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Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA

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ABSTRACT

The requirement of PTB, polypyrimidine tract binding protein, for internal initiation of translation has been tested using an RNA affinity column to deplete rabbit reticulocyte lysates of PTB. The affinity column was prepared by coupling CNBr-activated Sepharose with the segment of the 5′-untranslated region of encephalomyocarditis virus (EMCV) RNA previously shown to bind PTB. Lysates passed through this column were devoid of PTB, and were incapable of internal initiation of translation dependent on the EMCV 5′-untranslated region, while retaining the capacity for translation dependent on ribosome scanning. Full activity for internal initiation was restored by the addition of recombinant PTB at the physiologically relevant concentration of about 5 μg/mL. When various PTB deletion mutants were tested, it was found that this activity required virtually the full-length protein. Thus, PTB is an essential protein for internal initiation promoted by the EMCV 5′-untranslated region. However, the PTB-depleted lysate retained the capacity for internal initiation promoted by the 5′-untranslated regions of another cardiovirus, Theiler's murine encephalomyelitis virus, and of the unrelated hepatitis C virus, and in neither case did addition of recombinant PTB stimulate internal initiation. Therefore, PTB is not a universal internal initiation factor that is indispensable in every case of internal ribosome entry.

Keywords: cardiovirus; hepatitis C virus; internal ribosome entry segment (IRES); RNA affinity column; translation initiation factors

INTRODUCTION

The translation of picornavirus RNAs is initiated not by the usual 5′ end-dependent ribosome scanning mechanism that is thought to be operative with the vast majority of cellular and capped viral mRNAs, but by a mechanism of direct internal ribosome entry dependent on a ~450-nt segment of the viral 5′-untranslated region (5′-UTR) generally known as the IRES, for "internal ribosome entry segment" (for reviews, see Jackson et al., 1990, 1994; Agol, 1991; Kaminski et al., 1994b). An important question in the field is the extent to which the two mechanisms of translation initiation are similar in their requirements for cellular translation initiation factors. The evidence so far points to the conclusion that the same set of canonical initiation factors are required for internal initiation as for scanning-dependent initiation (Staehelin et al., 1975; Thomas et al., 1991, 1992; Scheper et al., 1992; Pause et al., 1994), with the caveat that only in the case of internal initiation can the requirement for eIF-4 (formerly known as eIF-4F) be fully satisfied by eIF-4 cleaved in its eIF-4γ component (Thomas et al., 1992; Liebig et al., 1993), a proteolytic cleavage that occurs following infection by certain members of the picornavirus family.

However, this conclusion concerning the canonical initiation factors does not rule out the possibility that internal initiation may require additional *trans*-acting cellular proteins. A common approach to search for possible factors required specifically for internal initiation is to carry out UV-crosslinking assays with cellular extracts and ^{32}P-labeled picornavirus IRES probes. It has long been recognized that, by the criterion of UV-crosslinking, all picornavirus IRESes bind a 58–60-kDa protein doublet present in extracts of HeLa cells, Krebs II ascites cells, etc, and also, at somewhat lower concentrations, in rabbit reticulocyte lysates (Borovjagin

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et al., 1990, 1991; Jang & Wimmer, 1990; Luz & Beck, 1990, 1991; Pestova et al., 1991; Borman et al., 1993; Hellen et al., 1993). This protein doublet is labeled more intensely in such assays if the probe is an IRES of the cardio-/aphthovirus subgroup of picornaviridae, than with the entero-/rhinovirus IRESes. In due course and to general surprise, this protein doublet was identified as PTB (Borman et al., 1993; Hellen et al., 1993), polypyrimidine tract binding protein, a mammalian pre-mRNA splicing factor that was originally thought to activate 3′ splice site selection (Gil et al., 1991; Patton et al., 1991), but more likely functions as a negative regulator of splicing (Lin & Patton, 1995; Singh et al., 1995).

However, the mere fact that PTB binds to all picornavirus IRESes cannot be taken as evidence that it is an obligatory *trans*-acting factor for internal initiation. Evidence for a functional role for PTB has previously been somewhat equivocal. HeLa cell extracts depleted of PTB by immunological methods were defective in IRES-dependent translation, but retained activity for translation of mRNAs by the conventional 5′ end-dependent scanning mechanism (Hellen et al., 1993). However, IRES-dependent translation was not restored by addition of recombinant PTB, an observation that raises some doubt as to whether PTB itself is the active entity or whether the critical component was some other protein that is complexed with PTB.

We describe here the use of an RNA affinity column method to deplete rabbit reticulocyte lysates of PTB and, by this strategy, we provide the first direct evidence that PTB is essential for internal initiation promoted by the IRES of the cardiovirus, encephalomyocarditis virus (EMCV).

RESULTS

Specific inhibition of internal initiation by subfragments of EMCV IRES

We first used PCR to divide the EMCV 5′-UTR segment from the poly(C) tract to the authentic initiation codon into three cDNA subclones, which were inserted into pGEM-1 vector to allow in vitro transcripts to be generated. The boundaries of the subclones were chosen so as to maintain RNA secondary structure domains (Fig. 1) according to the generally accepted secondary structure of this part of the EMCV 5′-UTR (Pilipenko et al., 1989; Duke et al., 1992). Besides the individual domains, we also made cDNA constructs of neighboring pairs, Domains 1 + 2 and Domains 2 + 3, as well as all three domains.

Domain 1 includes, near its 3′ end, the stem-loop structure (at nt 420) that has been identified as the main PTB-binding site in the EMCV 5′-UTR (Jang & Wimmer, 1990; Borovjagin et al., 1991; Witherell et al., 1993) and in the related foot-and-mouth disease virus 5′-UTR (Luz & Beck, 1990, 1991). Significantly, the top of the loop includes the sequence UCUU, a tetranucleotide sequence that was present, within a more random pyrimidine-rich background, in the majority of sequences obtained in SELEX experiments with PTB (I. Perez & J.G. Patton, in prep.). It also includes sequences further upstream, which are not absolutely essential for internal initiation but enhance its efficiency (Jang & Wimmer, 1990; Borovjagin et al., 1991). These upstream sequences include a stem-loop at nt 360, which has been suggested to be a second binding site for PTB (Borovjagin et al., 1991), although other authors, while agreeing that there is a PTB-binding site in this region, concluded that the binding site was more complex and disperse than just the stem-loop at nt 360 (Witherell et al., 1993). In the related FMDV 5′-UTR, there is no equivalent of the stem-loop structure of the EMCV 5′-UTR at nt 360; binding of PTB to sequences upstream of the main binding site has not been detected (Luz & Beck, 1991), nor are these upstream sequences part of the functional IRES (Belsham & Brangwyn, 1990; Kühn et al., 1990).

Domain 2 lies entirely within the IRES, and includes a highly conserved complex stem-loop at the top of a long but less conserved and less highly base paired stem (Pilipenko et al., 1989; Jackson et al., 1994; Kaminski et al., 1994b). Domain 3 is a smaller stem-loop, quite highly conserved among cardioviruses and aphthoviruses (Pilipenko et al., 1989). It includes the pyrimidine-rich tract near the 3′ end of the IRES, and ends one residue after the authentic initiation site, the AUG at nt 834. In the case of the FMDV IRES, this region has been shown to bind PTB, presumably at the oligopyrimidine tract, because mutations in the tract decreased PTB binding (Luz & Beck, 1991). However, binding of PTB to this site was inhibited by physiologically relevant KCl concentrations (Luz & Beck, 1991), and, apart from one exception (Witherell et al., 1993), PTB binding to the corresponding region of the EMCV IRES has not been observed (Jang & Wimmer, 1990; Borovjagin et al., 1991).

