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# Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA

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## ABSTRACT

**Initiation of translation of the animal picornavirus RNAs is via a mechanism of direct internal ribosome entry, which requires a substantial segment of the viral 5′-untranslated region, generally known as the IRES (for "internal ribosome entry site"). Because, however, translation of the RNAs of members of the enterovirus, and more especially, the rhinovirus subgroups of the *Picornaviridae* is restricted in the reticulocyte lysate system, but is greatly stimulated by the addition of HeLa cell extracts, the implication is that, in these cases, internal initiation also requires cellular *trans*-acting factors that are more abundant in HeLa cell extracts than in rabbit reticulocytes. This was used as the basis of a functional assay for the purification of the HeLa cell factors required for translation dependent on the human rhinovirus-2 (HRV) IRES. There are two such HeLa cell factors separable by ion-exchange chromatography, each of which is individually active in the assay, although their combined effect is synergistic. One of these activities is shown to be polypyrimidine-tract binding protein (PTB) on the grounds that (1) the activity copurifies to homogeneity with PTB and (2) recombinant PTB expressed in *Escherichia coli* stimulates HRV IRES-dependent translation with a specific activity similar to that of the purified HeLa cell factor. Furthermore, it is shown that recombinant PTB also stimulates the translation of RNAs bearing the poliovirus type 1 (Mahoney) IRES.**

**Keywords: autoantigen La; internal ribosome entry segment (IRES); picornavirus; poliovirus; poly(C) binding protein-2; RNA binding proteins**

## INTRODUCTION

Although the normal mode of initiation of translation of eukaryotic cellular and viral mRNAs is by the scanning ribosome mechanism (Kozak, 1989), a small subset of such RNAs is translated by a mechanism of direct internal ribosome entry (reviewed in Jackson & Kaminski, 1995). The best characterized examples, indeed the paradigm, of RNAs translated by internal ribosome entry are the animal picornavirus RNAs (Jang et al., 1988; Pelletier & Sonenberg, 1988; Jackson & Kaminski, 1995). Internal initiation of picornavirus RNA translation requires a substantial *cis*-acting RNA element, ~450 nt long, which is generally known as the IRES (for "internal ribosome entry segment/site"). IRESes are characterized and studied by the fact that if they are inserted between the two cistrons of a laboratory-generated dicistronic mRNA construct, the synthesis of translation product from the downstream cistron is increased from a very low yield (observed in the absence of any IRES insert) to a yield that frequently far exceeds that of the upstream, scanning-dependent cistron. With the exception of hepatitis A virus, the picornavirus family can be divided into two groups based on the conservation of primary and especially secondary structure of the IRES element: (1) the entero- and rhinoviruses; and (2) the cardio- and aphthoviruses. Within each group there is quite strong conservation of IRES primary nucleotide sequence, and even stronger conservation of secondary structure, but there is virtually no conservation between the two groups (reviewed in Jackson & Kaminski, 1995).

Aside from the problem of how these *cis*-acting RNA elements promote internal ribosome entry, there is also the associated question of whether this unusual mode of initiation of translation requires special cellular protein factors other than the canonical translation initiation factors. Here again, there seem to be distinct

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differences between the two major subgroups of picornaviruses. This is illustrated by the fact that whereas the cardio- and aphthovirus IRESes function efficiently in rabbit reticulocyte lysates, and in general show little tissue tropism, this is not true of the entero- and rhinovirus IRESes. Poliovirus RNA is translated quite efficiently in extracts of HeLa cells, but its translation in rabbit reticulocyte lysates is both inefficient and inaccurate (Brown & Ehrenfeld, 1979; Dorner et al., 1984), unless HeLa cell cytoplasmic extracts are added. The translation of poliovirus RNA is also restricted in *Xenopus* oocytes, and can likewise be rescued by coinjection of HeLa cell extracts (Pelletier et al., 1988; Gamarnik & Andino, 1996).

These observations imply that internal initiation dependent on IRESes of the entero-/rhinovirus group requires cellular factors that are missing in rabbit reticulocyte lysates, or are at least very much more abundant in HeLa cells than in rabbit reticulocytes. The identity of such factors remains somewhat a mystery. The only attempt so far to isolate and identify the activity using the functional translation assay as the basis of purification was made by Svitkin et al. (1988), who studied poliovirus RNA translation in reticulocyte lysates supplemented with fractions derived from Krebs II ascites cells. After a very partial purification, they proposed that the active entity might be initiation factor eIF2/eIF2B complex, but this was later refuted by the results of direct tests with purified eIF2/eIF2B (Svitkin et al., 1994).

All other efforts at identifying the relevant factors have been based on an approach of identifying proteins that can be shown to bind to the 5′-untranslated region in UV-crosslinking or gel-retardation assays, and are thus prejudiced by the assumption that proteins that bind to the IRES must necessarily play a role in internal initiation. Two such IRES-binding proteins identified in this way are the autoantigen La and polypyrimidine tract binding protein (PTB), a protein previously considered to be a constitutive pre-mRNA splicing factor but now thought to be a negative regulator of alternative splicing (Patton et al., 1991; Lin & Patton, 1995). In translation assays using rabbit reticulocyte lysate, addition of La does indeed stimulate poliovirus RNA translation, but only at such very high concentrations ($\sim$100 $\mu$g/mL) that it seems highly unlikely that the stimulation effected by HeLa or Krebs II ascites cell cytoplasmic extracts could be entirely due to the La in such extracts (Meerovitch et al., 1993; Svitkin et al., 1994). As for PTB, HeLa cell extracts immunodepleted of this protein lost the capacity for translation dependent on the IRES of encephalomyocarditis virus (a cardiovirus) or poliovirus, though they retained activity for translation of mRNAs via the scanning ribosome mechanism (Hellen et al., 1993). At face value this result would imply that PTB is required for internal initiation promoted by these two viral IRESes, but this conclusion was clouded with very considerable reservations by the fact that addition of recombinant PTB failed to rescue IRES-dependent translation in the depleted extract (Hellen et al., 1993).

As a continuation and completion of an earlier study (Borman et al., 1993), we report here the first successful purification that is based solely on an activity assay of enhanced IRES-dependent translation. (Although UV crosslinking was used as a secondary or correlative assay, the functional translation assay was used as the exclusive basis of the purification.) We show that there are two separable HeLa cell activities, each of which is individually active in promoting translation dependent on the human rhinovirus-2 IRES, although they show at least additive, and possibly synergistic, stimulation when tested together. We identify one of these factors as PTB and present a model to account for the fact that there are two such activities.

## RESULTS

### Assay system for the purification of *trans*-acting factors

The outcome of translation of the poliovirus and rhinovirus genomic RNAs in the reticulocyte lysate is a rather low yield of authentic viral products plus a large number of aberrant products, not synthesized during viral infection, that originate from initiation at incorrect sites (Brown & Ehrenfeld, 1979; Dorner et al., 1984). Supplementation of such assays with HeLa cell cytoplasmic extracts or a ribosomal salt-wash fraction stimulates the synthesis of the authentic products, and to varying degrees, decreases the yield of the aberrant products. This does not provide a particularly convenient assay for the purification of the active HeLa cell components because there is uncertainty as to whether activity should be judged by the enhanced yield of the correct products or the suppression of incorrect product synthesis (Jackson, 1989). The situation is further complicated by the proteolytic processing of the authentic viral polypeptides by virus-encoded proteases.

A rather more reliable assay is to use an artificial dicistronic mRNA with the poliovirus or rhinovirus IRES element as intercistronic spacer. The yield of the downstream IRES-dependent cistron product is very low in reticulocyte lysate, but is likewise stimulated by the addition of HeLa cell extracts. The advantage of this particular assay is that the purification of the relevant HeLa cell factors can be monitored by the specific stimulation of the downstream cistron product relative to that of the upstream scanning-dependent cistron product. Thus, the upstream cistron acts as an internal control for nonspecific stimulation or inhibition of translation. We have therefore chosen to use this as the assay system and have further chosen to use the rhinovirus IRES rather than the poliovirus equivalent since the activity of the rhinovirus IRES in unsupplemented rab-

bit reticulocyte lysate is particularly low, even lower than that of poliovirus, and therefore the stimulation effected by addition of HeLa cell extracts is even greater.

Consequently, throughout this work, the assays have been based on the translation of a dicistronic mRNA (XLJHRV10-611) in which the upstream cistron codes for the *Xenopus laevis* cyclin B2, the intercistronic spacer is the 5′-UTR of HRV-2 (complete apart from the first 9 nt), and the downstream cistron codes for a slightly truncated version of the influenza virus NS1 protein, designated here as NS′ (Borman & Jackson, 1992). The addition of increasing amounts of HeLa cell S10 cytoplasmic extract to rabbit reticulocyte lysate results in a strong stimulation of the downstream IRES-dependent cistron relative to the upstream cistron (Fig. 1). The apparent decrease in the yield of radiolabeled cyclin (translated from the upstream cistron) with increasing amounts of HeLa S10 added is probably due to isotope dilution by the unlabeled methionine in the (undialyzed) HeLa cell S10 extract. In contrast, the yield of radiolabeled IRES-dependent cistron product is influenced by increasing amounts of HeLa S10 in two opposing ways: increasing isotope dilution will decrease the apparent yield, but the stimulation of IRES-dependent translation counterbalances this up to 30% (by volume) HeLa cell S10 (or 20% at the lowest RNA concentrations), beyond which the isotope dilution effect predominates, probably because the stimulatory effect is saturated (Fig. 1).

Note that at any particular input of HeLa cell S10 extract, the efficiency of IRES-dependent translation shows an unusual relationship with the RNA concentration, in that above 25–50 $\mu$g/mL RNA, the yield of NS′ decreases, whereas the upstream cistron product yield actually increases over the same RNA concentration range (Fig. 1). The downturn is more severe at low HeLa cell S10 input, as can be readily visualized by comparing the relative intensity of the labeled NS′ band at 100 as opposed to 6.26 $\mu$g/mL at each different concentration of HeLa extract: at 10% HeLa extract, 6.25 $\mu$g/mL RNA gives a higher signal than 100 $\mu$g/mL, but the converse is true at 40% (v/v) HeLa S10. This paradoxical response to increasing RNA concentration, which is commonly seen when RNAs with the entero- and rhinovirus IRESes are translated in vitro, will be discussed later. To avoid the decreased response to HeLa cell factors associated with the higher RNA concentrations, all subsequent assays were carried out at an RNA concentration of 10 $\mu$g/mL, and the proportion of the HeLa cell supplement was maintained at 20% by volume.

