhancer-binding protein β are mediated by the N-terminal third of hnRNP K (Miau et al., 1998; Kim

et al., 2000). Finally, the interleukin-1-responsive kinase binds the C-terminal fragment of hnRNP K (Van Seuningen et al., 1995). Many of these interaction sites on hnRNP K are coincident with regions of alternative splicing, further supporting the complex interactions between the various PCBP isoforms and their functional potentials. Although likely, these specific assignments have yet to be directly supported.

POSTTRANSCRIPTIONAL CONTROL BY PCBPs: MULTIPLE BINDING SITES AND DIVERSE FUNCTIONS

Stabilization of cellular and viral mRNAs

PCBPs have been implicated in a wide spectrum of posttranscriptional controls. Initial insights into the roles emerged from studies of human α-globin mRNA stabilization (Weiss & Liebhaber, 1994, 1995). High-level stability of α-globin mRNA is essential to full expression of globin proteins during the 2–3 days of transcriptionally silent terminal erythroid differentiation. Stabilization of α-globin mRNA is tightly linked to formation of a binary complex between a single molecule of αCP and a pyrimidine-rich motif within the α-globin 3′ UTR ("α-complex"; Fig. 4A; Kiledjian et al., 1995; Wang et al., 1995; Chkheidze et al., 1999). Interruption of any of the three C-rich patches within this region disrupts the α-complex assembly in vitro and decreases α-globin mRNA stability in vivo (Wang et al., 1995; Weiss & Liebhaber 1995). αCP-1 and αCP-2 can independently bind to the human α-globin mRNA 3′ UTR to form the α-complex as demonstrated in vitro. This binding is specific to αCPs as hnRNP K lacks this binding activity (Chkheidze et al., 1999). The respective K_d values of recombinant αCP-1 (51 nM) and αCP-2 (0.8 nM)