In vitro transcripts of these three subclones were tested as specific inhibitors of internal initiation dependent on the intact EMCV IRES. For this assay, an uncapped dicistronic mRNA (also generated by in vitro transcription) was used in which translation of the upstream *Xenopus laevis* cyclin B2 cistron is by the usual 5′ end-dependent scanning mechanism, whereas translation of the downstream poliovirus 2A cistron is by internal initiation dependent on the EMCV IRES sequences that constitute the intercistronic spacer. In this system, selective and specific inhibition of IRES-dependent initiation would be shown if the yield of the 2A product decreased, but the yield of cyclin was unaffected. On a concentration basis, by far the most potent inhibitor was random poly(U,C), but, even though the translation of the downstream cistron was somewhat more

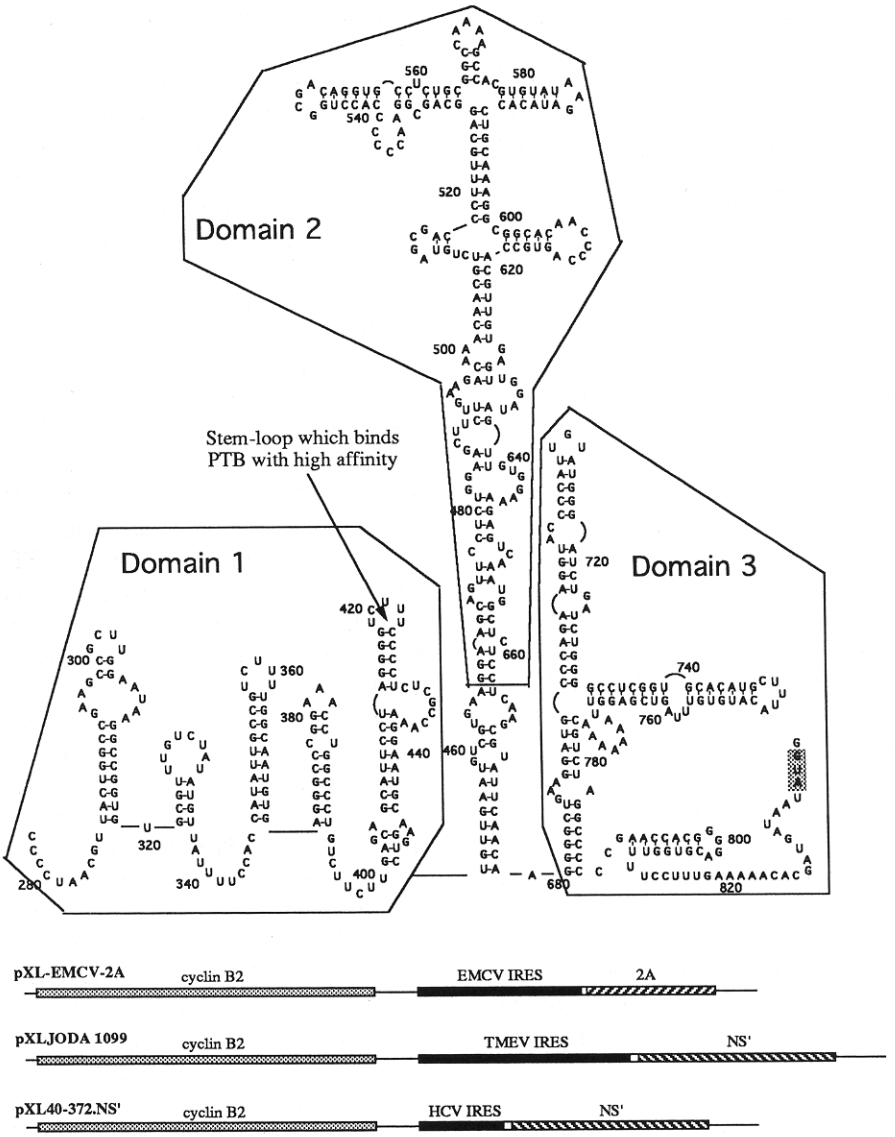

FIGURE 1. Domain structure of the EMCV 5′-UTR. The secondary structure downstream of the poly(C) tract, starting at nt 277 in the sequence of Duke et al. (1992) and extending to the first residue after the authentic initiation site (at nt 834), is shown based on a composite of the structures presented by Pilipenko et al. (1989) and Duke et al. (1992). The three subdomains cloned by PCR, as discussed in the text, are boxed. The arrow indicates the stem-loop that is the main high-affinity PTB-binding site as explained in the text. The lower part of the figure shows, approximately to scale, the dicistronic constructs used in this work. The upstream cyclin cistron coding region is stippled, and the downstream 2A or NS′ cistron coding region is crosshatched. Viral 5′-UTR segments that include the IRES elements are shown in black, and homologous viral coding sequences at the start of the downstream cistron are shown as open rectangles. Thin lines denote 5′- and 3′-UTR segments, which may include some polylinker sequences. Construction of pXLJODA 1099 is described in Hunt et al. (1993), pXL40-372.NS′ in Reynolds et al. (1995), and pXL-EMCV-2A in this paper.

sensitive to inhibition than the synthesis of cyclin, the window of selectivity was rather narrow, and cyclin synthesis was also inhibited at higher concentrations (Fig. 2). Poly(U) was a much less potent inhibitor, but was more selective for inhibition of specifically the downstream cistron translation (data not shown). The weaker inhibitory activity of poly(U) compared to poly (U,C) is consistent with the recently defined consensus PTB-binding site (Singh et al., 1995; I. Perez & J.G. Patton, in prep.).

The most selective inhibitor, however, was EMCV Domain 1, which reduced the yield of 2A from the downstream cistron, but actually increased the yield of cyclin (Fig. 2), probably as a consequence of the relief of competition between the two cistrons for limiting components of the translation machinery such as ribosomes or general initiation factors. The highest concentration tested (50 μg/mL, equivalent to a 27-fold molar excess of competitor over mRNA) almost completely abolished downstream cistron translation. By the same

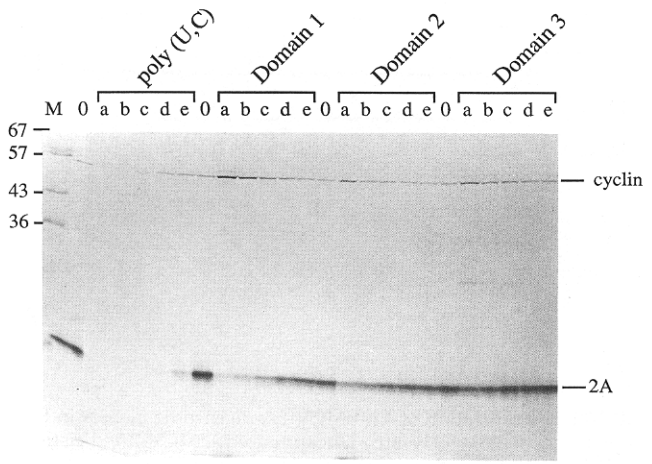

FIGURE 2. Specific inhibition of IRES-dependent translation by competitor RNAs. Uncapped XL-EMCV-2A dicistronic mRNA was translated at a final concentration of 25 μg/mL in the presence of either random poly (U,C), or uncapped EMCV Domain 1, Domain 2, or Domain 3 RNAs as potential inhibitors. Final concentrations (μg/mL) of inhibitory RNAs were: a, 50; b, 25; c, 12.5; d, 6.25; e, 3.13; and 0, none. Translation products were separated by gel electrophoresis and the resulting autoradiograph is shown. Track M was loaded with radioactive marker proteins of sizes as given in the left margin, and the positions of the cyclin (upstream cistron) and 2A (downstream cistron) products are shown in the right margin.

criteria, Domain 2 was a selective but much weaker inhibitor of internal initiation: 50 μg/mL of Domain 2 inhibited to about the same extent as 12.5 μg/mL Domain 1 RNA (Fig. 2). Domain 3, on the other hand, was virtually noninhibitory over this concentration range. Transcripts consisting of two or more contiguous domains, for example, Domains 1 + 2 or Domains 2 + 3, were no more inhibitory than the average of the two individual domains (data not shown).