Preliminary experiments showed that when the cytoplasmic S10 extract was fractionated into a post-ribosomal S100 supernatant by centrifugation at 100,000 $\times$ *g* under low-salt conditions, and the crude ribosomes then washed at high salt (0.5 M KCl), stimulatory activity was found in both the S100 and ribosomal salt-wash fractions (data not shown). Therefore we used a high-salt S100 extract, prepared by bringing S10 extract to 0.5 M KCl, removing the ribosomes by centrifugation at 100,000 $\times$ *g*, and dialyzing the supernatant against a low-salt buffer containing 100 mM KCl,

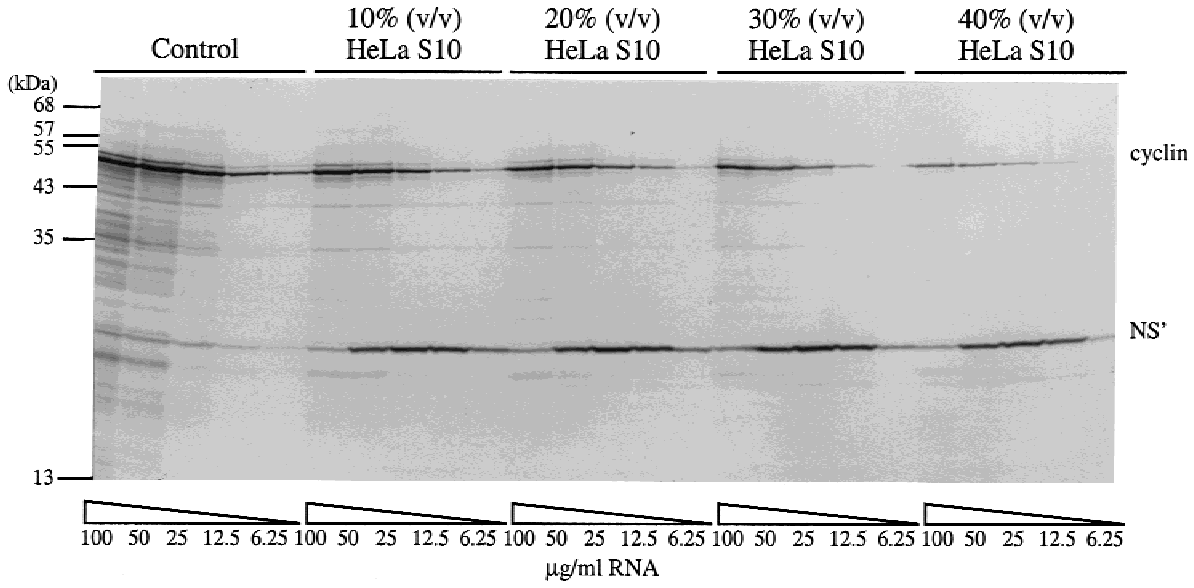


**FIGURE 1.** HeLa cell cytoplasmic extract stimulates HRV-2 IRES-dependent translation in rabbit reticulocyte lysate. Uncapped XLJHRV10-611 mRNA was translated at 100, 50, 25, 12.5, and 6.25 $\mu$g/mL in translation assays containing 75% (v/v) reticulocyte lysate (RRL; control lanes); 65% (v/v) RRL and 10% (v/v) undialyzed HeLa S10; 55% RRL and 20% HeLa S10; 45% RRL and 30% HeLa S10; or 35% RRL and 40% HeLa S10 under standard conditions (see Materials and Methods). An autoradiogram of the translation products, resolved by 20% SDS/polyacrylamide gel electrophoresis, is shown. The upstream cistron translation product (cyclin) and downstream cistron product (NS′) are indicated. The positions of protein molecular weight markers (in kDa) are indicated in the left margin.

as the starting material for the subsequent purification of the relevant factors. Figure 2 shows that this HS S100 material specifically stimulates IRES-dependent translation, and at this particular RNA concentration, saturation is attained at ~25% (by volume) HS S100. In contrast to our previous experience (Borman et al., 1993), we also found that nuclear extracts show a similar stimulatory activity, although in the unfractionated state they also effect a nonspecific inhibition of translation of both cistrons, particularly when added at higher concentrations (Fig. 2).

It should be appreciated that the efficiency of translation of both the upstream scanning-dependent cistron and the downstream IRES-dependent cistron is influenced by the KCl concentration in the assay, although the sensitivity to inhibition by high KCl concentration is different for the two cistrons. To avoid this complication in the assay of column fractions eluted with increasing KCl concentrations, the KCl concentration of each fraction was estimated by measuring the conductivity, and then aliquots of all column fractions were adjusted to the same KCl concentration by the addition of an appropriate volume of buffer (20 mM HEPES-KOH pH 7.5, 2 mM DTT) or buffer containing 1 M KCl. Hence, in all the assays shown here, the KCl concentration was constant across all column fractions.

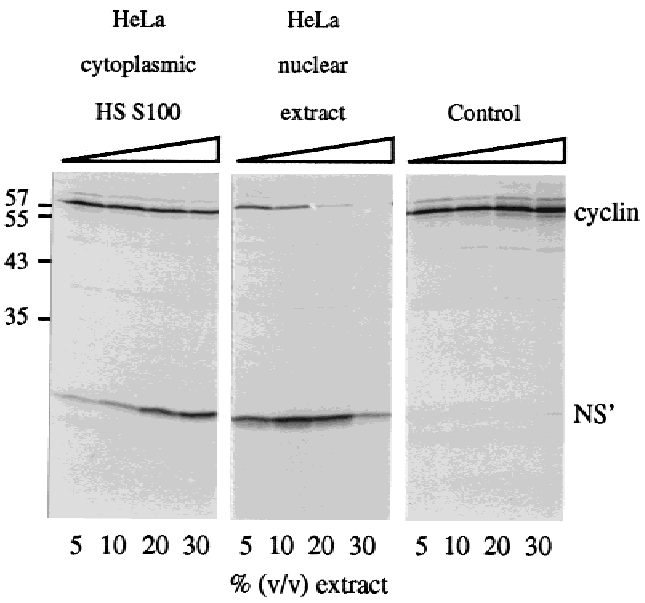


**FIGURE 2.** HeLa cell cytoplasmic and nuclear extracts contain factors that stimulate HRV IRES-dependent translation. Uncapped XLJHRV10-611 mRNA was translated in reticulocyte lysate at 10 $\mu$g/mL in the presence of HeLa cell HS S100 cytoplasmic extract or HeLa cell nuclear extract, each added at 5%, 10%, 20%, or 30% (v/v). Control reactions were supplemented with H100 buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl), also at 5%, 10%, 20%, or 30% (v/v). Reactions were carried out under standard conditions, with 90 mM added KCl and 0.5 mM added $MgCl_2$. An autoradiogram of the translation products, resolved by SDS/20% polyacrylamide gel electrophoresis, is shown. The upstream cistron translation product (cyclin) and downstream cistron product (NS′) are indicated. The positions of protein molecular weight markers (in kDa) are indicated in the left margin.

### The stimulatory activity has two components

We reported previously that when HeLa cell cytoplasmic extracts are passed over DEAE-Sepharose, the activity that stimulates HRV IRES-dependent translation separates into two components, which we named the A- and B-type activities (Borman et al., 1993). The A-type activity flowed through the column at 100 mM KCl. The B-type activity, however, was retained on the matrix at this salt concentration, but eluted with 200 mM KCl, and appeared to co-elute with a 97-kDa protein that was crosslinkable to the HRV 5′-UTR by UV irradiation (Borman et al., 1993). Figure 3A shows that a similar separation of two activities occurs when the HeLa HS S100 is fractionated by chromatography on heparin-Sepharose. By the criterion of retention on DEAE-Sepharose, the first peak to elute from the heparin-Sepharose column is the B-type activity, and the second is the A-type (data not shown). A similar separation into two activities was seen when the starting material was nuclear extract, although in that case, there seemed to be a higher ratio of the A-type activity to B-type than in the HS S100.

When fractions from the heparin-Sepharose column were subjected to UV-crosslinking assays with $^{32}$P-labeled HRV 5′-UTR as the probe, the B-type activity was seen to co-elute with a crosslinkable 97-kDa protein, as reported previously (Borman et al., 1993), and the A-type activity with a crosslinkable 57-kDa protein doublet (Fig. 3B). To confirm that this 57-kDa protein is PTB, we immunoblotted the heparin-Sepharose column fractions with anti-PTB antibodies (Fig. 3C). The elution profile of PTB exactly matches that of the crosslinkable doublet, showing that the elution of PTB correlates precisely with the A-type activity profile. By this criterion, however, the B-type, activity-enriched fractions were devoid of PTB.

Duplicate blots of the same fractions were also probed with anti-La antibodies. It was found that although La was spread across many fractions, much as reported by Stefano (1984), its elution profile did not coincide precisely with either of the two activities defined by the translation assay (Fig. 3D). Thus, although La has been shown to stimulate translation initiation at the authentic initiation site of poliovirus RNA, it does not seem likely that either of the two activities which stimulate HRV IRES-dependent translation can be attributed entirely to La. Moreover, using in vitro phosphorylation by the haem-controlled eIF2 kinase to locate eIF2$\alpha$ in these same column fractions, we found that neither activity co-eluted with eIF2 (data not shown), even though it had been previously proposed that eIF2/2B is part of the complex that specifically stimulates poliovirus RNA translation (Svitkin et al., 1988).