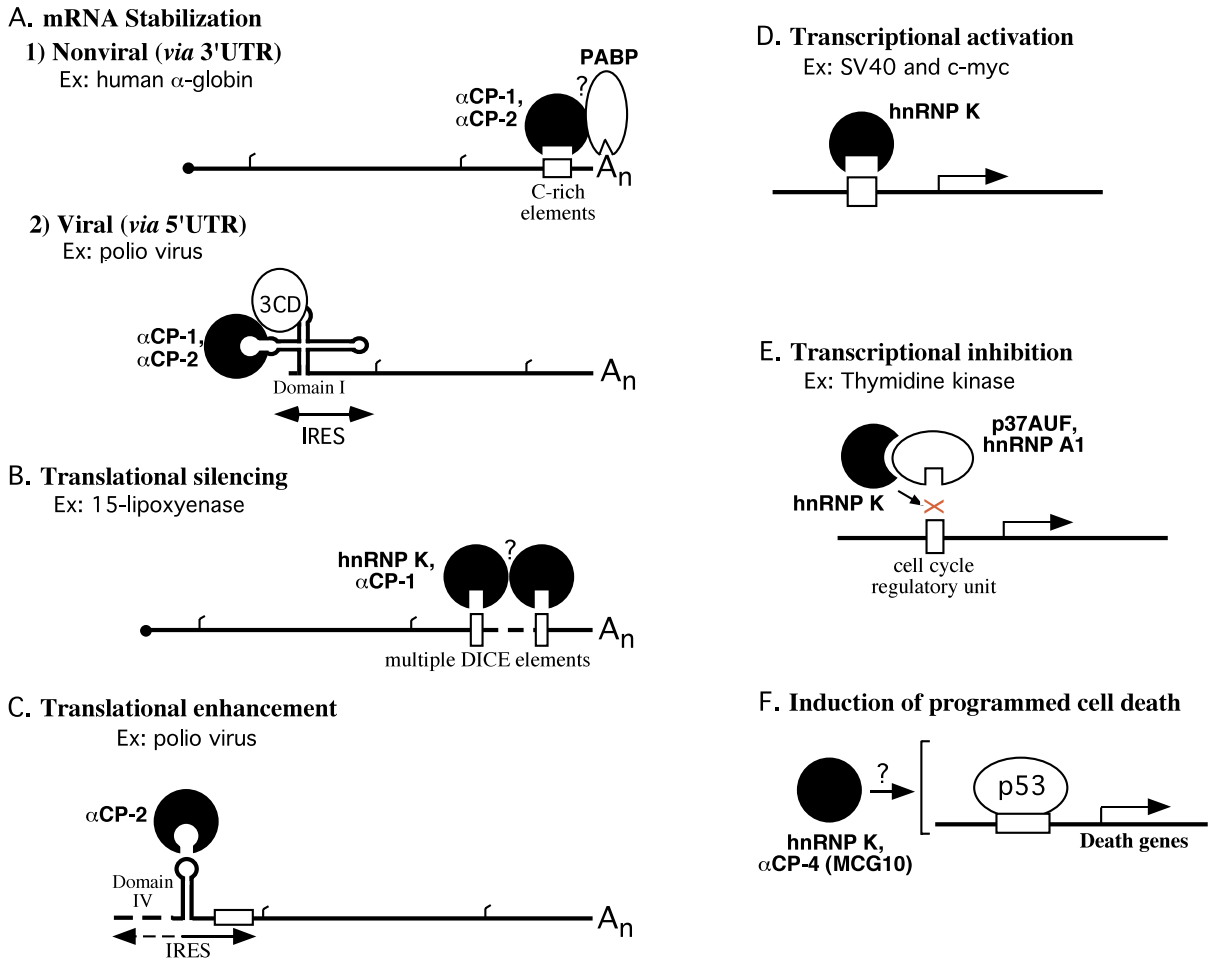

FIGURE 4. Examples of functions mediated by the PCBPs. Six distinct examples of PCBP function are shown. In each case, the PCBP isoform of interest is indicated as a filled circle. A specific example is given for each of these indicated functions and each is expanded upon in the text. The mRNAs are indicated in diagrammatic format with tick marks indicating the initiation and termination codons. The cellular mRNAs are also shown with cap (filled circles) and poly(A) tails. The polioviral mRNA lacks a cap structure; the two IRES structures of specific interest, secondary structures known as Domain I and Domain IV, are shown. Question marks indicate unidentified protein factors and/or assumed protein–protein interactions (as discussed in the text).

for α-complex assembly (Chkheidze et al., 1999) are in agreement with the apparent K_d (0.5 nM) determined for assembly of the α-complex by incubating α-globin 3′ UTR with total cytoplasmic extracts (Russell et al., 1998). The more recently discovered αCP-3 and αCP-4 proteins can also form an α-complex in vitro but their relationship to α-globin mRNA stability remains to be explored.

mRNA stabilization via αCP binding does not appear to be limited to α-globin mRNA. αCP-2 binds with high affinity ($K_d \sim 2$ nM) to a C-rich region within the 3′ UTR of collagen α1 (I) mRNA with a consequent increase in mRNA stability (Stefanovic et al., 1997). As is the case for α-globin mRNA, this interaction can occur in the absence of additional factors (Lindquist et al., 2000). αCP-1 and αCP-2 binding to 3′ UTR pyrimidine-rich motifs have been implicated in hypoxia-mediated stabilization of tyrosine hydroxylase (Paulding & Czyzyk-Krzeska, 1999) and erythropoietin (Czyzyk-Krzeska & Bendixen, 1999) mRNAs. αCPs are also constituents of a stabilizing complex on 3′ UTR of the β-globin mRNA that exhibits many of the properties of the α-complex (Yu & Russell, 2001). In addition, poly(C)-sensitive 3′ UTR complexes have been demonstrated to mediate increased stability of the heavy and light neurofilament mRNAs (Canete-Soler & Schlaepfer, 2000) although the identity of the corresponding proteins in this case has not been determined to date. These data suggest that binding of αCPs to 3′ UTR pyrimidine-rich determinants may represent a general mechanism for stabilization of long-lived cellular mRNAs (Holcik & Liebhaber, 1997).

