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# Members of the poly (rC) binding protein family stimulate the activity of the c-*myc* internal ribosome entry segment *in vitro* and *in vivo*

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**The 5′ untranslated region of the proto-oncogene c-*myc* contains an internal ribosome entry segment and c-Myc translation can be initiated by cap-independent as well as cap-dependent mechanisms. In contrast to the process of cap-dependent initiation, the *trans*-acting factor requirements for cellular internal ribosome entry are poorly understood. Here, we show that members of the poly (rC) binding protein family, poly (rC) binding protein 1 (PCBP1), poly (rC) binding protein 2 (PCBP2) and hnRNPK were able to activate the IRES *in vitro* up to threefold when added in combination with upstream of N-ras and unr-interacting protein. The interactions of PCBP1, PCBP2 and hnRNPK with c-*myc*-IRES-RNA were shown to be specific by ultraviolet crosslinking analysis and electrophoretic mobility shift assays, while immunoprecipitation of the three proteins using specific antibodies followed by reverse transcriptase–polymerase chain reaction showed that they were able to bind c-*myc* mRNA. c-*myc*–IRES-mediated translation from the reporter vector was stimulated by cotransfection of plasmids encoding PCBP1, PCBP2 and hnRNPK. Interestingly, the mutated version of the c-*myc* IRES that is prevalent in patients with multiple myeloma bound hnRNPK more efficiently *in vitro* and was stimulated by hnRNPK to a greater extent *in vivo*.**
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**Keywords:** c-*myc*; translation; internal ribosome entry; IRES; poly (rC) binding proteins

## Introduction

The protein products of the c-*myc* proto-oncogene are involved in both cell growth and cell death (Grandori *et al*., 2000). It is not surprising therefore that the expression of c-*myc* is controlled at multiple levels including transcription, protein stability, RNA stability and translation (Eisenman, 2001). The translational regulation of c-*myc* is complex and the synthesis of this protein can be initiated via two mechanisms (Nanbru *et al*., 1997; Stoneley *et al*., 1998; West *et al*., 1998). c-*myc* mRNA translation initiation can occur by cap-dependent scanning, which requires the binding of the multimeric complex eIF4F (which is comprised of the cap-binding protein eIF4E, the DEAD-box helicase eIF4A and the scaffold protein eIF4G) to the 7-methyl-G cap structure of the mRNA, followed by recruitment of the 40S ribosomal subunit, and scanning to the first AUG codon, which is in an adequate context (Gray and Wickens, 1998). Alternatively c-*myc* translation can be initiated by internal ribosome entry (Nanbru *et al*., 1997; Stoneley *et al*., 1998). Internal ribosome entry is brought about by a complex RNA structural element (termed an internal ribosome entry segment, IRES) and this allows recruitment of the ribosome to a site that is distant from the 7-methyl-G cap. The sequence encoding the c-*myc* IRES is downstream of the most commonly used promoter P2 (75–90% of all c-*myc* messages are initiated from this promoter (Marcu *et al*., 1992)), and thus the majority of c-*myc* mRNA in the cell has the potential for its translation to be initiated by internal ribosome entry (Stoneley *et al*., 1998).

Control of translation is important in regulating cellular levels of c-Myc and increased c-Myc expression can occur from aberrant translational regulation (West *et al*., 1995; Paulin *et al*., 1996). For example, in cell lines derived from patients with multiple myeloma (MM, an incurable disease that is characterized by bone marrow plasmacytosis, osteolytic lesions and secretion of a monoclonal immunoglobulin; Niesvizky *et al*., 1993) there is an up to 20-fold increase in c-*myc* protein levels that occurs by a translational mechanism (Paulin *et al*., 1996). This increased c-Myc protein expression in MM-derived cell lines correlates with a C-T mutation in the region of c-*myc* DNA that contains the IRES (Paulin *et al*., 1996) and RNA derived from the mutant IRES displays enhanced binding of protein factors (Paulin *et al*., 1998). The C-T mutation is also present in the cells derived from the bone marrow of MM patients (42%) and this mutation alters translation initiation via the IRES (Chappell *et al*., 2000) demonstrating that a single mutation in the c-*myc* IRES is sufficient to cause enhanced initiation of translation via internal ribosome entry.

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Cellular IRESes, including c-*myc*, function very inefficiently (if at all) *in vitro* (Nanbru *et al*., 1997; Stoneley *et al*., 2000). This is because cellular internal ribosome entry is aided by IRES *trans*-acting factors (ITAFs) that allow the RNA to attain the correct structure for entry. Several lines of evidence would suggest that the *trans*-acting factor requirements are different for each IRES. For example, cellular IRESes display considerable cell tropism thus, the N-*myc* and c-*myc* IRESes have comparable activity in HeLa cells yet the N-*myc* IRES is up to sevenfold more active than the c-*myc* IRES in cell lines of neuronal origin, (Jopling and Willis, 2001). In addition, while both the fibroblast growth factor 2 (FGF-2) and c-*myc* IRESes were active in developing embryos, only the FGF-2 IRES was shown to be active in adult brain, suggesting that certain ITAFs are not present in the fully differentiated cell types (Creancier *et al*., 2000, 2001). Finally, the *trans*-acting factors required by the cellular IRESes that have been studied so far have been found to differ. Thus, the XIAP IRES requires La (Holcik and Korneluk, 2000) and hnRNPC (Holcik *et al*., 2003) for activity, while the Apaf-1 IRES requires upstream of N-ras, (unr) and polypyrimidine tract binding protein/neuronal polypyrimidine tract binding protein (PTB/nPTB) for function ((Mitchell *et al*., 2001, 2003). Here, we have investigated the *trans*-acting factor requirement of the c-*myc* IRES and we show that the c-*myc* IRES, like several viral IRESes (Gamarnik and Andino, 1997; Andino *et al*., 1999; Walter *et al*., 1999), requires poly (rC) binding proteins for function. These proteins are members of the KH domain family of single-stranded nucleic acid binding proteins and bind to sequences that are C-rich (Ostareck-Lederer *et al*., 1998; Makeyev and Liebhaber, 2002). We show that c-*myc*–IRES-mediated translation is stimulated by poly (rC) binding protein 1 (PCBP1), poly (rC) binding protein 2 (PCBP2) and hnRNPK. Interestingly, there is a potential binding site for hnRNPK in the region of c-*myc* IRES RNA that contains the mutation that is prevalent in MM and the mutant version of the IRES shows enhanced binding of this protein.