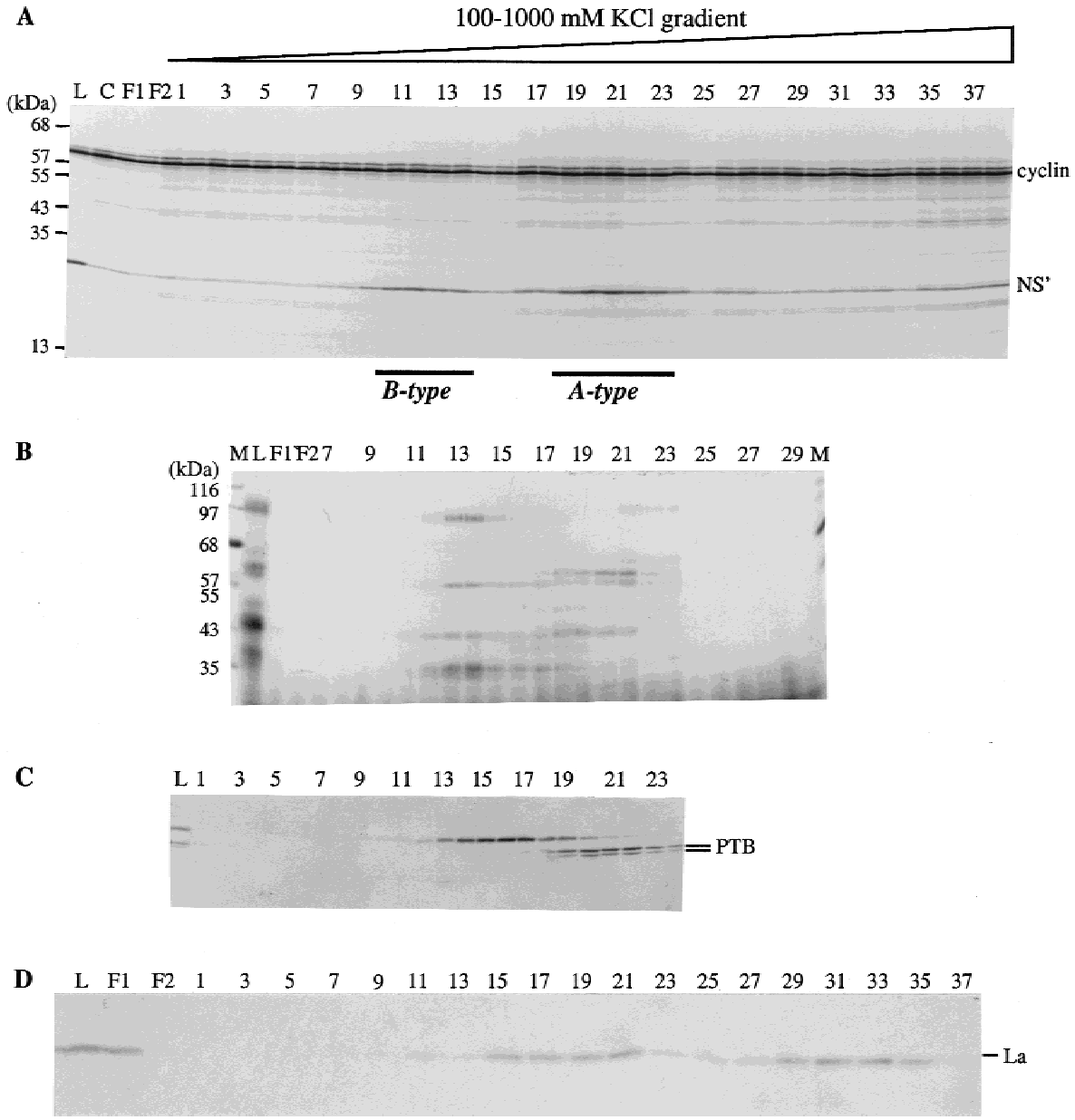


**FIGURE 3.** Fractionation of HeLa cell HS S100 extract by heparin-Sepharose column chromatography. Chromatography was carried out as described in Materials and Methods. **A**: Translation assays of uncapped mRNA XLJHRV10-611, supplemented with 20% (v/v) HeLa HS S100 (lane L), column flow-through fractions (lanes F1 and F2), eluate fractions 1–38 (each of which had been adjusted to 400 mM KCl), or H400 buffer (lane C). Reactions were carried out under standard conditions. An autoradiogram of the translation products, resolved by 20% SDS-polyacrylamide gel electrophoresis, is shown. **B**: UV-crosslinking assays of HeLa cell HS S100 (lane L), flow-through fractions F1 and F2, and fractions 7–29, using $^{32}$P-labeled JHRV10-605 RNA as probe. Reactions were carried out under standard conditions in the presence of 0.1 mg/mL heparin. A fluorogram of the $^{32}$P-labeled proteins, resolved by SDS/15% polyacrylamide gel electrophoresis, is shown. Lane M: protein molecular weight markers (sizes, in kDa, are indicated in the left margin). **C**: Immunoblot of SDS/15% polyacrylamide gel of HeLa cell HS S100 (lane L) and fractions 1–24, probed with rabbit anti-PTB antibodies and detected using alkaline phosphatase-conjugated anti-rabbit secondary antibody. The position of the PTB doublet is shown. The nature of the larger cross-reacting protein (fractions 13–20) is not known. **D**: Immunoblot of SDS/15% polyacrylamide gel of HeLa HS S100 (lane L) and alternate (odd-numbered) fractions 1–37, probed with rabbit anti-La antibodies and detected using alkaline phosphatase-conjugated anti-rabbit secondary antibody.

### The A-type activity is polypyrimidine-tract binding protein

After successive fractionation on DEAE-Sepharose and heparin-Sepharose, the A-type activity was purified on poly(U)-Sepharose (Fig. 4). The activity that specifically stimulated HRV IRES-dependent translation co-eluted with an abundant silver-stainable protein, a doublet of 57 kDa (Fig. 4A,B). Western blotting of alternate fractions with anti-PTB antibodies confirmed

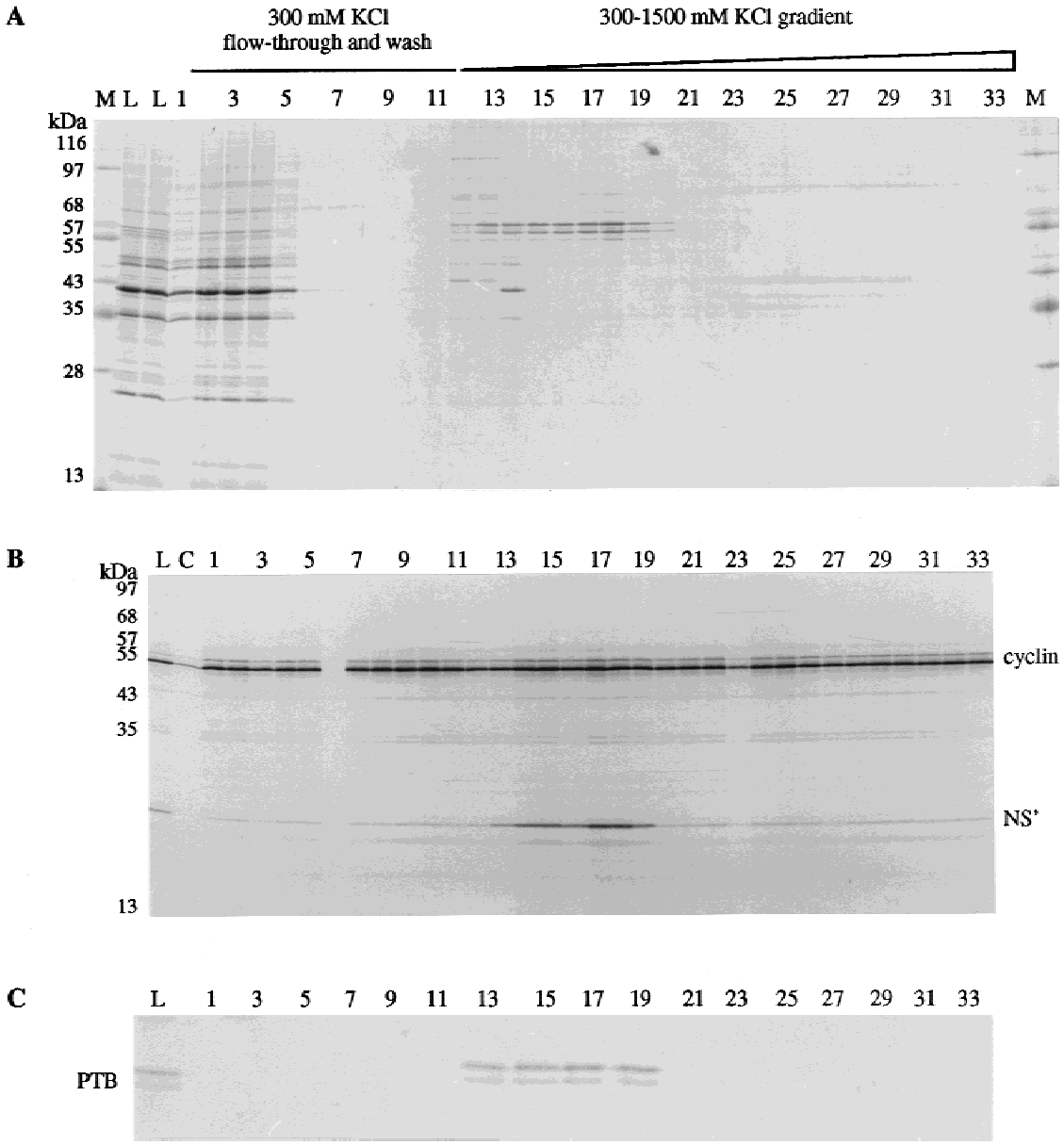


**FIGURE 4.** Purification of HeLa cell A-type activity on poly(U)-Sepharose. HeLa cell A-type activity was purified first on DEAE-Sepharose and then on heparin-Sepharose, and applied to a poly(U)-Sepharose column as described in Materials and Methods. **A**: Silver-stained SDS/15% polyacrylamide gel showing the column load (lane L), flow-through (fractions 1–5), 300 mM KCl wash (fractions 6–11), and salt-gradient eluate (fractions 12–33). Lane M: protein molecular weight markers (sizes indicated in kDa). **B**: Translation assays of uncapped XLJHRV10-611 mRNA in reticulocyte lysate supplemented with the column load (lane L), and fractions 1–33 (which had each been adjusted to 450 mM KCl) at 20% (v/v). A negative control assay without the addition of factors was also carried out (lane C). An autoradiogram of the translation products, resolved by SDS/20% polyacrylamide gel electrophoresis, is shown. **C**: Immunoblot of SDS/15% polyacrylamide gel of the load (lane L) and alternate (odd-numbered) fractions 1–33, probed with rabbit anti-PTB antibodies and detected using alkaline phosphatase-conjugated anti-rabbit secondary antibody.

that the activity precisely co-elutes with PTB and identified the silver-stainable doublet most likely as being PTB (Fig. 4C). The active fractions, however, were also shown by Western blot analysis to be devoid of La (data not shown).