A role for α-CP binding has been recently proposed for stabilization of viral mRNAs (Fig. 4A). αCP-1 and αCP-2 can interact with two independent structures of the polioviral 5′ UTR: a cloverleaf structure at the 5′ terminus (stem-loop I) and a more centrally located stem-loop IV (Fig. 4C; Blyn et al., 1996; Gamarnik & Andino, 1997). Both structures have short C-rich stretches exposed in the loop structures. αCP-2 binding to stem-loop IV is essential for efficient poliovirus translation (see below), whereas interaction of αCPs with the 5′ cloverleaf structure has recently been found to stabilize poliovirus mRNA (Murray et al., 2001). In contrast to the binary interactions of αCP with 3′ UTR motifs of cellular proteins (α-complexes; as detailed above), the binding of αCP to the polio viral 5′ cloverleaf is dependent on cooperative interactions with a polio-encoded protein, 3CD (precursor of the viral protease 3C and the viral polymerase 3D; Gamarnik & Andino, 1997; Parsley et al., 1997); binding of 3CD dramatically increases the binding affinity of αCP-2 (from K_d values of $\sim$95 to $\sim$1 nM; Gamarnik & Andino, 2000). This 3CD/αCP-containing complex is also required for initiation of positive- and negative-strand synthesis (Gamarnik & Andino, 1998). As a result, αCP protein not only enhances polio viral mRNA stability but also coordinates the switch from translation to replication of the polio viral genome.

A generalized picture that emerges from the above studies is that αCPs can stabilize distinct subsets of mRNAs by targeting specific binding sites. In cellular (capped) mRNAs, these sites appear to be limited to the 3′ UTR. Formation of complexes in this region would appear logical as this is the only site in these mRNAs at which RNP complexes can remain undisturbed during active translation. The 5′ UTR stabilizing complex in the polioviral RNA is located 5′ of the ribosomal entry site (IRES) and thus would be similarly protected from ribosome interference. Therefore, in both cases it appears that the stabilizing complex assembles at a position in which it can be maintained and shielded from scanning ribosomes or ribosome subunits.

αCP-mediated translational controls: Silencing and enhancement by the same protein

Translational silencing

A detailed study of translation control by αCPs has come from the analysis of 15-lipoxygenase (LOX) mRNA expression (Fig. 4B). LOX mRNA is synthesized in early erythroblasts and is then stored for several days in a translationally silent state until the terminal (reticulocyte) stage of differentiation. At that point, mRNA is translationally activated and the newly synthesized LOX protein degrades microsomal membranes as the reticulocyte matures into the red blood cell (for review, see Kuhn et al., 1999). Thus the timing of translational activation is a critical step in the control of LOX expression. Translational silencing of LOX mRNA is tightly linked to formation of a RNP complex at an evolutionarily conserved CU-rich repeated motif (DICE, differentiation control element) within the 3′ UTR. The complex that forms at this site contains two PCBPs; hnRNP K and one of the two major αCP isoforms. Addition or forced expression of hnRNP K, αCP-1, or αCP-2 can inhibit LOX mRNA translation in vitro and in transfected HeLa cells. This DICE-dependent translation inhibition is transferable to a reporter mRNA and acts on capped as well as uncapped reporter mRNAs (Ostareck et al., 1997). Whether the effects of hnRNP K, αCP-1, and αCP-2 are additive, synergistic, or redundant is not clear. It is also not clear if the same DICE complex contributes to the remarkable stability of the LOX mRNA and whether the translational and putative stability controls of the LOX mRNA are interdependent.

Mechanistic studies revealed that hnRNP K and αCP-1 proteins silence the DICE-containing mRNA by inhibiting 60S joining to the 40S complex at the initiation AUG (Ostareck et al., 2001). The manner by which the 3′ UTR complex mediates control of translational initiation at the 5′ end of the mRNA and the relative

contribution of the two distinct PCBPs to this process remain to be defined. It appears that hnRNP K and αCPs may regulate translation of cellular (LOX) RNA in response to as yet undefined cell-type specific and/or differentiation signals. Despite apparent similarity between translation-silencing DICE-complex and mRNA-stabilizing α-complex, translation of α-globin mRNA is not silenced by forced expression of hnRNP K or αCP. In actual fact, α-globin mRNA is actively translated in the same cells and at the same developmental time at which LOX mRNA is selectively silenced. Thus, binding of αCP to the C-rich region targets within the 3′ UTRs of α-globin mRNA (α-complex) and the LOX mRNA (DICE complex) have distinct and restricted effects in the same cell and within the same window of cell differentiation. This implies that despite their obvious similarities these two PCBP containing complexes activate distinct posttranscriptional control pathways.