## Results

### *PCBP-1, PCBP-2 and hnRNPK stimulate c-myc IRES activity in vitro*

The c-*myc* IRES is relatively inactive in all *in vitro* systems that we have tested to date (Stoneley *et al*., 2000). For example, the presence of the c-*myc* IRES on a monocistronic RNA encoding c-Myc reduces the amount of protein produced by 90% when compared to that which is produced by scanning from an RNA, which does not contain the IRES (Stoneley *et al*., 2000). At least six proteins from mammalian cell extracts have been found to interact specifically with c-*myc* IRES RNA (Paulin *et al*., 1998) and it is likely therefore that a combination of several factors working in concert are required to generate an *in vitro* system, where the c-*myc* IRES is functional. Thus, experiments were performed to assess the effects that known ITAFs have on the function of the c-*myc* IRES *in vitro* since we, and others, have shown previously that certain viral ITAFs are able to increase the activity of cellular IRESes (Holcik and Korneluk 2000; Mitchell *et al*., 2001, 2003). Rabbit reticulocyte lysates were primed with dicistronic RNAs generated from pRMF (Figure 1a), putative *trans*-acting factors were added and luciferase activity assayed. We have shown previously by ultraviolet (UV) crosslinking studies that unr binds to c-*myc* IRES RNA (Mitchell *et al*., 2001) and addition of unr to rabbit reticulocyte

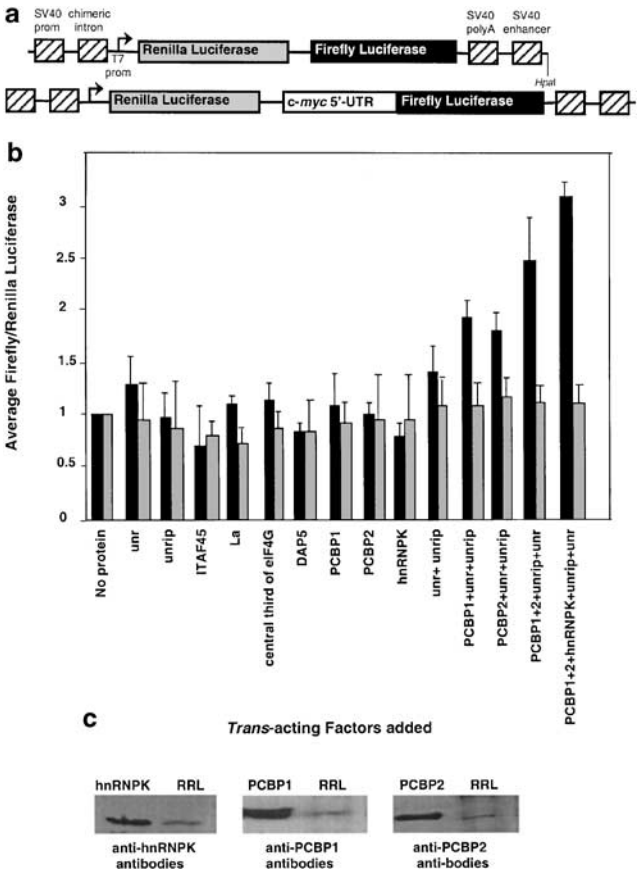


**Figure 1** Identification of *trans*-acting factors that increase c-*myc* IRES activity *in vitro* (**a**) Schematic diagram of the discistronic plasmids pRMF and pRF. These were digested with *Hpa*I and used to prime *in vitro* transcription reactions. (**b**) Rabbit reticulocyte lysates (8 *μ*l) were primed with capped dicistronic RNA derived from pRMF that contains the c-*myc* IRES fused in frame with the luciferase gene with the addition 250–500 ng of putative *trans*-acting factors where indicated. Firefly luciferase levels are increased 1.4-fold by unr but not by any other single factor. However, combinations of factors (PCBP1, PCBP2, hnRNPK, unr and unrip) are able to increase IRES function up to 3.2-fold. Solid bars represent the activity produced from pRMF and grey bars the activity produced from pRF. All values were normalized to the control that did not contain any protein. (**c**) Western blots of rabbit reticulocyte lysates. Reticulocyte lysates (8 *μ*l) were separated by SDS–PAGE in conjunction with 250 ng of PCBP1, PCBP2 or hnRNPK. These gels were immunoblotted and probed for the presence of PCBP1, PCBP2 or hnRNPK using specific antibodies. The addition of purified PCBP1, PCBP2 and hnRNPK to reticulocyte lysates increases the concentration of these proteins by approximately fivefold

lysates caused a small (1.4-fold), but reproducible increase in the translation of firefly luciferase (Figure 1b). However, addition of PCBP1, PCBP2, hnRNPK, the unr interacting protein, unrip (Hunt *et al*., 1999), ITAF45 (Pilipenko *et al*., 2000), La (Holcik and Korneluk, 2000), the central third of eIF4G (Lomakin *et al*., 2000), or DAP5 (Henis-Korenblit *et al*., 2002) alone had no effect (Figure 1b). Addition of unr and unrip together increased the activation of the IRES up to 1.6-fold. The addition of hnRNPK, PCBP1, PCBP2, unrip and unr simultaneously increased the IRES function to 3.2-fold. This is a similar degree of activation to that which is observed with the Apaf-1 IRES in the presence of unr and nPTB (Mitchell *et al*., 2001). In each case, addition of these proteins had no effect on the firefly luciferase produced from the control RNA derived from pRF, which does not contain the c-*myc* IRES (Figure 1a). Immunoblots were performed to test the relative abundance of these proteins in reticulocyte lysates. Rabbit reticulocyte lysates were separated by PAGE and then immunoblotted using either anti-PCBP1, PCBP1 or hnRNPK antibodies. As expected, all three proteins were present in reticulocyte lysates; however, addition of 250 ng of PCBP1, PCBP2 or hnRNPK to the assays would have increased the endogenous levels of each protein by approximately fivefold (Figure 1c).