To verify the identity of the A-type activity as PTB, we tested the effect of bacterially expressed recombinant PTB in the dicistronic mRNA translation assay (Fig. 5). His-PTB was found to specifically stimulate IRES-dependent translation, but to have no effect on translation of the scanning-dependent cistron. Maximal stimulation (an approximately fivefold increase in the absolute level of NS′ synthesized) was observed with 2–5 $\mu$g/mL His-PTB, and half-maximal stimulation with approximately 1 $\mu$g/mL.

It was considered important to verify that the specific stimulatory activity of the recombinant PTB was quantitatively comparable to that of the purified HeLa cell

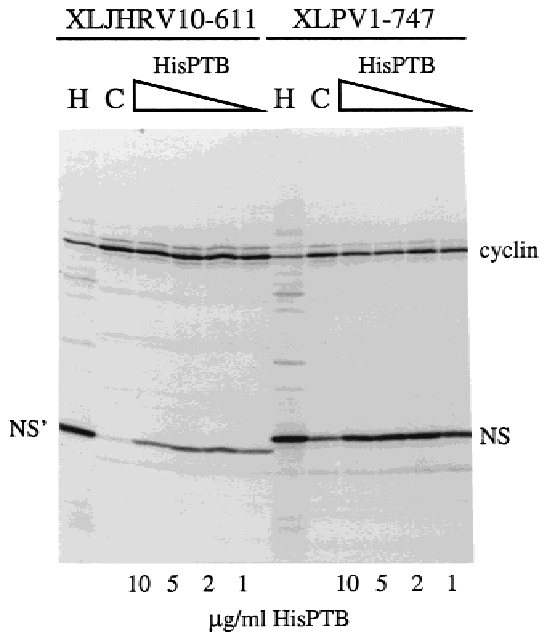


**FIGURE 5.** Recombinant PTB stimulates HRV and poliovirus IRES-dependent translation. Reticulocyte lysate translation assays, programmed with either 10 $\mu$g/mL uncapped XLJHRV10-611 mRNA or 10 $\mu$g/mL uncapped XLPV1-747 mRNA, were supplemented with 10, 5, 2, or 1 $\mu$g/mL recombinant His-PTB, as indicated below each lane. Negative control reactions with no addition of factors were carried out (lanes C), as were positive control reactions with the addition of 20% (v/v) HeLa HS S100 (lanes H). An autoradiogram of the translation products, analyzed by 20% SDS-polyacrylamide gel electrophoresis, is shown. The difference in size of the two NS-related products is because NS′ driven by the HRV IRES is a slightly truncated form of the full-length NS reading frame in the poliovirus construct, which also has a short linker between the initiation codon and the NS reading frame (see Materials and Methods).

PTB. For this purpose, it was necessary to estimate the concentration of PTB in the HeLa cell material at different stages of purification. We chose to do this using an assay based on UV crosslinking of PTB to the Theiler's murine encephalomyelitis virus (TMEV) (DA strain) IRES. PTB is almost exclusively the only HeLa cell protein labeled by crosslinking to this particular RNA (Kaminski et al., 1995), and this enabled us to estimate the endogenous content of PTB in any particular fraction by adding varying, but known, amounts of GST-PTB and carrying out crosslinking using a $^{32}$P-labeled TMEV IRES probe. Since the RNA probe was limiting under the conditions used for the assay, increasing amounts of GST-PTB resulted in increased labeling of this ~80-kDa protein at the expense of labeling of endogenous PTB (~57 kDa). The concentration of the endogenous PTB was thus estimated as the amount of GST-PTB that has to be added to give equivalent labeling of GST-PTB and endogenous PTB. In this way, we determined that recombinant His-PTB (and also GST-PTB, which is, incidentally, slightly less active than the His-tagged version because of the presence of the GST moiety), has a specific stimulatory activity very similar to that of the purified HeLa cell PTB from the heparin-Sepharose and poly(U)-Sepharose columns.

Using the same functional crosslinking assay, we also estimated the PTB concentration as 2–5 $\mu$g/mL in reticulocyte lysates (varying with the batch), 7–10 $\mu$g/mL in HeLa HS S100, and 20–30 $\mu$g/mL in HeLa nuclear extracts. Considering that the HeLa cell supplements are added to the translation assay at a concentration of 20% by volume, it is clear that recombinant PTB stimulates translation at quite low concentrations, likely to be physiologically relevant.

### Recombinant PTB acts synergistically with purified B-type activity in stimulating IRES-dependent translation

Although recombinant PTB mimics the activity of purified HeLa PTB in the dicistronic translation assay, there is still an appreciable difference in the maximal stimulation of downstream cistron yield achieved with these components compared with crude HeLa cell HS S100 extract (Fig. 5). Indeed, although the addition of HS S100 increases translation typically tenfold, purified HeLa cell PTB or recombinant PTB achieve a maximum of three- to fivefold stimulation. This suggests that at least part of the activity observed in crude HeLa preparations is attributable to some other component. The obvious candidate for the putative missing component is the B-type activity. We therefore tested the combined effect of the B-type activity together with the A-type/PTB. Translation assays were supplemented with either a saturating concentration (10 $\mu$g/mL) of recombinant PTB or purified HeLa cell PTB, or near-saturating levels of purified HeLa cell B-type activity, or a combination of both (Fig. 6). The results show that the two types of factor have a greater stimulatory effect together than either component alone, and in fact their combined effect is synergistic. In repeat assays of this type of experiment, we found that on occasion the combined effects of the two activities were additive rather than synergistic. Although we are unable to explain the variability between experiments, we can conclude the two active entities are not functionally redundant, and do not fulfill the same role.

### PTB also stimulates poliovirus IRES-dependent translation

The effects of recombinant PTB on poliovirus IRES-dependent translation were also tested (Fig. 5). A dicistronic RNA (XLPV1-747), similar to the rhinovirus dicistronic construct XLJHRV10-611 but containing the poliovirus type 1 (Mahoney) IRES in the intercistronic space and coding for a slightly longer form of the NS protein (see Materials and Methods), was translated in reticulocyte lysate in the presence or absence of re-

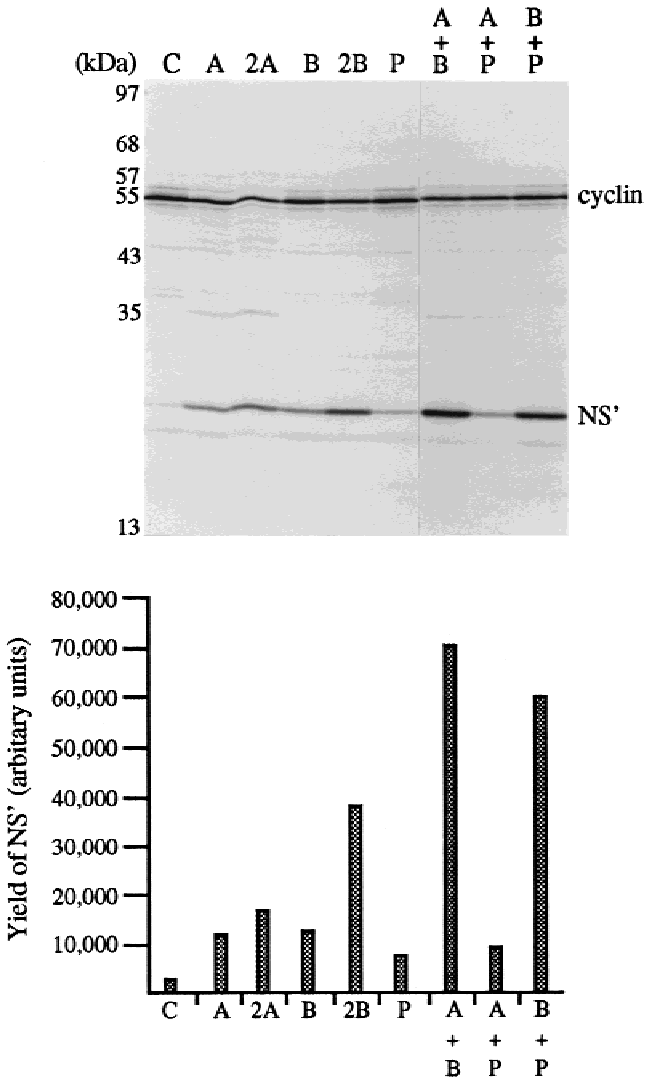


**FIGURE 6.** The combined effect of the HeLa A-type and B-type activities is at least additive. Translation assays of the uncapped mRNA XLJHRV10-611 were supplemented with A- or B-type activities from HeLa cell nuclear extract fractionated by DEAE-Sepharose (as described in Materials and Methods), and/or recombinant PTB. Additions were made as follows: lanes (A) and (2A): 10 and 20% (v/v) purified HeLa cell A-type activity, respectively; lanes (B) and (2B): 10 and 20% (v/v) partially purified HeLa cell B-type activity, respectively; lane (P): 10 $\mu$g/mL GST-PTB; lane (A+B): 10% (v/v) A-type activity and 10% (v/v) B-type activity ; lane (A+P): 10% (v/v) A-type activity and 10 $\mu$g/mL GST-PTB; lane (B+P): 10% (v/v) B-type activity and 10 $\mu$g/mL GST-PTB. A negative control lane with no factors added is indicated (lane C). Upper panel: an autoradiogram of the translation products, resolved by SDS/20% polyacrylamide gel electrophoresis. The sizes of protein molecular weight markers (in kDa) is indicated in the left margin. Lower panel: the yield of NS′ was quantitated by scanning densitometry of the autoradiogram shown in the upper panel, and is given in arbitrary scanner units.

combinant His-PTB. Although synthesis of the downstream cistron product in unsupplemented reticulocyte lysate is very much greater when translation is driven by the poliovirus IRES rather than the HRV-2 IRES, nevertheless the addition of His-PTB stimulated poliovirus IRES-dependent translation approximately twofold (Fig. 5).