Affinity column depletion of PTB

The above results suggested that EMCV Domain 1 could possibly be used in an affinity column approach to deplete the reticulocyte lysate of components required specifically for internal initiation. Accordingly, this RNA was coupled to an activated Sepharose matrix, namely cyanogen bromide-activated Sepharose. The RNA coupled to the column was ^{32}P-labeled at very low specific activity, to allow us to monitor leakage of the RNA from the column during its operation, but such leakage was found to be quite minimal. When samples of the flow-through lysate were counted, the recorded radiation was not significantly or consistently above background radiation. The column was operated under conditions approximating to the cell-free translation assays. Thus, the column was equilibrated in 100 mM KCl, 20 mM HEPES-KOH, pH 7.4, and the nuclease-treated lysate was made 100 mM in KCl and 0.5 mM in $MgCl_2$, before loading on the column. The standard procedure was to use a column of 0.5-mL bed volume, and to load 2–3 mL of lysate. The flow-through lysate was collected in individual fractions of 0.25 mL, and subsequently the column was eluted with KCl (up to 1.0 M), and/or SDS gel sample buffer.

The efficacy of the column to deplete the lysate of proteins that bind to the EMCV IRES was monitored by carrying out UV-crosslinking assays with the flow-through fractions and high specific activity ^{32}P-labeled EMCV Domain 1 (Fig. 3). These assays were conducted under relatively low stringency, using *Escherichia coli* rRNA as competitor but omitting the customary addition of the nonspecific heparin competitor. The results show that the crosslinkable PTB present in the lysate load was completely absent from the flow-through fractions. Crosslinkable PTB could be subsequently recovered from the column by elution with 1 M KCl, and a Coomassie-stained gel of this high salt eluate showed the presence of the typical 58–60-kDa PTB doublet (data not shown). If elution was made with SDS gel sample buffer, then the same Coomassie-stainable doublet of PTB was again observed. The conclusion is that the column has bound effectively all of the PTB in the load lysate, with the result that the flow-through fractions are essentially free of PTB. This conclusion was confirmed by SDS gel electrophoresis and subsequent western blotting of samples of the load lysate, the flow-through fractions, and the 1 M KCl or SDS gel sample buffer eluates (data not shown).

The possibility that PTB is actually present in the flow-through fractions, and that the apparent lack of

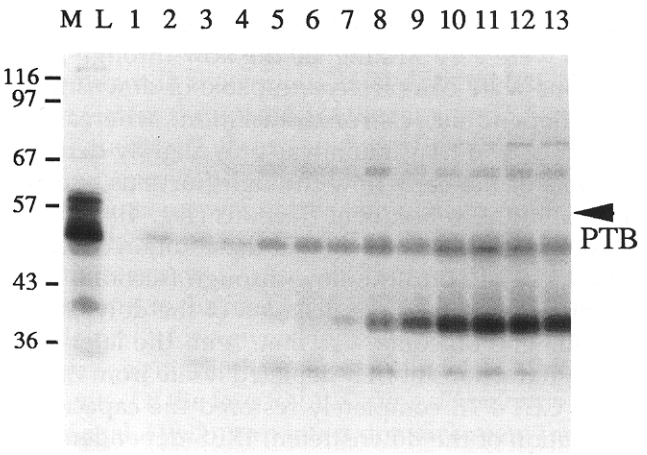

FIGURE 3. Proteins in normal and PTB-depleted reticulocyte lysate labeled by UV-crosslinking using a ^{32}P-labeled EMCV Domain 1 probe. Three milliliters of reticulocyte lysate was passed through a 0.5-mL affinity column and 13 fractions (0.25 mL) of flow-through lysate were collected. Each fraction and the column load lysate, L, was subjected to a UV-crosslinking reaction using a ^{32}P-labeled EMCV Domain 1 probe (Fig. 1). The labeled proteins were separated by gel electrophoresis, and the resulting autoradiograph is shown. Positions of radioactive marker proteins of the indicated sizes are given in the left margin, and the position of PTB in the right margin.

crosslinking to PTB in these fractions is due to the dilution of the high specific activity ^{32}P-labeled probe by very low specific activity ^{32}P-labeled EMCV Domain 1 RNA that has leaked from the column, can be discounted on two grounds. In the first place, as we show later (see Fig. 10), UV-crosslinking assays with ^{32}P-labeled EMCV IRES or Domain 1 and the column flow-through fractions supplemented with a low concentration of recombinant GST-PTB result in a strong signal of labeled GST-PTB. In addition, western blotting confirmed that PTB is truly absent from the flow-through fractions (data not shown).

The low stringency UV-crosslinking assays show that, whereas the PTB present in the load lysate is absent from all the lysate flow-through fractions, there are other proteins crosslinkable to EMCV Domain 1 that are absent from the very earliest flow-through fractions but are present in increasing concentrations in subsequent fractions, eventually, by fraction 6 of the flow-through, reaching the same apparent concentration as in the load lysate (Fig. 3). The possible significance of these proteins is discussed in the following section.

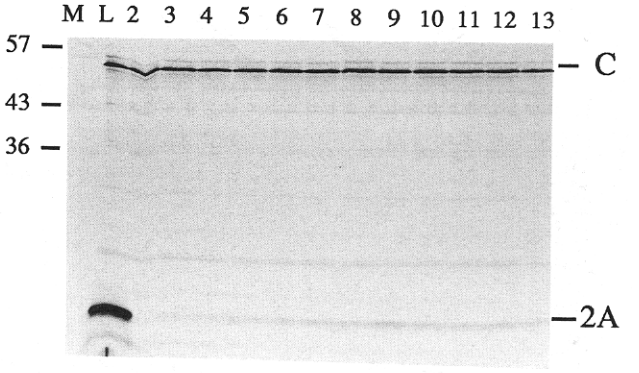

FIGURE 4. Translation of XL-EMCV-2A dicistronic mRNA in PTB-depleted reticulocyte lysate. Uncapped XL-EMCV-2A dicistronic mRNA was translated at a final concentration of 25 μg/mL in the column load lysate, L, and in the lysate flow-through fractions from the affinity column described in Figure 3. Translation products were separated by gel electrophoresis and the resulting autoradiograph is shown. Track M was loaded with radioactive marker proteins of sizes as given in the left margin, and the positions of the cyclin (upstream cistron) and 2A (downstream cistron) products are shown in the right margin as C and 2A, respectively.