### *PCBP1, PCBP2 and hnRNPK interact with the c-myc IRES in vivo*

Experiments were then performed to ensure that these three proteins bound to c-*myc* IRES RNA *in vivo*. Thus, HeLa cells were incubated with formaldehyde to form stable RNA–protein complexes, cells were lysed and PCBP1, PCBP2 or hnRNPK were immunoprecipitated from these cells using specific antibodies. After extensive washing of the immune complexes the RNA was extracted, cDNA was generated and this was used in polymerase chain reactions (PCRs) with primers specific either to c-*myc* or ribosomal protein S16 (Figure 2). A specific band was only obtained in the IP–PCR reactions when the primers specific for the c-*myc* IRES were used, but not those for ribosomal protein S16, showing that all three proteins bind RNA *in vivo* (Figure 2).

### *UV crosslinking analysis shows that the c-myc IRES interacts specifically with PCBP1, PCBP2 and hnRNPK*

To test the specificity of the interaction of the c-*myc* IRES with PCBP1, PCBP2 and hnRNPK, UV cross-linking was performed in the presence of competitor RNAs. Radiolabelled c-*myc* IRES RNA was incubated with PCBP1, PCBP2 or hnRNPK in the presence of increasing amounts of unlabelled competitor c-*myc* IRES RNA (Figure 3a) or GAPDH RNA (Figure 3b). Samples were exposed to UV light, treated with RNAses and then separated by SDS–PAGE. All three proteins interacted with the c-*myc* IRES RNA. Unlabelled c-*myc* IRES RNA, but not unlabelled GAPDH mRNA competed for the binding with these proteins, showing that these interactions were specific.

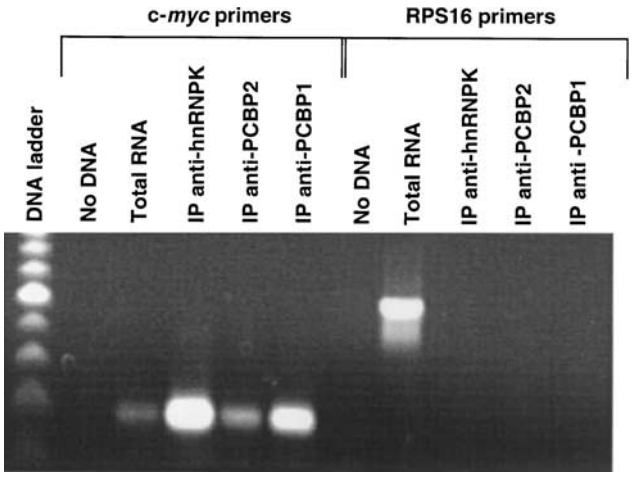


**Figure 2** Coimmunoprecipitation of c-*myc* RNA by hnRNPK, PCBP1 and PCBP2 from HeLa cells. The proteins, PCBP1, PCBP2 or hnRNPK were immunoprecipitated with the appropriate antibodies and the associated RNAs were reverse transcribed. The cDNAs were amplified by PCR using primers specific to either c-*myc* (that would give rise to a band of 200 bp) or ribosomal protein S16 (RPS16 that would give rise to a band of 450 bp). The products were separated on an agarose TBE gel. Each protein binds to the c-*myc* RNA but not to the control RNA

### *Electrophoretic mobility shift assays show that hnRNPK, PCBP1 and PCBP2 interact differently with the c-myc IRES*

It has been shown previously that members of the poly (rC) binding protein family contribute to many protein–protein interactions, and that PCBP1, PCBP2 and hnRNPK can form homodimers (Kim *et al*., 2000). Thus, to investigate further the interactions of c-*myc* IRES RNA with PCBP1, PCBP2 and hnRNPK, electrophorectic mobility shift assays (EMSAs) were performed. Radiolabelled c-*myc* IRES RNA was incubated with increasing amounts of each of these proteins and the products separated on a TBE–polyacrylamide gel (PAGE). A shifted band was observed with each of the proteins tested (Figure 4). The addition of an equimolar amount of active hnRNPK to c-*myc* IRES RNA generated a band of a size consistent with the binding of a single protein, and a twofold molar excess of active protein over RNA produced a band that corresponded with two hnRNPK proteins binding (Figure 4a). Therefore, either there is more than one hnRNPK binding site for this protein on the c-*myc* IRES RNA or else it is binding as a dimer. In the case of PCBP2, there was only a single shifted band at a position that would be expected for the binding of a monomer when an equimolar amount of active protein was used (Figure 4b). In contrast, when an equimolar amount of active PCBP1 was added to the RNA, a smear was always observed (Figure 4c). However, at a two to one protein : RNA ratio a band was observed at a

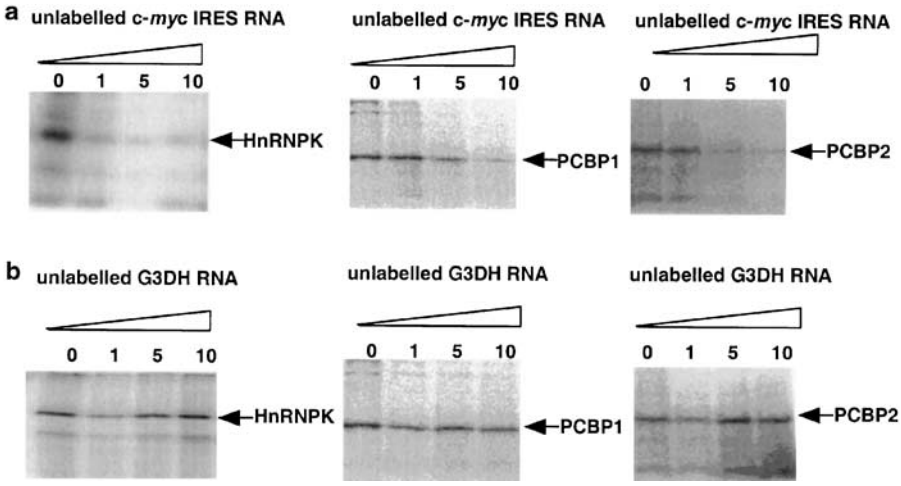


**Figure 3** Crosslinking of hnRNPK with PCBP1 and PCBP2 to the c-*myc* IRES. (**a**) HnRNPK, PCBP1 or PCBP2 were UV crosslinked to radiolabelled c-*myc* IRES RNA in the presence of the molar excess of unlabelled RNAs as shown. All three proteins are able to bind directly to the c-*myc* IRES. This binding is competed by unlabelled c-*myc* IRES RNA when added in a fivefold molar excess. (**b**) G3DH used as a control does not compete in this reaction at the concentration used