Human papillomavirus type 16 L2 mRNA appears to be silenced via binding to PCBPs (Collier et al., 1998). This binding occurs at the 3′ end of the coding region of the L2 mRNA. Of note, the L2 sequence does not possess a high content of cytosine; in fact it contains only 22% cytosines and only three sites in the L2 sequence contain four or more cytosine repeats. Despite this fact, the PCBPs display a high affinity for the L2 mRNA. hnRNP K, αCP-1, and αCP-2 can each individually inhibit L2 mRNA translation, and data suggest that translational silencing may be mediated by the same pathway as outlined for LOX mRNA. It is interesting to note that upon papilloma virus infection, L2 protein synthesis is restricted to the upper layers of stratified epithelium despite the fact that its mRNA is also present in lower layers (Collier et al., 1998) and the PCBP binding partners are ubiquitous. Thus, additional factors must cooperate with the PCBPs to effect this tissue specificity.

αCP homologs in *Xenopus* have been implicated in developmentally controlled activation of mRNA translation. *Xenopus* αCP-2 (Table 1) binds specifically to the C-rich cytoplasmic polyadenylation element (CPE) in the 3′ UTR of mRNA encoding the catalytic subunit of phosphatase 2A. This αCP-2/CPE complex appears to be involved in the developmentally controlled poly(A) elongation and translational activation of the target mRNA during *Xenopus* embryogenesis (Paillard et al., 2000). This function of αCP is reminiscent of its ability to stabilize the poly(A) tail of long-lived mRNAs (Morales et al., 1997; Wang et al., 1999). Whether these two functions reflect similar rate-limiting controls is not at present known.

Translational enhancement

A role for αCPs in translational enhancement is best established for polio viral mRNA (for review, see Andino et al., 1999, and Belsham & Sonenberg, 2000; Fig. 4C). Binding of αCP-2 to stem-loop IV within the central region of the IRES is essential to efficient poliovirus translation in HeLa cell extracts (Blyn et al., 1996, 1997; Gamarnik & Andino, 1997). This translational enhancement by αCP-2 is specific to the picornavirus IRES elements as it has no appreciable effect on the translation of capped reporter mRNAs (Gamarnik & Andino, 1998). Although recombinant αCP-1 and αCP-2 can both bind to stem-loop IV in vitro (Gamarnik & Andino, 1997), only recombinant αCP-2 is able to restore poliovirus IRES activity in αCP-depleted HeLa cell lysate (Blyn et al., 1997). The structural basis for this specificity and underlying mechanisms of action of αCP-2 in this setting remains undetermined.

The role of αCP-2 in translational enhancement may be shared by other picornaviruses. Cap-independent translation initiation within the picornavirus family is mediated by two major classes of IRESs (type I and type II). These IRESs differ in their sequences, secondary structures, and biological properties (Wimmer et al., 1993; Ehrenfeld, 1996). The enteroviruses and rhinoviruses utilize type I IRESs, and the cardioviruses and aphthoviruses utilize the type II IRES elements. Although αCP-2 is capable of interacting in vitro with both type I IRESs (from coxsackievirus strain B and human rhinovirus) and type II IRES (from encephalomyocarditis virus and foot-and-mouth disease virus), an effect on translation of the cardiovirus and aphthovirus has not been demonstrated. These data suggest that a functional role for αCP-2 in binding to viral 5′ UTRs appears to be limited to type I IRES elements (Walter et al., 1999).