Translation activity of the flow-through lysate

The flow-through fractions from the affinity column were tested for their translation activity using the same uncapped dicistronic mRNA as used for the inhibition studies described in the first section: an upstream cyclin B2 cistron translated by the normal 5′ end-dependent scanning mechanism, and a downstream poliovirus 2A cistron whose translation is by internal initiation dependent on the EMCV IRES element present as the intercistronic spacer. The results of these assays were very striking: all the flow-through lysate fractions were completely incapable of downstream, IRES-dependent, cistron translation, whereas upstream cistron translation was only slightly defective in some of the early flow-through fractions and was normal in the subsequent fractions (Fig. 4).

We therefore tested whether the addition of recombinant GST-PTB to the flow-through fractions could specifically rescue the translation of the downstream cistron. The outcome was that, with the later flow-through fractions of PTB-depleted lysate from the column, GST-PTB completely restored the capacity for translation of the downstream, IRES-dependent, cistron, with little effect on translation of the upstream cistron (Fig. 5). A dose-response assay showed that the translation of the downstream cistron was completely rescued by a final concentration slightly in excess of 5 μg/mL GST-PTB (Fig. 5). This corresponds to a concentration of about 70 nM, roughly threefold excess over the template RNA (2,880 nt), a ratio consistent with a K_d in the nanomolar range typical of specific RNA/protein interactions. We regard this to be a physiologically relevant concentration, i.e., close to the concentration of PTB in the load lysate. The concentration of PTB in load lysate was estimated by carrying out UV-crosslinking assays with this load lysate and a low concentration of high specific activity ^{32}P-labeled Theiler's murine encephalomyelitis virus (TMEV) IRES, or EMCV 5′-UTR, or Domain 1, together with varying concentrations of added GST-PTB. Because the concentration of labeled probe was near limiting, addition of increasing concentrations of GST-PTB resulted in increasing labeling of the 85-kDa GST-PTB at the expense of decreasing labeling of endogenous (58–60 kDa) reticulocyte PTB (data not shown, but the principle of the method can be seen in Figs. 7B and 10). By this method, the endogenous concentration of PTB in the load reticulocyte lysate can be estimated as either (1) the concentration of added GST-PTB required to reduce the labeling of the endogenous PTB by 50%, or (2) the concentration of added GST-PTB required to give equal labeling of the added 85-kDa GST-PTB and the endogenous 58–60-kDa PTB doublet. Both methods gave an estimate for the endogenous PTB concentration in the reticulocyte lysate load of 3–4 μg/mL (55–70 nM).

The data of Figure 5 demonstrate that, although translation of the downstream EMCV IRES-dependent cistron is fully restored by addition of GST-PTB to the later flow-through fractions (fraction 6 and subsequent fractions), fraction 2, the first fraction with a protein concentration close to that of the load lysate, shows absolutely no response to GST-PTB. Fractions of flow-through lysate following fraction 2 show a gradually increasing response, until the full response is reached by about fraction 6 (Fig. 5). This observation suggests that, apart from PTB, the affinity column is also capable

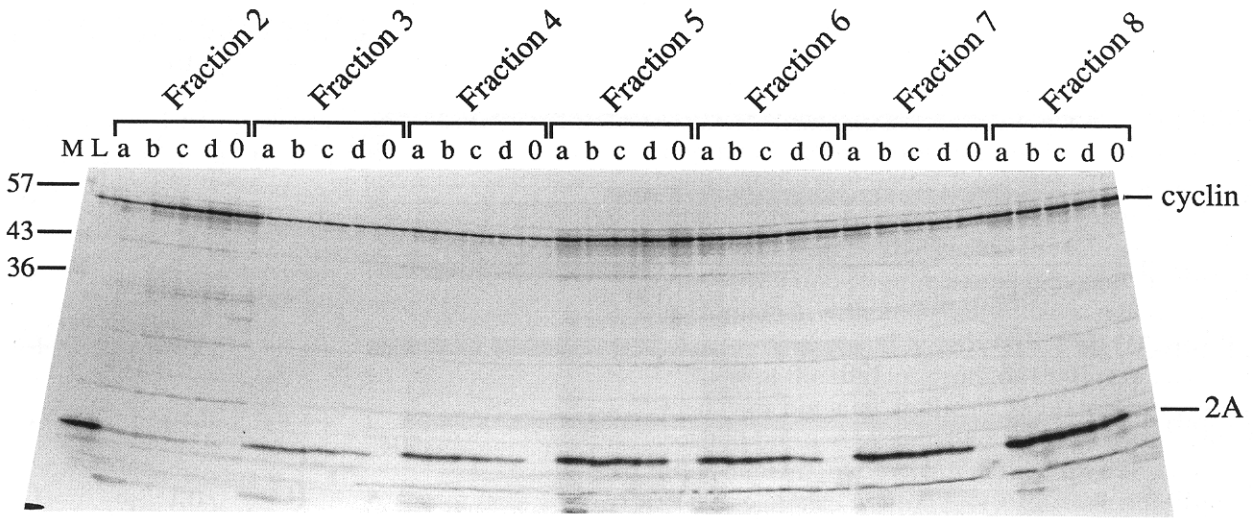

FIGURE 5. Effect of recombinant GST-PTB on translation of XL-EMCV-2A dicistronic mRNA in PTB-depleted lysates. PTB-depleted lysate was prepared as described in Figure 3, except that the volume of lysate loaded was 2 mL. Uncapped XL-EMCV-2A dicistronic mRNA was translated at a final concentration of 25 μg/mL in the column load lysate, L, and in the designated fractions of flow-through lysate. Recombinant GST-PTB was added to the translation assays at final concentrations (μg/mL) of: a, 20; b, 10; c, 5; d, 2.5; and 0, none. Translation products were separated by gel electrophoresis using a 10–30% polyacrylamide gradient gel (which explains the pronounced curvature of the dried gel), and the resulting autoradiograph is shown. Track M was loaded with radioactive marker proteins of sizes as given in the left margin, and the positions of the cyclin (upstream cistron) and 2A (downstream cistron) products are shown in the right margin.

of absorbing other reticulocyte lysate components that are essential for internal initiation driven by the EMCV IRES, but not absolutely essential for the 5′ end-dependent scanning mechanism, because even the early flow-through fractions are capable of translation of the upstream (cyclin) cistron. We do not know the nature of the other factor(s) necessary for IRES-dependent translation, but it is evident that any such factor(s) are easily displaced from the affinity column. The UV-crosslinking assays (Fig. 3), in fact, revealed several crosslinkable proteins that behave as predicted for such a factor: they are absent from the very first flow-through fractions, but gradually emerge in increasing concentrations until, by about fraction 6, they are present in the flow-through fractions at about the same concentration as in the load lysate (Fig. 3).

Experiments with truncated PTBs

The experiments described in the previous section provide a functional assay for PTB activity, indeed the only functional assay specifically for PTB activity in isolation, for, although PTB was originally thought to activate 3′ splice site selection (Gil et al., 1991; Patton et al., 1991), it is now considered more likely to function as a negative regulator of splicing by competition with constitutive splicing factors (Lin & Patton, 1995; Singh et al., 1995).

It was therefore of obvious interest to test various PTB mutants for activity in promoting IRES-dependent translation in the PTB-depleted reticulocyte lysate. These mutant PTBs were all deletion mutants, and were produced as GST-fusion proteins (designated GST-ptb in contrast to the full-length GST-PTB fusion protein) by overexpression in *E. coli*, exactly in the same way as the active full-length GST-PTB used in the experiments described above. The deletions in each of the mutants are depicted in Figure 6, which shows the positions of the four RNA-binding domains that have been identified in the protein sequence (Ghetti et al., 1992) and also summarizes the results obtained with each mutant in the various assays attempted.