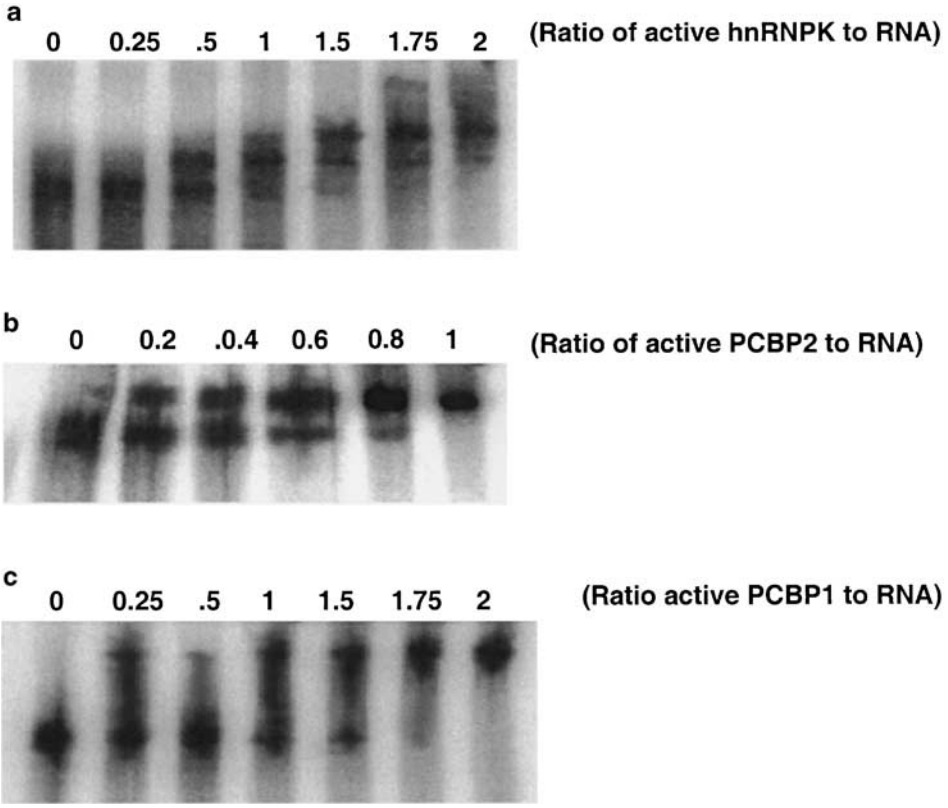


**Figure 4** hnRNPK and PCBP1 form complex associations with c-*myc* IRES RNA. EMSAs were performed by incubating increasing amounts of PCBP1, PCBP2 or hnRNPK protein with c-*myc* IRES RNA and separating the products on TBE acrylamide gels. (**a**) The interaction of hnRNPK produces multiple bands implying that hnRNPK is binding to the RNA at more than one site. (**b**) In contrast, PCBP2 appears to bind at a molar ratio at a single site. (**c**) The binding of PCBP1, however, produces a single high shifted band of a size that implies that this protein is binding as a dimer

position that was much greater than that produced for the shifted position of PCBP2 (Figure 4b), although these proteins have approximately the same molecular weight. Therefore, it is possible that two PCBP1 proteins are binding per RNA to the same site (Figure 4c).

*Location of binding sites for PCBP1, PCBP2 and HnRNPK on c-myc IRES RNA*

To identify the regions to which PCBP1, PCBP2 or hnRNPK could bind, a series of shorter radiolabelled c-*myc* IRES RNA fragments were generated by *in vitro* transcription using the plasmid pSKML digested with the enzymes shown or PCR products (Figure 5a). None of the proteins bound to the shortest fragment of only 34 nucleotides (nt) (Figure 5b). PCBP2 interacted with the 98 nt fragment and therefore requires the region 34–98 nt for binding. No consensus binding sites for PCBP2 are found in this region (Thisted *et al*., 2001); however, it is known from structural studies (LeQuesne *et al*., 2001) that this region of c-*myc* IRES RNA is highly folded and therefore the binding site may not be a linear sequence. Both PCBP1 and hnRNPK interact with the 211 nt fragment of the IRES (Figure 5b). The PCBP1 consensus binding site has not been determined. However, a consensus site has been described for the α-complex, which includes PCBP1 and PCBP2 and is involved in mRNA stabilization (Holcik and Liebhaber, 1997). The α-complex consensus is (C/U)CCA $N_x$ CCC(U/A) $Py_x$ UC(C/U)CC, and like the known PCBP2 consensus is CU rich and consists of a series of three C repeat regions. A C/U region fitting this general consensus is present in the c-*myc* IRES at 127–150 nt. This is contained within the 158 nt fragment that did not shift with PCBP1, but it is possible that the protein may need additional sequences after this site to adopt the correct secondary structure for protein binding.

The hnRNPK consensus sequence as defined by Thisted *et al*. (2001) is $UC_{3-4}$(UA/AU). However, a more recent study using a three-hybrid screen and hnRNPK as 'bait' showed that several additional short stretches of nucleotides are also important giving a longer less constrained consensus of CAUC(N)5 C(N)2 CCC (N)18 UCANCC (Ostrowski *et al*., 2002). There are several potential hnRNPK sites in the c-*myc* IRES. In the region 158–211 nts, these are ACCCUU at 166–171 nt and GCCCAU at 191–196 nt. In addition, there are putative hnRNPK binding sites in the region of RNA from 212 to 396. Thus, this section of RNA was also generated to determine whether there were additional sites for this protein in the 3′ end of the IRES. HnRNPK was found to bind to the 3′ end of the RNA (Figure 5b) and one putative site present in this section of RNA is from 254–259 UCCCGA. Interestingly, we have shown previously that in 42% of patients with MM this site is mutated (Chappell *et al*., 2000) to UCUCGA. Further studies were therefore carried out to investigate the binding of PCBP1 and PCBP2 to the c-*myc* IRES and to test the whether the mutated version of the IRES had a different affinity for hnRNPK.