### PTB also stimulates translation of HRV-2 and poliovirus genomic RNAs

A recent publication from this laboratory showed that the PTB-dependence of the encephalomyocarditis virus (EMCV) IRES is quite profoundly influenced by the nature of the downstream reporter cistron; the activity of the EMCV IRES was highly dependent on PTB if a heterologous reporter was used, but became largely PTB-independent if viral coding sequences served as the reporter (Kaminski & Jackson, 1998). We were therefore concerned as to whether the high PTB-dependence we have observed for the HRV IRES might likewise be influenced by the nature of the reporter sequences. To address this issue, the response to PTB of RNAs bearing authentic viral coding sequences was tested. The transcripts used for these experiments were derivatives of the rhinovirus and poliovirus genomic RNAs that were truncated at the 3′-end by linearization of the respective plasmid templates prior to transcription with *Nde*I. The HRV-2/*Nde*I transcript encodes nt 1–3603 of the HRV-2 genome and the T71/*Nde*I transcript encodes nt 1–3381 of poliovirus type I (Mahoney). Since the truncated polyprotein product does not undergo extensive proteolytic processing (apart from the rhinovirus HRV-2/*Nde*I translation product, where autocleavage of the C-terminal 2A peptide from the P1 capsid precursor does take place), the results of these assays are more easily interpreted than equivalent assays with the full-length viral RNAs (which are also complicated by the synthesis of aberrant products from incorrect initiation sites). Translation of both these monocistronic RNAs was stimulated by supplementing the lysate with recombinant PTB (Fig. 7). This provides reassuring evidence that our results demonstrating a PTB-dependence for HRV and poliovirus IRES-dependent translation are not peculiarities of the laboratory-generated dicistronic mRNA used in the assay system.

### Neither activity corresponds to poly(C) binding protein 2 (PCBP-2), nor can PCBP-2 functionally substitute for either

While this work was in progress, it was reported that poly(C) binding protein 2 (PCBP-2) plays an important role in internal initiation directed by the poliovirus IRES (Blyn et al., 1996, 1997). In addition, Gamarnik and Andino (1997) reported that both PCBP-1 and PCBP-2 bind to the poliovirus IRES, and antibodies specific to either protein inhibited poliovirus RNA translation in *Xenopus* oocytes injected with HeLa cell cytoplasmic extract. In view of the similar structures of the poliovirus and rhinovirus IRESes and the quite close phylogenetic relationship between the viruses, these findings raise the question of whether either HeLa cell activity corresponds to PCBP-2 (or PCBP-1). Western blotting, using

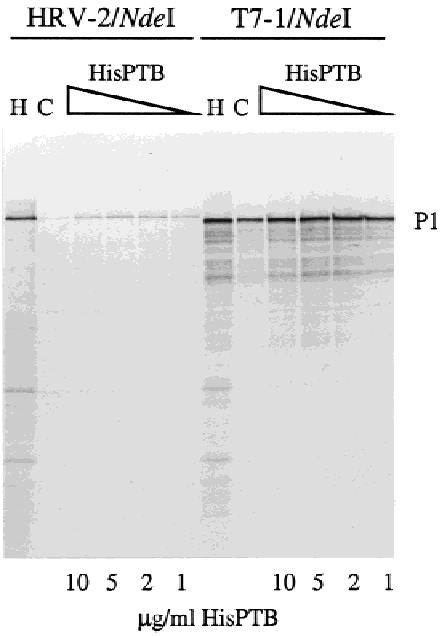


**FIGURE 7.** The reporter coding sequence does not alter the PTB-dependence of HRV and poliovirus IRES-dependent translation. Reticulocyte lysate translation assays, programmed with either 10 $\mu$g/mL uncapped HRV-2/*Nde*I mRNA or 10 $\mu$g/mL uncapped T7-1/*Nde*I mRNA, were supplemented with 10, 5, 2, or 1 $\mu$g/mL recombinant His-PTB, as indicated below each lane. Negative control reactions were supplemented with H100 buffer (lanes C), and positive control reactions with 20% (v/v) HeLa HS S100 (lanes H). An autoradiogram of the translation products, analyzed by 20% SDS-polyacrylamide gel electrophoresis, is shown.

an antiserum that recognizes both PCBPs, showed, not unexpectedly, that the purified A-type activity is devoid of PCBP (data not shown), and we will show elsewhere that the purified B-type activity is not PCBP (S.L. Hunt, J.J. Hsuan, N. Totty & R.J. Jackson, in prep.). In fact, Western blotting across column fractions shows that the B-type activity separates from PCBP-1 and PCBP-2 quite early in the purification; there was no stimulatory activity associated with the fractions enriched in PCBPs (data not shown). This is confirmed by the addition of recombinant proteins to reticulocyte lysate translation assays (Fig. 8A). In the case of the dicistronic mRNA with the HRV IRES, addition of recombinant PCBP-2 on its own failed to stimulate IRES-dependent translation over the buffer control, and did not significantly augment the stimulation caused by PTB. A similar negative result of no stimulation by PCBP-2 on its own was seen using the construct with the poliovirus IRES, but in this case there was a distinct synergy between PTB and PCBP-2 (Fig. 8A).

To verify that the recombinant PCBP-2 used in these experiments is indeed active in supporting IRES-dependent translation, the experiment shown in Figure 8B was performed, in which the dicistronic mRNA with the poliovirus IRES was translated in reticulocyte lysate supplemented with HeLa HS S100. A strong inhibition of translation specifically of the IRES-dependent cistron was seen on addition of the stem-loop structure with the high affinity PCBP-2 binding site, stem-loop IV in the nomenclature used by Blyn et al. (1996, 1997). This specific inhibition could be completely reversed by addition of recombinant PCBP-2, with absolute specificity in that the efficiency of translation of the upstream cistron was unaffected (Fig. 8B). A similar inhibition, which could be reversed by recombinant PCBP-2, was seen when the system was programmed with the dicistronic mRNA with the HRV IRES (data not shown).

The failure of recombinant PCBP-2 to stimulate the activity of the poliovirus (or HRV) IRES (Fig. 8A), unless steps are taken to effectively deplete the system of PCBP-2 by adding stem-loop IV (Figure 8B), implies that the reticulocyte lysate contains sufficient PCBP-2 to be not limiting. This was confirmed by Western blotting with anti-(human)PCBP-2 antibodies (which also recognize PCBP-1). This showed two cross-reacting proteins of 43–45 kDa in both HeLa cell extracts and reticulocyte lysates. The ratio of the signals given by the two bands differs between HeLa and reticulocyte extracts; the species of lower apparent molecular mass predominates in reticulocyte lysates, and the larger form in HeLa cell extracts. However the combined signals of the two bands is at least as great for reticulocyte lysates as HeLa cell extracts (data not shown).

From these results we conclude that neither of the two HeLa cell activities that we detect by our translation assay corresponds to PCBP-1 or PCBP-2. Furthermore PCBP-2 cannot functionally substitute for either activity.

## DISCUSSION

We have shown that when HeLa cell extracts are fractionated to purify the factors that stimulate rhinovirus IRES activity in the rabbit reticulocyte lysates, two separable activities are obtained. Each of these is individually active in stimulating IRES-dependent translation, and their combined effect is at least additive, and on some occasions synergistic. We have identified one of the two HeLa cell factors as PTB. We will describe elsewhere the purification, characterization and cloning of the components of the B-type activity, and the expression and assay of the recombinant proteins. Suffice it to state here that the B-type activity corresponds to none of the proteins previously associated with picornavirus IRES activity: PTB, the autoantigen La (Meerovitch et al., 1993; Svitkin et al., 1994), poly(C) binding protein 2 (Blyn et al., 1996, 1997), eIF2/2B complex (Svitkin et al., 1988), or the C-terminal cleavage product of initiation factor eIF4G, which has been shown to stimulate translation dependent on entero- and rhinovirus IRESes (Ohlmann et al., 1995, 1996; Ziegler et al., 1995; Borman et al., 1997).

Although it may seem surprising in view of the apparently widespread assumptions about the involve-