αCP plays a role in the translation of viruses in addition to those in the picornavirus family. Although the IRES element of hepatitis A virus (HAV) cannot easily be classified as a type I or type II IRES (Brown et al., 1994), αCP-2 is required to facilitate HAV internal ribosome entry similarly to type I IRESs (Graff et al., 1998). Along the same lines, the hepatitis C virus (HCV) IRES has been difficult to classify as a type I or type II IRES due to its unique structure (Reynolds et al., 1995). Whereas αCP-1 and αCP-2 can bind specifically to the HCV 5′ UTR (Spangberg & Schwartz, 1999), it has not been possible to document an effect of this binding on HCV translation (Fukushi et al., 2001). In this respect, the HAV IRES behaves in a manner reminiscent of a type I IRES, whereas HCV IRES resembles a type II IRES.

Thus, αCP binding can contribute to both silencing and enhancement of translation. The former action is mediated by 3′ UTR complexes and the latter by complexes within the 5′ IRES segments of picornaviruses. In neither case are the downstream events that control the rates of ribosome loading clearly defined. In both cases, the complexes are formed at substantial distances from the sites of translation initiation. Whether the two processes converge on a common pathway remains an interesting possibility. Delineation of the

relevant pathways should be highly informative as they may define novel aspects of translational biochemistry and long-range interactions mediated by mRNP complexes.

TRANSCRIPTIONAL CONTROLS: DIVERSE ROLES AND MECHANISMS

In addition to their roles in mRNA stability and translational controls, the PCBPs appear to have diverse functions in the more proximal events in gene expression. hnRNP K has been implicated in multiple aspects of transcriptional regulation. hnRNP K has a specific binding site on the SV40 early promoter (Gaillard et al., 1994) and in the pyrimidine-rich strand of the CT element in the promoter of human c-myc gene (Fig. 4D; Tomonaga & Levens, 1996). In both of these cases, this interaction activates transcription in in vitro systems apparently by an hnRNP K-dependent assembly of the TFIID complex at target promoters (Michelotti et al., 1996). A competition/displacement model has been suggested to explain the observed differences in the effects of hnRNP K on Sp1- and Sp3-mediated transcriptional activation of the neuronal nicotinic acetylcholine receptor promoter (Du et al., 1998). According to this model, hnRNP K selectively blocks access of Sp1, but not Sp3, to an E2 element. In the case of the thymidine kinase promoter, hnRNP K itself cannot physically interact with promoter but may repress transcription by inhibiting the binding of other *trans*-factors to the cell cycle regulatory determinant of this promoter (Fig. 4E; Lau et al., 2000). In addition, hnRNP K has been reported to directly interact in vitro and in vivo with zinc-finger transcriptional repressor Zik-1 (Denisenko et al., 1996) and with other structurally related transcriptional repressors such as Kid-1 and MZF-1 (Bomsztyk et al., 1997). Thus, the abilities of hnRNP K to interact with both nucleic acid and protein targets is essential for its transcriptional functions.

hnRNP K has recently been identified among a limited number of proteins binding specifically to the homopyrimidine strand of $d(GA \cdot TC)_n$ sequences (Garcia-Bassets et al., 1999). Alternating $(GA \cdot TC)_n$ DNA sequences are abundant in eukaryotic genomes, can form non-B-DNA conformers in vitro, and are often found in gene regulatory regions. Regions of single-stranded DNA in non-B-DNA conformers have a tendency to restore their regular double-stranded sequence. By stabilizing a single-stranded bubble in such a region, hnRNP K may increase DNA flexibility and allow other DNA-binding proteins to align more easily with the basal transcriptional machinery (Garcia-Bassets et al., 1999). In these capacities, hnRNP K may serve as an "architectural" transcription factor. Such a role has been specifically suggested for hnRNP K in regulation of the c-myc promoter (Michelotti et al., 1996). A similar structural function may be attributed by hnRNP K binding at the telomere; in vitro studies reveal high-affinity binding of this protein to a cytosine-rich oligonucleotide that mimics a special intramolecular folded structure called "i-DNA" in the telomere (Lacroix et al., 2000). Whether this telomere interaction occurs in vivo is not clear.