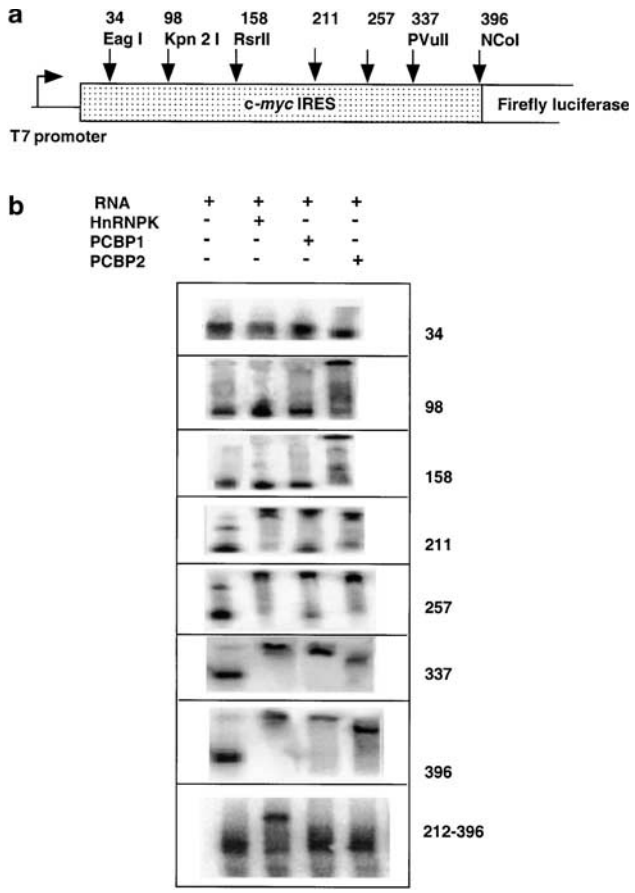


**Figure 5** Identification of the PCBP1, PCBP2 and hnRNPK binding regions within the c-*myc* IRES. (**a**) Schematic diagram of the sizes of c-*myc* IRES fragments generated. (**b**) EMSAs with radiolabelled c-*myc* IRES fragments in the presence of 0.2 μg of PCBP1 and PCBP2 and 0.15 μg of hnRNPK. These data show that PCBP2 requires the region between 34 and 98 nts for binding. However, PCBP1 and hnRNPK require nucleotides from 158 to 211 for binding. Only hnRNPK binds to the 212–396 fragment

*Mutated form of the IRES binds more tightly to hnRNPK*

Additional EMSAs were performed to obtain dissociation constants for PCBP1, PCBP2 and hnRNPK with c-*myc* IRES RNA. The radiolabelled c-*myc* IRES RNA was titrated with increasing amounts of protein. In each case, the fraction of bound RNA was assessed using a PhosphorImager, and was plotted on a graph relative to the amount of protein added to the reaction (Black *et al*., 1998). With PCBP1 a straight line was obtained and the dissociation constant for this interaction is $2.4 \times 10^{-6}$ M (Figure 6a). In the case of PCBP2, a straight line was also obtained with a value of $y = 15.7x$-0.5614 (Figure 6b). From this information, we can infer that the c-*myc* IRES contains a single PCBP2 binding site, and the $K_d$ for this interaction is $1.6 \times 10^{-5}$ M. Since the previous data suggest that there were two binding sites for hnRNPK on the c-*myc* IRES RNA, the dissociation constants were calculated for each site. The $K_d$ for the hnRNPK site present in the 1–211 fragment is $7.8 \times 10^{-6}$ M (Figure 6c). The hnRNP K binding site in the 212–396 wild-type fragment has a $K_d$ of $1.3 \times 10^{-5}$ M and the mutant a $K_d$ of $9.2 \times 10^{-6}$ M (Figure 6di and ii). Thus, hnRNPK binds more strongly to the sequence of c-*myc* IRES RNA that is mutated in MM.

### *PCBP-1, PCBP-2 and HnRNPK stimulate the c-myc IRES* in vivo

To investigate the effects of these proteins on c-*myc* IRES activity *in vivo* HeLa cells were cotransfected with the c-*myc* IRES containing plasmid pRMF (which contains the c-*myc* IRES between cistrons for firefly and Renilla luciferase; Figure 1a) with increasing amounts of plasmid DNA containing genes encoding PCBP1 (pcPCBP1), PCBP2 (pcPCBP2) or hnRNPK (pSG5-hnRNPK). In each case, the levels of the corresponding proteins expressed were increased by approximately threefold when the plasmids were transfected (Figure 7ai–ci). We were unable to obtain higher expression than this and it is possible that there is some additional mechanism for regulating the levels of these proteins. When the IRES activity was determined, the c-*myc* IRES was stimulated maximally twofold with PCBP1, 1.6-fold with PCBP2 (Figure 7aii, bii) and hnRNPK stimulated the 'wild-type' IRES 2.5-fold (Figure cii). In contrast, cotransfection had no effect on the control plasmid pRF (data not shown). These experiments were then repeated with the mutant version of the IRES. Interestingly, cotransfection with the hnRNPK encoding plasmid stimulated the mutant version of the IRES up to 3.7-fold (Figure ciii). These activations are relatively small and could in part reflect the high endogenous levels of these proteins.