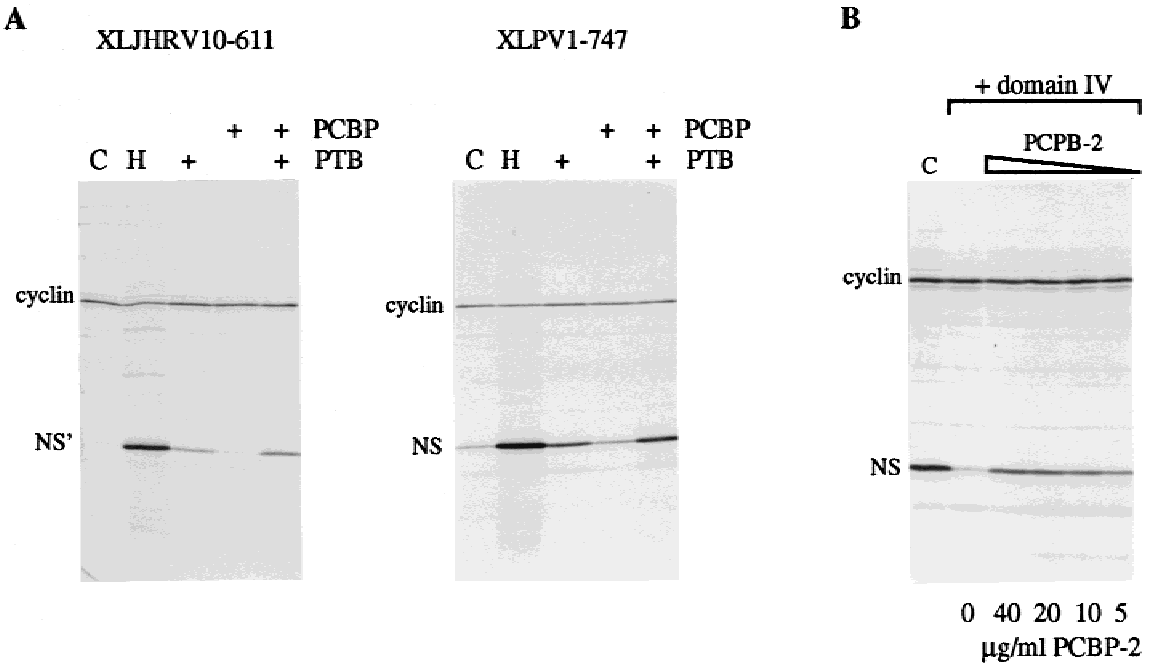


**FIGURE 8.** The effect of recombinant PCBP-2 on HRV IRES activity. **A**: Translation assays were programmed with either 10 $\mu$g/mL uncapped XLJHRV10-611 or uncapped XLJPV1-747 dicistronic mRNA and supplemented with either 10 $\mu$g/mL His-PTB, 20 $\mu$g/mL PCBP-2, or a combination of both, as indicated above each lane. Negative control reactions with no added factors (lanes C) and positive control reactions with 20% (v/v) HeLa cell HS S100 extract added (lanes H) were also carried out. **B**: Uncapped dicistronic mRNA XLJPV1-747 was translated in rabbit reticulocyte lysate supplemented with 20% (v/v) HeLa cell HS S100 extract. HRV-2 5′-UTR Domain IV competitor RNA was added at a 30-fold molar excess (relative to mRNA concentration), and then the assays were supplemented with either 40, 20, 10, 5, or 0 $\mu$g/mL recombinant His-PCBP-2, as indicated below each lane. A control reaction with no competitor RNA added is shown (lane C).

ment of PTB in internal initiation of picornavirus RNA translation, this is actually the first direct demonstration that PTB is required for the function of entero- and rhinovirus IRESes, in the sense that it is the first report showing that addition of purified PTB, whether recombinant or from mammalian sources, stimulates in vitro translation dependent on these IRESes. The prevailing assumptions have been based in part on the fact that PTB binds to and can be crosslinked by UV irradiation to the poliovirus IRES (Hellen et al., 1994), but our recent work on the role of PTB in cardiovirus IRES function provides a strong warning against drawing the conclusion that proteins that bind to an IRES must necessarily be required for the function of that IRES (Kaminski & Jackson, 1998). As for more direct evidence, it is true that when HeLa cell extracts were immunodepleted of PTB, the capacity for translation dependent on the poliovirus IRES was lost, but the failure to rescue this activity by addition of recombinant PTB left in some doubt whether it was actually PTB that was the required entity (Hellen et al., 1993). It has also been shown that poliovirus IRES activity is inhibited by addition of that part of the EMCV IRES that encompasses the high affinity PTB binding site, and was not inhibited by mutants of this EMCV structure that had lost PTB binding (Pestova et al., 1991), but here again the lack of an add-back experiment left open whether it was PTB itself that was important.

Because of these uncertainties surrounding earlier experiments, we have attached great importance to verifying whether the A-type activity is due to PTB itself or to a complex of PTB with other proteins. Our results point unambiguously to the active entity being singular PTB. Not only does the activity copurify with PTB itself, but, more importantly, human recombinant PTB exhibits a similar specific activity in stimulating IRES-dependent translation, as does the purified HeLa protein. Moreover, the concentrations of PTB sufficient to achieve this effect encourages our belief that our results are of physiological significance. In assays supplemented with ~10 nM dicistronic mRNA, half-maximal stimulation is seen with ~1 $\mu$g/mL HisPTB (approximately 20 nM), and maximal stimulation was seen with 5 $\mu$g/mL HisPTB (approximately 100 nM). These numbers are of the order expected for typical RNA-binding proteins with dissociation constants in the 1–10 nM range. Taking into account the concentrations of PTB measured for HeLa cell cytoplasmic extracts (7–10 $\mu$g/mL), one has reasonable confidence that it is the PTB in these crude extracts (together with the B-type activity) that is responsible for the observed stimulation of IRES-dependent translation.

Neither activity copurifies with the autoantigen La, which may be surprising in view of the fact that the addition of recombinant La to rabbit reticulocyte lysates stimulates translation of poliovirus RNA (Meero-

vitch et al., 1993; Svitkin et al., 1994). We believe that one reason why our assays failed to pick up La as a stimulatory factor is that the concentration of La in HeLa cell extracts is not sufficiently great in relation to the very high concentrations required to stimulate poliovirus IRES activity in vitro. The amount of recombinant La required to effect stimulation (100 $\mu$g/mL, or 2 $\mu$M) is approximately 100-fold molar excess over the RNA, which is not consistent with the reported binding constant for La of $\sim$5 nM. In a collaborative study, Y.V. Svitkin and N. Sonenberg (pers. comm.) observed that addition of La to rabbit reticulocyte lysates programmed with our dicistronic test mRNA (XLHRV10-611) in an uncapped form caused a very strong inhibition of upstream (cyclin) cistron translation, but absolutely no increase in the very low yield of IRES-dependent cistron product. The outcome therefore was an increase in the ratio of yield of downstream cistron product relative to the yield of the upstream one, and so if this ratio is used as a measure of IRES activity, it could be claimed that La caused an increase in such activity. However, given that the increase in yield ratio was entirely due to the dramatic decrease in cyclin yield, we do not believe that such an interpretation is valid. Interestingly, when the experiment was repeated using an analogous construct with the poliovirus IRES as intercistronic spacer, La again caused a drastic decrease in the yield of cyclin, but in this case there was a small but perceptible increase in the yield of the downstream cistron product. Thus, irrespective of the question of whether the influence of La on picornavirus IRES function is of physiological significance, it seems as though its impact on in vitro translation is not precisely the same for entero- as opposed to rhinovirus IRESes. This may seem surprising in view of the close phylogenetic relationship between these viruses and the similarity in their IRES structures, but in fact a similar difference in impact appears to hold for the B-type activity that has a much greater influence on the activity of the rhinovirus than the poliovirus IRES (Hunt et al., in prep.).

The results described here raise two further questions. (1) If the contribution of rabbit reticulocyte lysate PTB to the assay is 1–3 $\mu$g/mL, why does supplementation with only 1–5 $\mu$g/mL recombinant PTB or 20% (by volume) of HeLa cell HS S100 containing 7–10 $\mu$g/mL PTB and thus contributing only 1.4–2 $\mu$g/mL PTB to the final assay system elicit a significant stimulation of IRES-dependent translation? (2) How is it that there are two separable HeLa cell factors, each of which is individually active in stimulating IRES-dependent translation, yet which act at least additively and in some assays synergistically when both are added? In addition, there is a third question which we believe is related certainly to the second and perhaps to both of the above two: what is the explanation of the paradoxical dose response whereby as RNA concentrations are increased there comes a point where the yield of IRES-dependent translation products does not just reach a plateau but actually starts to decrease quite sharply (Fig. 1)? We will propose a model which we believe to be a viable explanation that embraces both of the second and third of these questions and offers at least a partial answer to the first.

Nevertheless, it seems appropriate first to examine the most obvious alternative explanation for the existence of two separable and individually active HeLa cell factors, namely, that these are two functionally redundant proteins which each carry out the same role. However, this argument does not explain the observation that when the rabbit reticulocyte lysate has been supplemented with saturating concentrations of recombinant PTB (i.e., A-type activity), the addition of purified B-type activity elicits a further stimulation of IRES-dependent translation that is at least additive to the stimulation effected by recombinant PTB, and is probably somewhat synergistic (Fig. 6).

As for the paradoxical dose-response, this has been observed before in many different laboratories. It is very obvious in Figure 1 of Svitkin et al. (1994) for poliovirus virion RNA translation in rabbit reticulocyte lysates with or without supplementation with a high concentration of recombinant La, and has been observed in assays of poliovirus virion RNA and in vitro transcripts of full-length poliovirus cDNAs carried out in this laboratory (Jackson, 1989). It was also very evident when a dicistronic mRNA (similar to the ones used in our work) with the echovirus type 25-IRES was translated in rabbit reticulocyte lysate supplemented with HeLa cell cytoplasmic extract, and, significantly, it was shown in this case that the more HeLa cell extract that is added, the greater is the critical RNA concentration allowable before the downturn in IRES activity is evident (Fig. 5 of Bailly et al., 1996). Competition between the two cistrons for limiting components cannot be the full explanation for such an anomalous dose response (1) because in our case as well as well as Bailly et al. (1996), the decrease in yield of the IRES-dependent product (NS) is larger than any increase in the yield of the upstream cistron product, cyclin (Fig. 1), and (2) because it would be necessary to invoke a change in the competitive parameters with changing RNA concentration.

Our preferred model, which explains not only this paradoxical dose-response but also accounts for the existence of two distinct HeLa cell factors, is depicted in Figure 9. The essential postulate of the model is that the activity of the HRV or enterovirus IRESes depends on two cellular proteins, designated as A/a and B/b (equivalent to what we have previously referred to as the A-type and B-type activities), which bind to the entero-/rhinovirus IRES in a noncooperative or only weakly cooperative manner. A further postulate is that rabbit reticulocyte lysates contain both factors but in

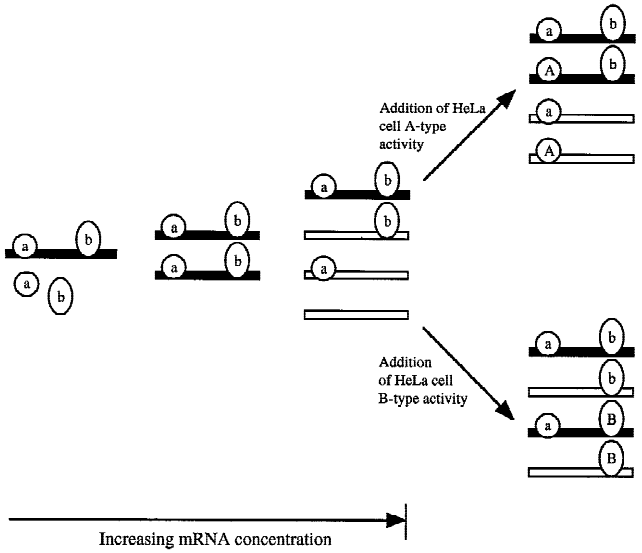


**FIGURE 9.** A model for *trans*-acting factor binding to the HRV-2 IRES. HeLa cell stimulatory activities are designated A and B. The model assumes that the binding of both factors, the A- and B-type activities, to the IRES is required to support internal initiation, and that the binding of these factors is noncooperative. It is further assumed that reticulocyte lysates contain both types of factor (designated a and b), but at lower concentrations than in HeLa cell extracts. Active IRES elements (those bound to both types of factor and therefore competent for translation initiation) are represented by solid black bars, and inactive IRESes (those bound to only one or neither of these factors) by open bars. Further explanation is given in the text.

rather limited amounts. This postulate is certainly true of the A-type activity (PTB). It remains to be proven in respect of the B-type activity, although in UV-crosslinking assays using $^{32}$P-labeled HRV 5′-UTR in unsupplemented rabbit reticulocyte lysate, we have observed weak labeling of a protein of 97 kDa, which is resistant to nonspecific competition by heparin just as is the case with the crosslinkable 97-kDa protein that copurifies with HeLa cell B-type activity.