One test for the function of the mutant GST-ptb proteins was a direct UV crosslinking of the purified protein with high specific activity ^{32}P-labeled EMCV Domain 1, and the outcome was scored by the degree of labeling of the mutant protein (Figs. 6, 7A). Two trends emerged from these assays: (1) the labeling was approximately proportional to the fraction of the full-length protein retained; but (2) for a given length, C-terminal fragments gave quite significantly greater labeling than N-terminal fragments. Of the four RNA binding domains noted by Ghetti et al. (1992), two intact domains seem the minimum to give a detectable signal in the UV-crosslinking assay, and the C-terminal pair give a considerably stronger signal than the N-terminal pair. Among the N-terminal fragments, only GST-ptb 1, which represents the first 68% of the protein, was labeled significantly in these assays, whereas GST-ptb 10, the first 62% of the protein, was only weakly labeled (Figs. 6, 7A). Of the C-terminal fragments, GST-ptb 2, which has both the C-terminal RNA binding domains but

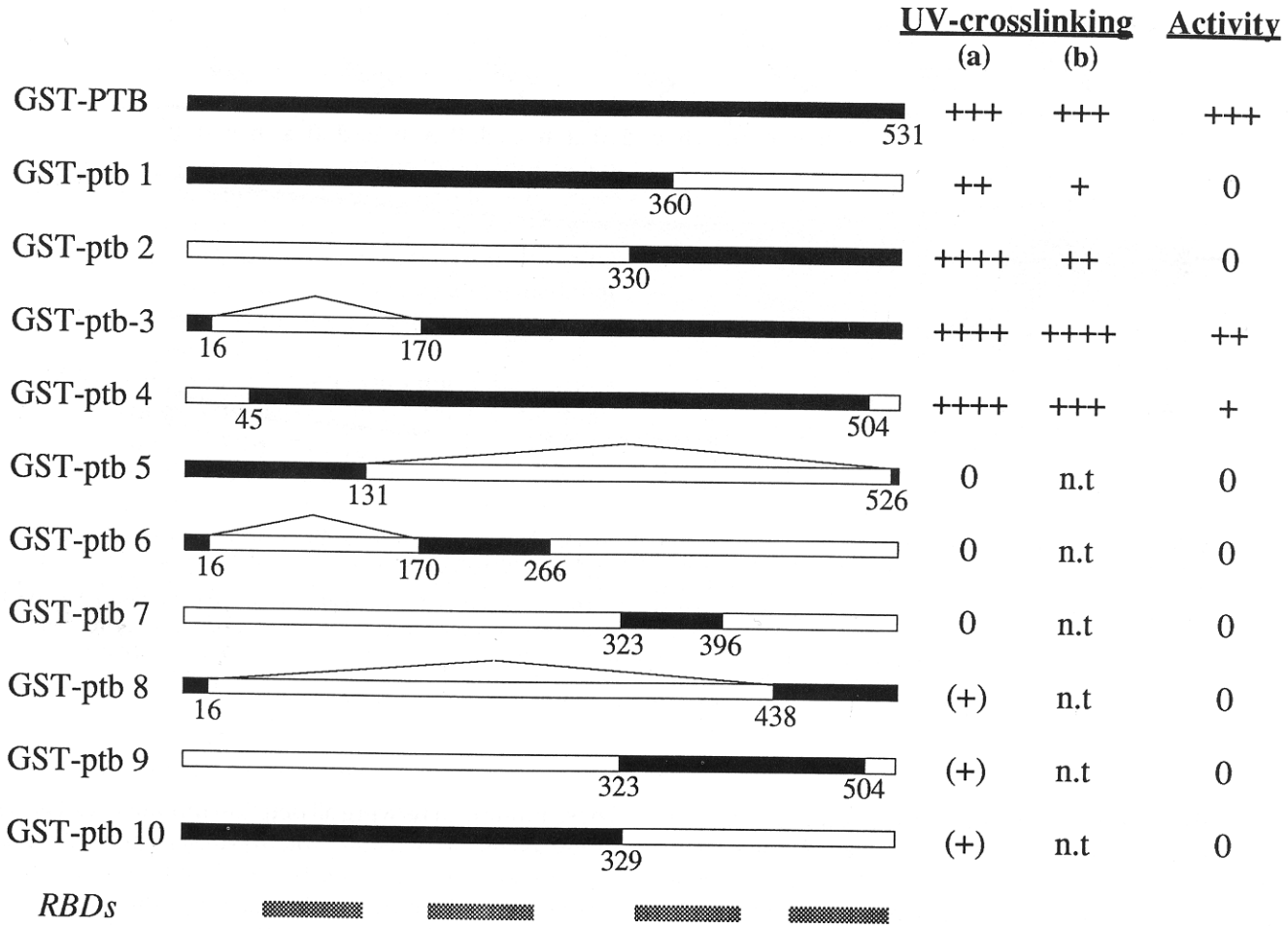

FIGURE 6. Nature and properties of the PTB deletion mutants tested. Deletions are shown approximately to scale: black bars indicate the sequences retained (with the end points designated according to the amino acid numbering system of the complete protein), and open bars show the sequences deleted. Positions of the four RNA-binding domains (*RBDs*) described by Ghetti et al. (1992) are shown as shaded bars. Also shown in semi-quantitative form is the intensity of labeling of each protein in UV-crosslinking assays using ^{32}P-labeled EMCV Domain 1 carried out under two conditions and shown in Figure 7: (a) crosslinking with purified recombinant protein (see Fig. 7A); or (b) crosslinking observed when the mutant proteins were added to PTB-depleted flow-through lysate from the affinity column (Fig. 7B). The extreme right-hand column summarizes the activity of the recombinant proteins in specifically stimulating the EMCV IRES-dependent translation of the downstream cistron in PTB-depleted flow-through lysate from the affinity column (Fig. 8).

does not extend much beyond the upstream of the two, was labeled quite strongly, whereas GST-ptb 9, which has a similar N-terminal boundary but lacks a complete RNA binding domain at the C-terminus, was labeled only weakly (Figs. 6, 7A). Some of the mutants, such as GST-ptb 3 and GST-ptb 4, which retain both RNP motifs and a considerable fraction of the N-terminal half of the protein, were actually consistently labeled more strongly than the full-length GST-PTB (Figs. 6, 7A). Similar relative intensities of labeling of the different mutant proteins were observed when a ^{32}P-labeled probe was used that is virtually identical to B3P3 described by Patton et al. (1991), a fragment of α-tropomyosin intron 2, including the long polypyrimidine tract (data not shown).