## Discussion

Internal ribosome entry on cellular IRESes would appear to be a complex process requiring several *trans*-acting factors that work in concert to allow the RNA to attain the correct structural conformation for the ribosome to bind. Our data and that of others would suggest that while cellular IRESes utilize some of the same *trans*-acting factors for function there is probably a unique set of proteins that is required for each IRES

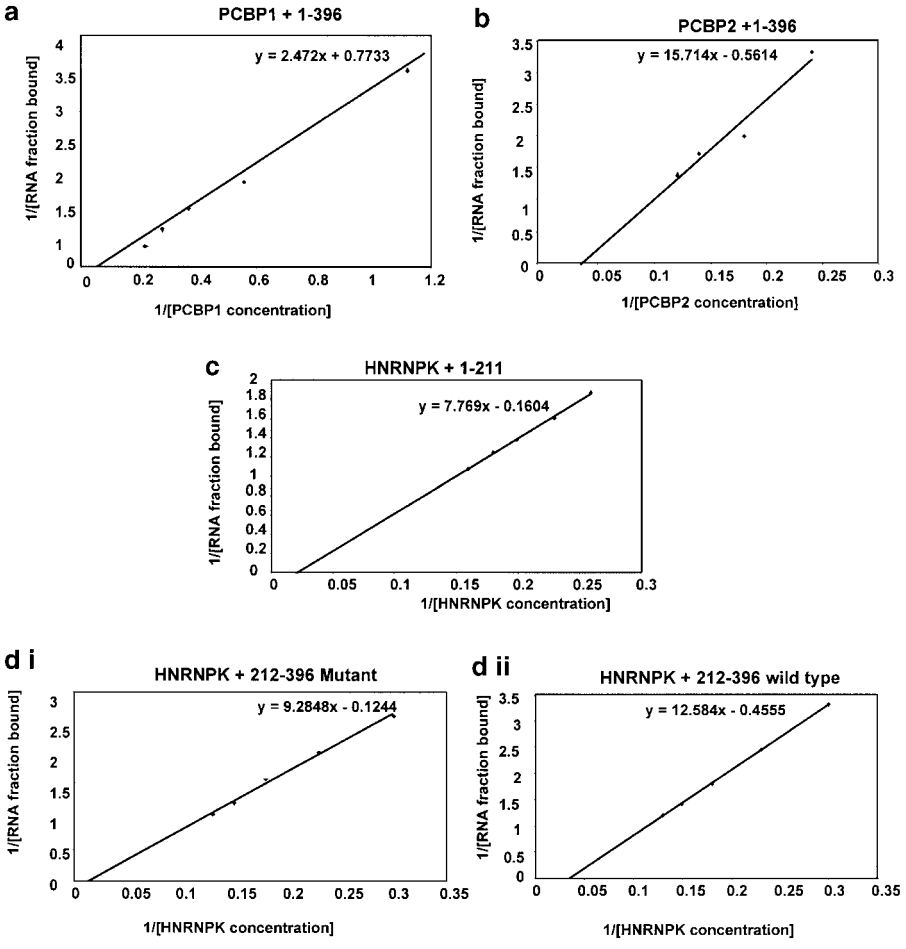


**Figure 6** Determining the $K_d$ values for PCBP1, PCBP2 and hnRNPK interactions with c-*myc* IRES RNA. EMSAs were performed using the concentrations of proteins shown and the bound fraction of RNA determined using the PhosphorImager. (**a**) A graph of the reciprocals of bound RNA against protein concentrations of PCBP1 produces a straight line trend for binding at a single site. The $K_d$ of this interaction can be calculated as $2.4 \times 10^{-6}$ M. (**b**) PCBP2 binding is weaker and the $K_d$ for this is $1.6 \times 10^{-5}$ M. (**c**) hnRNPK binds to the fragment of c-*myc* IRES RNA from 1 to 211 with a $K_d$ value of $7.8 \times 10^{-6}$ M. (**d**) hnRNPK binds to the wild-type fragment of c-*myc* IRES RNA from 212 to 396 with a value of $1.3 \times 10^{-5}$ M and to the mutant with a value of $9.2 \times 10^{-6}$ M showing that hnRNPK interacts more strongly with the mutated version of the IRES

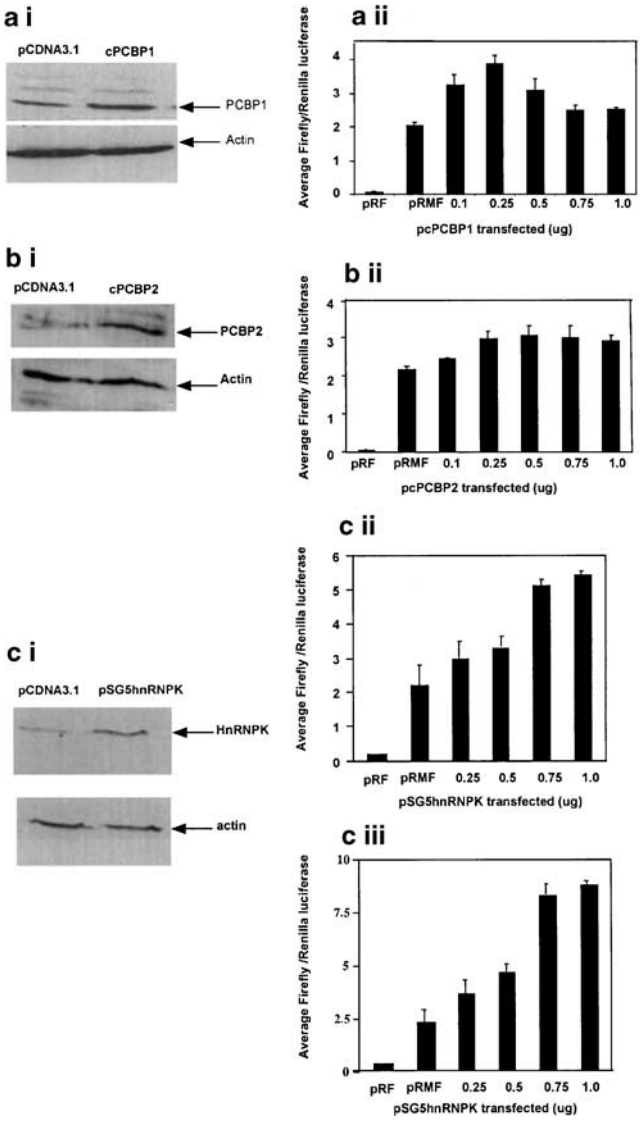


**Figure 7** Cotransfection of PCBP1, PCBP2 and hnRNPK into HeLa cells increases c-*myc* IRES activity. HeLa cells were cotransfected with the dicistronic plasmids pRMF harbouring the c-*myc* IRES or the control plasmid pRF and increasing amounts of DNA from those plasmids expressing PCBP1 (pcPCBP1), PCBP2 (pcPCBP2) and hnRNPK (pSG5hnRNPK) in addition to DNA from pCDNA3.1 so that the same amount of DNA was transfected in each case. Western blots confirmed an increase in protein expression (**ai**–**ci**) and luciferase activity was determined. It is possible to stimulate IRES function in HeLa cells with plasmids expressing PCBP1 (**aii**) and PCBP2 (**bii**) and hnRNPK (**cii**). The mutant version of the IRES is stimulated more by hnRNPK than the wild-type version up to a maximum of 3.7-fold