We will designate the endogenous reticulocyte factors by lower case a and b, and the HeLa cell equivalents by upper case letters. For simplicity we will assume that the effective endogenous levels of a in the reticulocyte lysate is the same as that of b. When very low concentrations of RNA are added to unsupplemented rabbit reticulocyte lysate, we reason that the two endogenous reticulocyte factors are present in sufficient excess that virtually all the input RNA will bind both a and b, and thus the IRESes will be functional (Fig. 9). As the RNA concentration is increased, there will come a point where it equals the endogenous level of a or b. If the binding of the two factors is noncooperative (or only weakly cooperative), further increases in RNA concentration above this equivalence point will result in a decrease in the absolute number (not just the relative proportion) of IRESes that bind both factors simultaneously, and thus there will be an absolute decrease in the yield of IRES-dependent translation product, consistent with the anomalous dose-responses noted above. In fact the model predicts that if the RNA concentration is raised to twice the critical level that allows maximum IRES activity, the absolute yield of IRES-dependent product should decrease to half this maximum, and for every further doubling the yield will decrease by a further half. This prediction is fairly close to the actual observations in Figure 1 of Svitkin et al. (1994) and Figure 5 of Bailly et al. (1996), though, if anything, the observed decrease in IRES activity with increasing RNA concentration is even more acute than the expectation.

If, at any RNA concentration above the critical level that allows maximum IRES activity in an unsupplemented assay, we add *either* HeLa cell A-type activity *or* HeLa cell B-type individually we would expect to see a stimulation of IRES-dependent translation in both cases, as the added HeLa cell factor binds to those IRESes to which only one of the endogenous reticulocyte factors, b or a, respectively, was bound (Fig. 9). Addition of both HeLa cell activities together should produce a stimulation that is at least additive (if the amount of HeLa cell A and B added is just sufficient to complement all the endogenous reticulocyte a and b), or apparently synergistic if the two HeLa cell factors are added in excess.

This model thus accounts for the anomalous dose-response and for the fact that there are two separable HeLa cell activities, each of which is individually active on its own. To a certain extent it can also account for the fact that addition of a relatively small amount of PTB (in relation to endogenous levels present in the rabbit reticulocyte lysate) can elicit a detectable stimulation; IRESes that have bound only reticulocyte B-type activity but not reticulocyte A-type (PTB) can be activated by addition of HeLa cell PTB. There is, however, a problem over the quantitative aspects of the stimulation. According to the model, addition of a quantity of HeLa cell or recombinant PTB that only doubles the total PTB in the system (without changing the concentration of B/b) cannot achieve more than a doubling of the yield of downstream translation product. In fact the results shown here suggest that adding a saturating amount of PTB to the reticulocyte lysate assays does elicit rather more than a doubling in IRES activity.

There are two explanations that would account for this minor discrepancy. One possibility is that the interaction between PTB and the IRES that activates internal initiation might be more complex than a simple binary PTB/RNA interaction, and thus might result in a response that is more closely related to the square (or higher power) of PTB concentration. In this respect it is interesting to note that PTB may exist as a homodimer, and may bind to RNA as a dimer (Pérez et al., 1997b; Oh et al., 1998).

The other explanation is that the endogenous reticulocyte PTB might be much less active in promoting

HRV IRES-activity than the HeLa cell equivalent, either for reasons of species specificity or because the endogenous PTB is somehow in a form that is not available for supporting IRES activity. Neither of these latter hypotheses is particularly satisfactory. While some species specificity in terms of specific stimulatory activity would be plausible, there is no precedent for a general RNA-binding protein showing very stringent species specificity approaching an "all or none" scenario in which the rabbit reticulocyte PTB would be virtually inactive. It should be noted that the translation of poliovirus RNA (which has an IRES that is quite similar in primary sequence and very similar in predicted secondary structure to the human rhinovirus IRES) is stimulated by extracts of mouse L-cells (Pelletier et al., 1988) or mouse Krebs II ascites cells (Svitkin et al., 1988), though admittedly it remains to be proven that the stimulation effected by these mouse cell extracts was due to a combination of both A-type activity (i.e., PTB) and B-type activity, rather than just B-type activity alone.

Moreover, in respect to either the species specificity or the unavailability theories that are based on the premise that the endogenous reticulocyte PTB is unable to support HRV IRES activity, it is worth noting that such arguments would have to include the additional caveat that they specifically refer only to the IRESes of rhinoviruses (and probably enteroviruses), since the endogenous reticulocyte PTB is fully available and fully active in supporting the function of those cardiovirus IRES derivatives which require it (Kaminski & Jackson, 1998).

If PCBP-2 and/or PCBP-1 is also required for the activity of the HRV IRES, as is the case with the poliovirus IRES (Blyn et al., 1996, 1997; Gamarnik & Andino, 1997), then there may be as many as three different RNA binding proteins that support HRV IRES activity: PTB, PCBP, and the B-type activity (ignoring La for the moment). Thus the model depicted in Figure 9 is an oversimplification, and the true situation is too complex to allow quantitative predictions without more information on relative abundances and binding constants. Nevertheless the principles inherent in that model still hold: that there is an RNA concentration above which any further increase will result in a decrease in absolute yield of IRES-dependent translation product; and that at a suitable range of RNA concentrations, any one of the proteins can elicit a stimulation, even though they do not each carry out the same function.

## MATERIALS AND METHODS

### Plasmid constructs

cDNA constructs were cloned in pGEM or Bluescript-based vectors, in a positive orientation with respect to the T7 promoter such that transcription with bacteriophage T7 RNA polymerase generates sense transcripts. pXLJHRV10-611 has been described previously (Borman & Jackson, 1992). It encodes a dicistronic mRNA consisting of the *Xenopus laevis* cyclin B2 open reading frame, followed by nt 10–611 of the human rhinovirus-2 5′-untranslated region and a slightly truncated derivative (NS′) of the influenza virus NS1 protein coding sequence, and the complete NS 3′-UTR. The initiation codon of the NS′ reading frame occupies the same position relative to viral 5′-UTR sequences as does the viral polyprotein initiation codon. pXLPV1-747 is similar, except that it contains nt 1–747 of the poliovirus type 1 (Mahoney) 5′-UTR in the intercistronic space followed by a 34 nt polylinker spacer and the unadulterated NS1 open reading frame and 3′-UTR. Translation of the downstream cistron is initiated at the viral initiation codon, and gives rise to a product that is larger than that of the NS′ cistron of pXLJHRV10-611. Both pXLJHRV10-611 and pXLPV1-747 were linearized with *Eco*RI prior to transcription to generate the dicistronic mRNAs.

pJHRV10-605 encodes nt 10–605 of the human rhinovirus-2 5′-UTR, fused via a 5-nt polylinker, to the NS′ coding sequence (Borman & Jackson, 1992; Borman et al., 1993). (The polylinker sequence shares the sequence of nt 606–611 of the HRV-2 5′-UTR, save for a C-to-T substitution at position 607.) It was linearized with *Bam*HI prior to transcription to generate transcripts consisting of the HRV 5′-UTR (from nt 10) and the initiation codon but no other coding sequences, which were used for UV-crosslinking assays. Domain IV of the HRV-2 5′-UTR (nt 232–431) was subcloned by polymerase chain reaction amplification of the template pJHRV10-605, with sense primer 5′GGGG*GAATTC*CAAAGCGCCTA3′ (*Eco*RI site is shown in italics) and antisense primer 5′GGG G*GGATCC*CAAAGCGAGCA3′ (*Bam*HI site is shown in italics). The resulting PCR product was digested with *Eco*RI and *Bam*HI and subcloned into pGEM3Zf(+) (Promega) that had likewise been digested with *Eco*RI and *Bam*HI. The resulting plasmid was digested with *Bam*HI prior to transcription with T7 RNA polymerase to generate domain IV competitor RNA.

pHRV-2 (described previously by Skern et al., 1985) encodes a cDNA of the full-length human rhinovirus-2 genome. Transcripts comprising nt 1–3603 of the viral RNA were generated by linearizing pHRV-2 with *Nde*I and then transcribing with T7 RNA polymerase. These truncated versions of the HRV-2 genome encode the viral 5′-UTR, followed by the P1 region of the polyprotein coding sequence, 2A and part of 2B; the resulting 110-kDa primary translation product undergoes *cis*-cleavage of the 2A moiety from the C-terminal end. pT7-1 encodes a cDNA of the poliovirus type 1 (Mahoney) genome in pGEM1 (Ypma-Wong & Semler, 1987). pT7-1 was linearized with *Nde*I, then transcribed with T7 RNA polymerase, generating transcripts encoding nt 1–3381 of the poliovirus genome, translation of which gives rise to a single polypeptide comprising most of the P1 region.

All plasmids were propagated according to standard procedures (Sambrook et al., 1989).