The above UV-crosslinking assays were done under the somewhat artificial conditions of a purified protein and a high specific activity labeled probe, with no other macromolecular components that might act as competitors. Because our ultimate aim was to try to correlate crosslinking with activity in promoting IRES-dependent initiation, the crosslinking assays were repeated in the PTB-depleted reticulocyte lysate in the case of those mutant proteins that were labeled to any significant extent in the assays with purified protein alone. Flow-through lysate from the affinity column was supplemented with GST-ptb mutant protein at the same concentration as is used in translation assays, and the crosslinking to ^{32}P-labeled EMCV Domain 1 reassayed under conditions similar to those used for translation

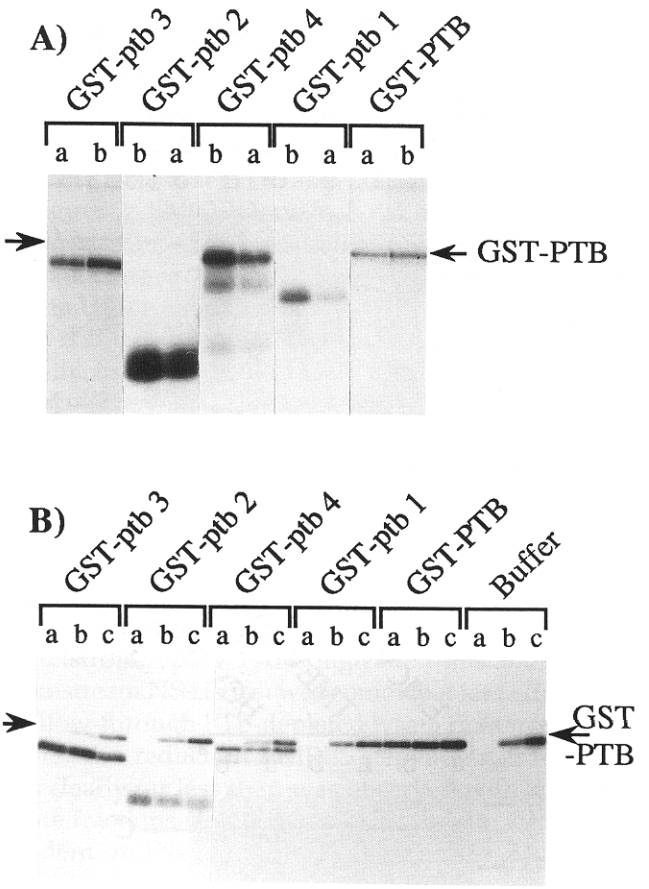

FIGURE 7. Labeling of PTB deletion mutant proteins by UV-crosslinking using a ^{32}P-labeled EMCV Domain 1 probe. **A:** The designated deletion mutants (as GST-fusion proteins) and full-length GST-PTB were subjected to standard UV-crosslinking reactions using ^{32}P-labeled EMCV Domain 1 as probe and final protein concentrations of: a, 0.5 μg/mL; or b, 1 μg/mL. **B:** UV-crosslinking assays were carried out with PTB-depleted lysate (pooled flow-through fractions from the affinity column) and ^{32}P-labeled EMCV Domain 1 under standard conditions, except that the assays were supplemented with the designated PTB deletion mutants (as GST-fusion proteins) at a final concentration of 0.12 μM (the molar equivalent to 10 μg/mL full length GST-PTB), together with full-length GST-PTB at: a, 0; b, 5 μg/mL; and c, 10 μg/mL. In both experiments, labeled proteins were separated by gel electrophoresis and the resulting autoradiograph is shown. The position of the full-length GST-PTB fusion protein is shown in each panel.

assays. The results shown in Figure 7B, and summarized in Figure 6, demonstrate that all the mutants that were labeled in assays of isolated purified protein were also crosslinkable in the background of the depleted lysate, although the labeling relative to that of full-length GST-PTB was somewhat reduced compared to assays of the purified isolated proteins, particularly in the case of GST-ptb 1 and GST-ptb 2.

Because the intensity of labeling of a protein in such UV-crosslinking assays is not a direct measure of binding affinity but only of crosslinkability, we also carried out the UV-crosslinking assays of mutant GST-ptb proteins in the depleted lysate in the presence of full-length GST-PTB competitor added at a molar ratio of 0.5 or 1.0 with respect to the concentration of mutant GST-ptb. Because the radioactive RNA probe is near limiting in such assays, labeling of the added full-length GST-PTB competitor results in a decrease in labeling of the mutant GST-ptb (Fig. 7B), from which a rough indication of relative affinity can be deduced. The results suggest that each of the four most efficiently crosslinked mutant proteins examined in this way binds to Domain 1 with an affinity that is not vastly different from that of full-length GST-PTB (Fig. 7B).

None of the mutant proteins that were labeled only weakly or not at all in the crosslinking assay with the isolated purified protein had any activity in promoting IRES-dependent downstream cistron translation in the PTB-depleted flow-through lysate (data not shown). Of the four mutant proteins that were labeled quite efficiently in the crosslinking assay, two (GST-ptb 1 and GST-ptb 2) were totally inactive in promoting IRES-dependent translation initiation, one (GST-ptb 4) had very weak activity, and only GST-ptb 3 was significantly active, albeit considerably less active than the full-length GST-PTB (Fig. 8). Interestingly, GST-ptb 4 retains a larger fraction of the full-length protein than GST-ptb 3 (Fig. 6), yet was less active in promoting IRES-dependent translation. What GST-ptb 3 has that GST-ptb 4 lacks is the extreme C-terminal sequences of the wild-type protein, again highlighting the impor-

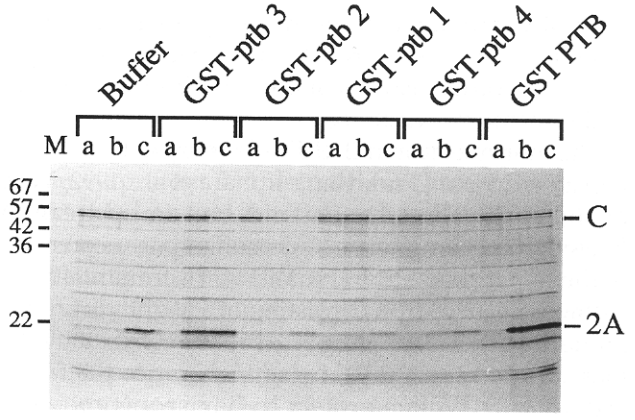

FIGURE 8. Effect of PTB deletion mutants on translation of XL-EMCV-2A dicistronic mRNA in PTB-depleted lysate. Uncapped XL-EMCV-2A dicistronic mRNA was translated at a final concentration of 25 μg/mL in PTB-depleted lysate (pooled flow-through fractions from the affinity column). Translation assays were supplemented with either full-length GST-PTB or the designated PTB deletion mutants (as GST-fusion proteins) as follows: a, no GST-PTB or PTB deletion mutant added; b, the designated PTB deletion mutant alone at 15 μg/mL final concentration or GST-PTB, where indicated, at 15 μg/mL; and c, as for b, but full-length GST-PTB also present at a final concentration of 5 μg/mL. Translation products were separated by gel electrophoresis and the resulting autoradiograph is shown. Track M was loaded with radioactive marker proteins of sizes as given in the left margin, and the positions of the cyclin (upstream cistron) and 2A (downstream cistron) products are shown in the right margin as C and 2A, respectively.

tance of the C-terminus for PTB function. It is also worth noting that, when UV-crosslinking was conducted in the depleted lysate under conditions similar to those of translation assays, GST-ptb 3 was labeled more than GST-ptb 4 (Fig. 7B), which correlates with their respective activities in the translation assays. However, the correlation breaks down when the most active mutant, GST-ptb 3, is compared with full-length GST-PTB: labeling of GST-PTB in the crosslinking assay is less than the labeling of GST-ptb 3 (Fig. 7B), yet its activity is very much higher (Fig. 8).