ROLES IN APOPTOTIC AND DEVELOPMENTAL PATHWAYS

Progress toward an understanding of hnRNP K functions in development have been significantly aided by analysis of the *Drosophila* ortholog, Hrb57A, the product of *bancal* gene (Table 1). Hrb57A possesses similarity in its structure and in nucleotide binding specificity to mammalian hnRNP K (Hovemann et al., 2000). Null and weak alleles of *bancal* result in *Drosophila* adults with shortened appendages as a consequence of a reduction in the number of cell divisions in the imaginal discs; remarkably this developmental phenotype can be reversed by expression of the human hnRNP K transgene (Charroux et al., 1999). Moreover, overexpression of either Hrb57A or human hnRNP K induced cell death in imaginal discs and this effect can be overcome by coexpression of the anti-apoptotic P35 protein (Charroux et al., 1999). Of note in this regard, forced expression of human hnRNP K decreases the level of the endogenous Hrb57A. These data indicate that hnRNP K is functionally involved in the control of cell division and apoptotic pathways and that its critical level may be controlled by negative feedback mechanisms (Charroux et al., 1999).

In marked contrast to the studies of the hnRNP K homologs, there are no clear phenotypes associated with deletion mutants of the αCP homolog (*mub)* in *Drosophila* (Table 1; Fig. 2B; Grams & Korge, 1998).

Analysis of human PCBPs supports their involvement in cell growth control. hnRNP K is a defined target of epidermal cell growth factors produced by human breast cancer cells and in this context hnRNP K can exert positive controls over the rate of cell proliferation (Mandal et al., 2001). A protein encoded by a splice variant of the αCP-4 transcript, MCG10, has recently been reported to participate in an apoptotic pathway in human cells (Fig. 4F). Elevated levels of MCG10 were induced in a p53-dependent manner in cells following DNA damage and forced expression of MCG10 induced apoptosis and cell cycle arrest at G_2-M (Zhu & Chen, 2000). These data suggest that an αCP-4 isoform, MCG10, is a potential mediator of p53 tumor suppression.

PCBP BIOLOGY: WHERE WE ARE AND WHERE WE MIGHT GO

The five PCBP genes encode a well-defined subset of single-strand nucleic acid-binding proteins. These pro-

teins are structurally related by their triplicated KH domains, by their common evolutionary origins, and by a shared preference for C-rich binding sites. Current data clearly demonstrates that these proteins play important and diverse roles in gene expression at both transcriptional and posttranscriptional levels. A number of general models have been put forward to unify the actions of one or more of these proteins. For example, it has been suggested that the seemingly disparate functions of hnRNP K at multiple levels of gene can be unified by considering it as a RNA- and DNA-regulated docking platform linking sequence-specific nucleic acid binding with specific protein–protein interactions and serving as a fulcrum for multilateral molecular cross-talk (Bomsztyk et al., 1997). Although of significant utility, this model is difficult to fully reconcile with the observation that mammalian and *Drosophila* hnRNP K proteins demonstrate functional equivalence despite their apparent absence of structural homology in the auxiliary domains responsible for protein–protein interactions.

Another model suggests that the family of PCBPs may serve the general role of chaperones that stabilize complex secondary and tertiary RNA structures or single-stranded DNA structures within promoters or at telomeres to facilitate single-strand-dependent interactions with a variety of *trans*-factors. This model has been proposed, for example, in the regulation of the c-myc promoter by hnRNP K (Michelotti et al., 1996) and for facilitation of internal ribosome entry on the picornavirus IRES (Belsham & Sonenberg, 2000). However, recent studies from our laboratory indicate that some members of this family, in particular αCP-1 and αCP-2, can function in their mRNA stabilization role in the total absence of their RNA binding function (J. Kong & S.A. Liebhaber, in prep.). Thus, the chaperone functions of the PCBPs, if important in mRNA stabilization, would only represent one facet of their function.

From all these considerations, it becomes apparent that the functional diversity and spectrum of actions of the PCBPs are quite wide and may not lend themselves to unifying models at present. The flip side of the apparent shortfall is that there remains a rich mine of structural and mechanistic questions yet to be explored. These studies should reveal, in the not too distant future, the complexity of interactions and pathways utilized by this family in its extensive and multiple contributions to the control of cell function.

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