### HeLa cell culture and extract preparation

Human HeLa S3 cells were grown in suspension to $5 \times 10^5$ cells/mL, then harvested and resuspended in 2 vol (with respect to packed cell volume) homogenization buffer (20 mM MOPS-KOH, pH 7.2, 10 mM KCl, 1.5 mM $Mg(OAc)_2$, 2 mM EGTA, and 2 mM DTT) containing 10 $\mu$g/mL soybean trypsin inhibitor, 2 $\mu$g/mL leupeptin, 2 $\mu$g/mL aprotonin, 0.2 mM phe-

nylmethylsulphonyl fluoride (PMSF). The cells were lysed with 15 strokes of a glass Dounce B homogenizer (Wheaton Instruments). Nuclei were pelleted by centrifugation for 15 min at 4,000 rpm in a Sorvall SS34 rotor (the supernatant from this step was used to prepare HeLa cytoplasmic S10 extracts, see below), resuspended in 0.5 vol (with respect to the nuclear pellet volume) low-salt buffer (20 mM HEPES-KOH, pH 7.9, 20 mM KCl, 1.5 mM $MgCl_2$, 25% glycerol, 2 mM DTT, and 0.2 mM PMSF), and then stirred continuously at 4 °C while 0.5 vol (also with respect to the nuclear pellet volume) high-salt buffer (20 mM HEPES-KOH, pH 7.9, 1.2 M KCl, 1.5 mM $MgCl_2$, 25% glycerol, 2 mM DTT, and 0.2 mM PMSF) was added dropwise. Stirring was continued for a further 30 min and then the extract was centrifuged for 30 min at 8,500 rpm in a Sorvall SS34 rotor. The supernatant, the nuclear extract, was dialyzed against H100 (20 mM HEPES-KOH, pH 7.5, and 100 mM KCl) for 5 h at 4 °C, clarified by centrifugation for 20 min at 8,500 rpm in a Sorvall SS34 rotor, and stored at −80 °C.

Meanwhile, the postnuclear cytoplasmic supernatant was centrifuged again for 30 min at 8,500 rpm in an SS34 rotor to generate the S10 extract. This was made 6 mM in $Mg(OAc)_2$, and then brought to 0.5 M KCl by the dropwise addition of 4 M KCl on ice. After 30 min incubation on ice, the extract was layered onto a 5-mL, 25% glycerol cushion in 20 mM MOPS-KOH, pH 7.2, 0.5 M KCl, 6 mM $Mg(OAc)_2$, 2 mM EGTA, and 2 mM DTT, and centrifuged for 3.5 h at 28,000 rpm (100,000 × *g*) in a Beckman SW40Ti rotor at 4 °C. The resulting high-salt S100 (HS S100) extract was dialyzed overnight at 4 °C against H100 and stored at −80 °C.

## Column chromatography

All chromatography was carried out at 4 °C using buffers containing 20 mM HEPES-KOH, pH 7.5, 2 mM DTT and KCl at the concentration specified (i.e., H100 contains 20 mM HEPES-KOH, pH 7.5, 2 mM DTT, plus 100 mM KCl). The salt concentration of each column fraction was determined by measuring its conductivity, and then all fractions were adjusted to the same KCl concentration by the addition of the appropriate volume of either H0 or H1000 buffer. Two microliters of this salt-adjusted material were then used for supplementation into translation or UV-crosslinking assays. Fractions were stored frozen at −80 °C between successive columns.

### *Partial purification of the A- and B-type activities*

The heparin-Sepharose column depicted in Figure 3 was prepared and run as follows: a 4-mL heparin-Sepharose column, equilibrated with H100, was loaded at 0.2 mL/min with ~18 mL HeLa HS S100 extract (~180 mg protein) using a Pharmacia FPLC system. The column was washed with 5 column volumes H100, and then eluted with a 32-mL 100–550 mM linear KCl gradient (in 20 mM HEPES-KOH, pH 7.5, 2 mM DTT) and finally with 6 mL H1000. The flow-through material and washes were collected in two aliquots, and the eluate in 1 mL fractions. The A- and B-type activities, as used in Figure 6, were purified by fractionation of HeLa cell nuclear extract on DEAE-Sepharose (Fast Flow) (Pharmacia Biotech). The A-type activity flowed through at 100 mM KCl, and the B-type activity was eluted with 200 mM KCl.

### *Purification of the A-type activity*

Twenty-three milliliters of HeLa cell nuclear extract (400 mg protein) were applied under gravity to a 5-mL DEAE-Sepharose (Fast Flow) column (Pharmacia) that had been equilibrated with H100. The flow-through material, containing the A-type activity, was then applied to a 4-mL heparin-Sepharose column, which was run essentially as described above using a Pharmacia FPLC system, except that elution was carried out using a 20-mL, 100–550-mM KCl linear gradient, followed by a 10-mL, 550–1,000-mM linear KCl gradient. Those fractions enriched in A-type activity (~330–460 mM KCl eluate) were pooled, diluted to 300 mM KCl with H0, and loaded, under gravity, onto a 1.5-mL poly(U)-Sepharose (Pharmacia) column that had been equilibrated with H300. This was washed with 10 column volumes H300 (20 mM HEPES-KOH, pH 7.5, 300 mM KCl, and 2 mM DTT) and sequentially eluted with 2.5 mL each of H550, H750, and H1000, collecting 0.5-mL fractions. The A-type activity was eluted with 550 mM KCl.

## In vitro transcription and translation assays

Uncapped mRNAs for use in translation assays were generated by in vitro transcription of the linearized plasmid template with T7 RNA polymerase, as described previously (Dasso & Jackson, 1989; Kaminski et al., 1990). Trace amounts of [$\alpha^{32}$P]UTP (PB10163, Amersham International) were included in the reaction to facilitate the quantitation of the RNA yield, as described previously (Dasso & Jackson, 1989). Translation assays were carried out in reticulocyte lysate that had been treated with micrococcal nuclease according to the protocol of Jackson and Hunt (1983). All reactions were carried out in 10-$\mu$L volumes containing 10 mM creatine phosphate, 0.5 mM $MgCl_2$, 0.1 mM each amino acid (with the exception of methionine), 5 $\mu$Ci [$^{35}$S]methionine (SJ1515, Amersham International), and 4 mM 2-aminopurine, and were incubated at 30 °C for 1.5 h. Assays were carried out at 90 mM added KCl (including any contribution by the column fractions being assayed) and at an mRNA concentration of 10 $\mu$g/mL, unless otherwise stated. Typically, reactions contained 55–75% (v/v) reticulocyte lysate, although the amount was adjusted to take into account the volume of the other assay components. For assaying HeLa cell extracts, column fractions or recombinant protein, these components were added, typically, at 20% (v/v). Translation products were analyzed by 20% polyacrylamide gel electrophoresis and autoradiography. Quantitation was carried out by densitometry of the autoradiogram using Phoretix software.

## UV-crosslinking assays

High-specific activity $^{32}$P-labeled RNA probes were synthesized in 10-$\mu$L reactions, essentially as described in Borman et al. (1993), but with the following modified conditions: 0.5 $\mu$g linearized plasmid DNA, 40 U T7 RNA polymerase, 50 U RNAguard (Pharmacia), 1.25 mM rATP, 1.25 mM rCTP, 1.25 mM rGTP, 45 $\mu$M rUTP, 50 $\mu$Ci [$\alpha^{32}$P]UTP (PB10163, Amersham International), 40 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, and 12.5 mM DTT. The yield of RNA was quantitated as described by Dasso and Jackson (1989). UV-crosslinking

reactions were carried out in 96-well microtiter plates and contained 10 mM HEPES-KOH, pH 7.2, 3 mM $MgCl_2$, 5% glycerol, and 1 mM DTT. Typically, 2 $\mu$L protein solution (either HeLa cell extract or column fractions) were preincubated in a 10 $\mu$L reaction at 30 °C for 10 min with 15 fmol (~200,000 cpm) $^{32}$P-labeled RNA probe in the presence of 0.1 mg/mL *Escherichia coli* rRNA competitor (Sigma) and at a KCl concentration of 90 mM. Heparin (a nonspecific competitor) was then added to a final concentration of 0.1 mg/mL, and incubation continued for a further 5 min. Reactions were subsequently placed on 3-cm deep ice in a Stratalinker XL-1500 (Stratagene) and irradiated with three doses of UV light at an energy setting of 0.8 J/cm$^2$ (2.4 J/cm$^2$ in total). Reactions were digested with 4 $\mu$g RNaseA and 0.8 U cobra venom ribonuclease VI (Pharmacia) at 37 °C for 30 min, and then analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

### Immunoblotting

Five-microliter aliquots of extract or column fractions were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Hybond-C nitrocellulose membrane (Amersham International) by electrophoresis in 192 mM glycine, 25 mM Tris base, 20% methanol, and 0.01% SDS for 2 h at 0.4 A using a Biorad transblot apparatus. The membrane was blocked by overnight incubation at 4 °C in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing 4% (w/v) nonfat dried milk powder. The membrane was then incubated for 2 h at room temperature with primary antibody diluted 1:5,000 in 2% (w/v) nonfat dried milk powder in TBST, washed extensively with TBST, and incubated with anti-rabbit alkaline-phosphatase coupled secondary antibody (Sigma) that had been diluted 1:5,000 in 2% (w/v) nonfat dried milk powder in TBST. Finally, the membrane was washed as before with TBST and the alkaline phosphatase detection reaction carried out as outlined in Harlow and Lane (1988).

### Overexpression of recombinant human PTB in *E. coli*

pET28aPTB (Pérez et al., 1997a) for overexpression of recombinant His-tagged human PTB was provided by J.G. Patton. His-tagged PTB was overexpressed in *E. coli* BL21(DE3) cells and purified using Ni-NTA Sepharose (Qiagen) in accordance with the supplier's recommendations. pGEX3XPTB (Patton et al., 1991), also provided by J.G. Patton, was used for the overexpression of GST-PTB fusion protein, which was purified as described by Smith and Corcoran (1990). The purified His-PTB and GST-PTB were extensively dialyzed against H100 buffer prior to supplementation into translation and UV-crosslinking assays. Recombinant His-tagged PCBP-2 was purified according to Parsley et al. (1997).

### Silver-staining protein gels

Protein samples were analyzed by SDS/polyacrylamide gel electrophoresis, the gel soaked overnight in 50% methanol (AR grade), washed for 30 min in double-distilled water, and then silver stained according to the method of Wray et al. (1981).

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