It should be noted that, of the two inactive mutants, GST-ptb 1 and GST-ptb 2, certainly the former, and possibly both, appeared to inhibit the stimulation by full-length GST-PTB (Fig. 8). There also seemed to be a similar, though probably smaller, inhibition in the case of the weakly active GST-ptb 4 (Fig. 8). This correlates with the results of the competitive UV-crosslinking assays conducted in PTB-depleted lysates (Fig. 7B), which implied that the mutant proteins, or certainly GST-ptb 2 and GST-ptb 4, bind to the IRES with an affinity comparable to that of full-length GST-PTB. We infer this to mean that, in the translation assay (Fig. 8), the mutant proteins were actually binding to the usual site, in competition with GST-PTB, but were incapable of promoting internal initiation.

PTB is not required for internal initiation promoted by some other IRESes

The PTB-depleted lysate obtained by affinity chromatography provided us with an opportunity to test whether PTB is required for internal initiation promoted by other IRES elements, both of picornaviral and non-picornaviral origin.

The non-picornaviral IRES examined was that of hepatitis C virus (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The test construct has an upstream *X. laevis* cyclin cistron, an intercistronic spacer consisting of the hepatitis C virus (HCV) 5′-UTR from nt 40, and a downstream cistron consisting of a slightly truncated influenza virus NS-coding region, as described by Borman and Jackson (1992), fused in-frame to the first 11 codons of HCV core protein coding sequence, which we find necessary for efficient internal initiation (Reynolds et al., 1995). The product of downstream cistron translation is therefore the truncated NS protein preceded by the N-terminal 10 amino acids of HCV core, assuming that the initiating methionine residue is removed by aminopeptidase activity. Translation of this dicistronic mRNA in the parent load lysate and in the flow-through fractions shows that IRES-dependent downstream cistron translation is unaffected by the absence of PTB in the flow-through lysate fractions, and is not stimulated by the addition of recombinant GST-PTB to these fractions (Fig. 9A). In order to rule out the possibility that HCV IRES function requires PTB but

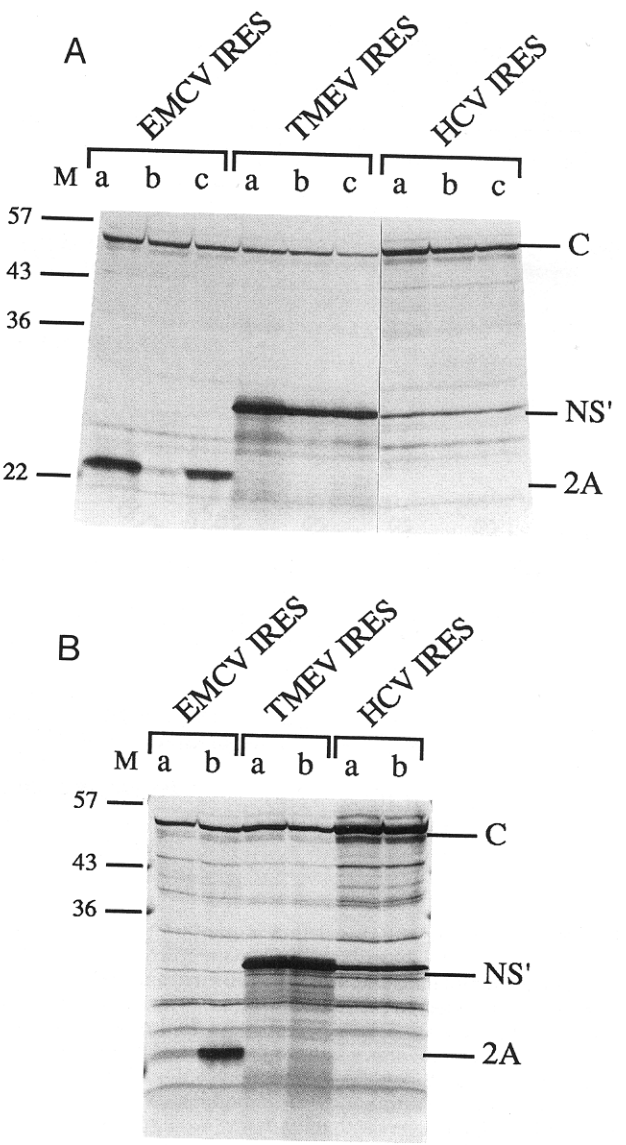

FIGURE 9. Translation of dicistronic mRNAs with different IRESes in PTB-depleted lysates. Uncapped dicistronic mRNAs with the EMCV IRES (XL-EMCV-2A), TMEV IRES (XLJODA 1099), or HCV IRES (XL40-372.NS′) were each translated at a final concentration of 50 μg/mL. **A:** Translation assays were carried out in: a, column load lysate; b, PTB-depleted lysate (pooled flow-through fractions from the affinity column); and c, PTB-depleted lysate supplemented with GST-PTB at 10 μg/mL. **B:** PTB-depleted lysate from the experiment shown in A was passed through a second affinity column. Flow-through fractions were collected and used in translation assays of the same dicistronic mRNAs: a, without further additions; and b, with GST-PTB added at a final concentration of 10 μg/mL. Translation products were separated by gel electrophoresis and the resulting autoradiograph is shown. Track M was loaded with radioactive marker proteins of sizes as given in the left margin, and positions of the cyclin (upstream cistron), and NS′ and 2A (downstream cistron) products are shown in the right margin as C, NS′, and 2A, respectively.

only at very low concentrations, such as might be present in the flow-through fractions by leakage, we took the depleted lysate flow-through fractions and passed them through a second affinity column. How-

ever, internal initiation dependent on the HCV IRES was unimpaired by this double depletion, and there was still no stimulation by recombinant PTB (Fig. 9B). We conclude that HCV IRES function does not require the participation of PTB, a conclusion that is in accord with the facts that (1) in assays similar to that shown in Figure 2, EMCV Domain 1 did not inhibit downstream cistron translation dependent on the HCV IRES (data not shown); and (2) there is minimal labeling of PTB in UV-crosslinking assays with a high specific activity ^{32}P-labeled probe comprising the HCV 5′-UTR plus the first 11 codons of HCV core protein coding sequence (Fig. 10).

We also tested internal initiation dependent on the IRES of another cardiovirus, TMEV. The dicistronic mRNA is similar to that described above for the HCV IRES, with the same upstream cyclin cistron and a downstream cistron consisting of a slightly truncated NS cistron preceded by first 13 codons of TMEV polyprotein coding sequences, and with the TMEV IRES as intercistronic spacer. Although the translation of the downstream NS cistron was somewhat less efficient in the flow-through PTB-depleted lysate than in the load lysate, the reduction in IRES-dependent translation was clearly far less than was observed with the same lysate fractions translating a downstream cistron dependent on the EMCV IRES (Figure 9A). Moreover, addition of recombinant GST-PTB to the depleted lysate did not stimulate internal initiation dependent on the TMEV IRES, not even if the lysate was passed through two affinity columns to ensure complete depletion of PTB (Fig. 9B). We conclude that there is no evidence that PTB is required for internal initiation dependent on the TMEV IRES; the reduced translation of the downstream cistron in the flow-through lysate (Fig. 9A) is most likely to be due to the removal of protein(s) other than PTB.

This result was surprising in view of the fact that PTB is labeled very strongly in UV-crosslinking assays with ^{32}P-labeled TMEV IRES, and labeling is at least as intense as with ^{32}P-labeled EMCV IRES (Fig. 10). UV-crosslinking assays with flow-through lysate fractions from either a single (Fig. 10) or double (data not shown) passage through the affinity column confirmed the absence of crosslinkable PTB, but efficient crosslinking to GST-PTB could be observed if the recombinant protein was added to these flow-through fractions (Fig. 10).