(Creancier *et al*., 2000, 2001). Thus, the c-*myc* IRES is similar to the Apaf-1 IRES in that unr is able to enhance IRES function albeit with a much lower degree of activation in this case (Mitchell *et al*., 2001, 2003), but it also differs since members of the poly (rC) binding protein family also interact, possibly as part of a complex as all three proteins are required to stimulate the IRES *in vitro* (Figure 1). We have shown by UV crosslinking analysis, EMSAs and immunoprecipitation–RT–PCRs that PCBP1, PCBP2 and hnRNPK all interact specifically with the c-*myc* RNA (Figures 2–6). The data suggest that PCBP1 interacts with the IRES as a dimer, while PCBP2 binds as a monomer and there are two separate binding sites for hnRNPK (Figures 4–6). We have also shown that a mutated version of the c-*myc* IRES that is found in 42% of patients with MM binds more tightly to hnRNPK (Figure 6). Cotransfection of plasmids expressing PCBP1, PCBP2 or hnRNPK with the IRES containing plasmid pRMF stimulated c-*myc* IRES function *in vivo* in HeLa cells (Figure 7). Moreover, cotransfection with hnRNPK stimulated the mutated version of the IRES to a greater extent. In patients with MM and in cells lines derived therefrom which harbour this mutation, there is increased expression of c-Myc. This increase in expression is due to an upregulation in c-*myc* IRES function and these data would suggest that this is in part due to the enhanced binding of hnRNPK to the mutated sequence.

The data presented would strongly suggest that members of the poly (rC) binding protein family interact with the c-*myc* IRES and are able to stimulate its function.

Since the initiation of translation of c-*myc* can occur by cap-dependent mechanisms as well as by internal ribosome entry the structure of the c-*myc* IRES RNA must be sufficiently flexible to allow ribosome scanning (Le Quesne *et al*., 2001). The RNA binding chaperones may be required to maintain the RNA in the correct conformation for internal ribosome entry to occur. In this regard, the binding sites for all three proteins lie within a region that we have shown previously to be highly structured (Le Quesne *et al*., 2001). A complex set of factors must be required, however, as the level of firefly luciferase produced *in vitro* via the IRES in the presence of five interacting proteins is clearly well below that observed in *vivo* (Figure 1). The c-*myc* IRES is almost completely inactive when present in dicistronic mRNAs introduced directly into the cytoplasmic compartment, suggesting that a nuclear experience is an essential prerequisite for internal initiation mediated by the c-*myc* IRES (Stoneley *et al*., 2000). It has been found that hnRNPK (Michael *et al*., 1997), PCBP1 and PCBP2 (Makeyev and Liebhaber, 2002) are able to shuttle between the nucleus and the cytoplasm and indeed hnRNPK contains a classical bipartite basic NLS at its N-terminus (Michael *et al*., 1997). Therefore, it is possible that PCBP1, PCBP2 and hnRNPK interact with the c-*myc* IRES in the nucleus and that the formation of a nuclear RNA/protein complex renders the IRES competent for internal initiation. It has been shown that hnRNPK interacts with the CT element in the promoter of the c-*myc* gene (Michelotti *et al*., 1996a, b). We suggest that certain proteins required for transcription of IRES containing genes may also necessary for their translation, and that this is mediated by their recruitment to the RNA during transcription.

## Materials and methods

### *Materials*

Media and Serum were purchased from GIBCO BRL, Luciferase assay kits 'Stop & Glo' and Flexi rabbit reticulocyte lysates were purchased from Promega. Galactolight plus assay system was purchased from Tropix. HeLa cells were obtained originally from American Tissue Type Culture Collection. All other chemicals were purchased from Sigma (Poole, UK).

### *Plasmid constructs*

The plasmid pSKML is a bluescript-based vector that contains the c-*myc* IRES fused in frame with the firefly luciferase gene; pRMF and pRF are as described (Stoneley *et al*., 2000). A 600 bp fragment encoding part of the GAPDH gene was subcloned into pSK + bluescript to generate pSK + GAPDH. The cDNAs encoding unr, PTB, La, central third of eIF4G, DAPS, $ITAF_{45}$ were subcloned into the PET28a vector, PCBP1 and PCBP2 were cloned into PET21a and hnRNPK was cloned in PET16b (Ostareck *et al*., 1997), which enabled proteins to be expressed in *Escherichia coli* and purified. For expression in tissue culture cells cDNAs were additionally subcloned into pCDNA3.1 with the exception of HnRNPK, which was in the vector pSG5-hnRNPK (Ostareck *et al*., 1997).

### *Protein expression*

All proteins were overexpressed in *E. coli* from PET28a, PET21a and PET16 vectors by the addition of IPTG to the growth medium. All proteins contained a His-tag and were purified using a Nickel affinity column using the manufacturer's protocol (Qiagen). The activity of each protein was determined in EMSAs (see below). HnRNPK was 90% active, PCBP1 was 81% active and PCBP2 was 63% active.

### *SDS–PAGE and Western blotting*

For analysis of PCBP1, PCBP2 and hnRNPK proteins, cell extracts (equal cell numbers per lane, or rabbit reticulocyte lysates, 8 $\mu$l of extract) were analysed by SDS–PAGE electrophoresis and electroblotted as described in Mitchell *et al*. (2001). HnRNPK antibody was generated as described previously (Van Seuningen *et al*., 1995), PCBP2 antibody was generated in the Willis laboratory and PCBP1 antibody was a kind gift from Dr R Andino. Blots were then incubated with peroxidase-conjugated secondary antibodies raised against mouse or rabbit immunoglobulin and developed using the chemiluminescence reagent 'Illumin 8' (generated by Dr M Murray, Department of Genetics, University of Leicester).

### *Cell Culture and transient transfections*

HeLa cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO-BRL) containing 10% foetal calf serum in a humidified atmosphere containing 5% $CO_2$.

Calcium phosphate-mediated DNA transfection of mammalian cells was performed essentially as described in Jordan *et al*. (1996) with minor modifications (Stoneley, 1998). All transfections were performed in triplicate on at least three independent occasions.

The activity of firefly and Renilla luciferases in lysates prepared from transfected cells were measured using a Dual-luciferase reporter assay system (Promega) and light emission was measured over 10 s using an OPTOCOMP I luminometer.

The activity of $\beta$-galactosidase in lysates prepared from cells transfected with pcDNA3.1/HISB/LacZ (invitrogen) was measured using a Galactolight plus assay system (Tropix).

### In vitro *transcription and translation*

Vector DNA (pRMF, pRF, pSKML, pSK + GAPDH) was linearized by restriction digestion using a site downstream of the sequence of interest. Transcripts were synthesized in a reaction containing 1 × transcription buffer (40 mM HEPES-KOH, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 10 mM NaCl), 40 U of RNasin (1 mM ATP, 1 mM UTP, 1 mM CTP, 1 mM GTP), 1 $\mu$g of DNA template and 20 U of T7, T3 or SP6 RNA polymerase in a final volume of 50 $\mu$l. For radiolabelled RNAs 50 $\mu$Ci of $^{32}$P CTP was included in the reactions. After incubation of the reaction for 1 h at 37°C, the RNA was purified by phenol extraction, followed by ethanol precipitation.

RNA (5 ng/$\mu$l) was used to prime Promega rabbit reticulocyte lysate *in vitro* translation system in a final volume of 12.5 $\mu$l according to the manufacturer's instructions, with the addition of 250–500 ng of purified proteins as indicated. All experiments were performed in triplicate on at least three independent occasions.

### *UV crosslinking assay*

Radiolabelled RNA transcripts (2.4 nmol; $4.5 \times 10^5$ c.p.m.) were incubated with 0.25 $\mu$g of PCBP1, PCBP1 or 0.5 $\mu$g of hnRNPK in a 30 $\mu$l buffer mix (containing 10 mM HEPES pH 7.4, 3 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 1.3 mM ATP, 6% glycerol, 0.1 $\mu$g/$\mu$l yeast tRNA) in the presence or absence of unlabelled competitor transcripts, for 10 min at room temperature in a 96-well microtitre plate (Falcon). Samples were UV irradiated on ice for a period of 30 min using a 305 nM UV light source. RNase A (0.2 mg/ml) and RNAseT1 (1 U) were added to each of the samples that were then incubated at 37°C for 30 min to allow degradation of any unprotected RNA species. An equal volume of 2 × SDS sample buffer was added to the samples prior to separation by SDS–PAGE (10% gels). Gels were then dried and the results visualized on a Molecular Dynamics phosphorimager.

### *Electrophoretic mobility shift assays*

Approximately $8.6 \times 10^{-8}$ mol. labelled transcript (23 000 c.p.m.) was incubated in a 20 $\mu$l buffer mix containing 5 $\mu$l 5 × transcription buffer (200 mM Tris-HCl pH8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 0.75 $\mu$l DTT (1 M), 1.5 $\mu$l tRNA (10 mg/ml), 1 $\mu$l rATP (10 mM), and 40 U of RNAsin. Proteins (0.05–0.5 $\mu$g) were then incubated with the RNA at room temperature for 10 min. TBE loading buffer (3 $\mu$l of 10 × ) was added and samples loaded directly onto 5% acrylamide gels made using 1 × TBE filter sterilized buffer. Samples were then electrophoresed at 150 V for 2 h in 1 × TBE filter sterilized buffer. Gels were dried under vacuum at 80°C for 2 h and exposed on a phosphorimager. For the calculation of dissociation constants, experiments were carried out as in Black *et al*. (1998). After the working concentration of protein was assessed, EMSAs were run using various concentrations of protein (PCBP1, PCBP2 and hnRNPK 0.2–5 $\mu$l (0.3–7 $\mu$M) and the fraction of bound RNA determined on the PhosphorImager.

### *Immunoprecipitation and RT–PCR*

The method used was that described by Niranjanakumuri *et al*. (2002). Briefly, $2 \times 10^6$ HeLa cells were incubated in 10 ml of

phosphate-buffered saline containing 1% (V/V) formaldehyde for 10 min at room temperature. The reaction was quenched by the addition of 0.25 M glycine, the cells harvested and the pellet resuspended in RIPA buffer (50 mM Tris-Cl pH 7.5, 1 % NP40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) and sonicated to lyse the cells. Insoluble material was removed by centrifugation (16 000 *g*) and the lysates were precleared by incubating with protein A/G beads (Santa Cruz) a nonspecific antibody, and nonspecific competitor tRNA (100 $\mu$g/ml). HnRNPK antibody was generated as described previously (Van Seuninge *et al*., 1995), PCBP2 antibody was generated in the Willis laboratory and PCBP1 antibody was a kind gift from Dr R Andino. The samples were centrifuged and the proteins were immunoprecipitated from the supernatant overnight at 4°C by addition of antibody and protein A/G beads. The beads were harvested and washed six times in RIPA buffer, additionally containing 0.5 M NaCl and 1 M urea. Finally, the beads were resuspended in 100 $\mu$l of 50 mM Tris-Cl, 5 mM EDTA and 10 mM DTT. The crosslinks were reversed by heating the sample to 70°C for 45 min and the RNA was extracted using Tri-reagent (SIGMA). The RNA was then used to generate cDNA using oligo-dT and MMLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was performed using either primers specific to the coding region of c-*myc* or primers specific to ribosomal protein S16 (GCGCGGTGAGGTTGTCTAGTC and GAGT TTTGAGTCACGATGGGC).

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