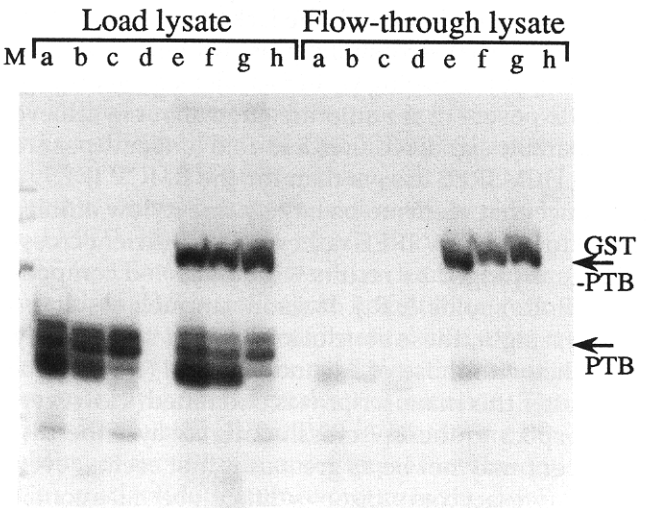

FIGURE 10. Proteins in normal and PTB-depleted reticulocyte lysate labeled by UV-crosslinking using various ^{32}P-labeled IRES probes. UV-crosslinking reactions were carried out using the affinity column load lysate or PTB-depleted lysate (pooled flow-through fractions from the affinity column), as designated. GST-PTB was added at a final concentration of 2 μg/mL to the assays in lanes e–h, inclusive. ^{32}P-labeled probes were as follows: a, e, EMCV Domain 1; b, f, EMCV 5′-UTR, the complete structure shown in Figure 1; c, g, TMEV IRES, the entire TMEV sequence from XLJODA 1099; d, h, HCV IRES, the entire HCV sequence from XL40-372.NS′. Labeled proteins were separated by gel electrophoresis, and the resulting autoradiograph is shown. Positions of GST-PTB and endogenous reticulocyte PTB are shown in the right margin.

DISCUSSION

In the five years since a protein doublet in the size range 57–60 kDa was first reported to bind and to be crosslinked by UV-irradiation to the EMCV IRES (Borovjagin et al., 1990; Jang & Wimmer, 1990), an observation that was extended first to the FMDV IRES (Luz & Beck, 1990) and subsequently to other picornavirus IRESes, the question of whether the binding of this protein is essential for internal initiation, has remained controversial and largely unresolved. The identification of this protein doublet as PTB (Borman et al., 1993; Hellen et al., 1993) did not in itself help to resolve this issue, though it did permit experiments using recombinant protein. Hitherto, the argument in favor of a direct role in internal initiation has rested mainly on three observations. Perhaps the most compelling result is that mutations that disrupted the base paired stem of the main PTB binding site in the EMCV IRES abolished both internal initiation, and crosslinking of PTB, with both properties largely restored by compensating mutations that recreated the base paired stem (Jang & Wimmer, 1990). Secondly, HeLa cell extracts depleted of PTB by immunological methods were defective in the IRES-dependent translation of a monocistronic construct, but retained activity (in a separate control assay rather than in the more rigorous type of co-translation internal control) for translation of mRNAs by the conventional 5′ end-dependent scanning mechanism (Hellen et al., 1993). However, IRES-dependent translation was not restored by addition of recombinant PTB, an observation that implied that singular PTB is not the active principle, and raised some doubt as to whether the active entity is a complex between PTB and some other protein, or whether the PTB is actually

irrelevant and it is only the putative PTB-associated protein that is essential. Finally, it has been noted that EMCV 5′-UTR fragments that include the main PTB-binding site can act as quite potent inhibitors of translation initiation dependent on the EMCV IRES, and that such inhibition can be partially reversed by addition of recombinant PTB (Borovjagin et al., 1994). The inhibition and the effect of added PTB were said to be specific for internal initiation, because the translation of tobacco mosaic virus RNA, in a separate control assay rather than as a co-translated internal control, was less strongly inhibited, though there was only about a twofold difference between the susceptibility of translation of the two types of mRNA to inhibition by a given concentration of EMCV 5′-UTR fragment (Borovjagin et al., 1994).

The experiments reported here provide the first direct unequivocal evidence that PTB itself is necessary for internal initiation at least when such initiation is promoted by the EMCV IRES. The approach that was successful in this instance, affinity chromatography using a specific RNA-Sepharose, may prove to be generally useful to deplete extracts of other specific RNA-binding proteins. The high specificity of this method is shown by the fact that only a Domain 1 affinity column was effective in depleting lysates of PTB; lysates passed through affinity columns with covalently bound EMCV 5′-UTR Domain 2, or adenovirus VA_I RNA, or *X. laevis* pre-$tRNA^{Phe}$ were not depleted of PTB and, in translation assays, gave a similar ratio of yields of products from the upstream and downstream (IRES-dependent) cistrons as the parent lysate (data not shown). By operating the affinity column at 100 mM KCl, nonspecific binding of other RNA-binding proteins or general translation initiation factors seems to have been reduced to quite low levels as witnessed by the fact that the flow-through lysate retains activity for translation of the upstream cistron, which acts as an internal control in our experimental procedure.

Our results show that the function of PTB in internal initiation of translation dependent on the EMCV IRES requires virtually the complete full-length protein. Truncated versions of the protein exhibited only weak activity, the best being GST-ptb 3, which retains both of the critical C-terminal RNA-binding domains plus one of the two such domains present in the N-terminal half. This and some of the other mutants that exhibited essentially no activity in the translation assay could nevertheless be efficiently crosslinked to Domain 1 (Fig. 7B), or to the complete EMCV IRES (data not shown), even in the presence of competing reticulocyte lysate proteins. Thus, binding per se is not sufficient for activation of internal initiation. Similar observations have been made with respect to the stimulation of poliovirus RNA translation in reticulocyte lysates effected by addition of the autoantigen La in high concentration (~100 μg/mL): mutants of La lacking the C-terminus were capable of binding to the poliovirus RNA, but did not stimulate translation (Svitkin et al., 1994a). The main PTB-binding site in the EMCV IRES consists of a stem closed by a loop consisting of five pyrimidines, which include the sequence UCUU, the motif that was present, within a more random pyrimidine-rich background, in the vast majority of sequences obtained in SELEX experiments with PTB (I. Perez & J.G. Patton, in prep.). In addition, there is a bulge of eight residues, five of them pyrimidines, half way up the stem (Fig. 1). It seems unlikely that all four RNA-binding domains of PTB could be simultaneously engaged with the loop and the bulge, which leaves open the possibility that at least one, and more probably two, of the RNA-binding domains would be free to make contact with other sites in the EMCV 5′-UTR. If this is the case, then our results with the PTB deletion mutants imply that these additional contacts are very important for the function of PTB in internal initiation, because mutants that retain two RNA-binding domains and obviously bind efficiently to the main PTB-binding site in the EMCV IRES are inactive in promoting internal initiation.

It is abundantly clear that PTB is not required for internal initiation of translation promoted by the HCV IRES in this system. Not only does recombinant PTB fail to stimulate internal initiation dependent on this IRES in the PTB-depleted lysate, but the HCV IRES functions with virtually the same efficiency in the depleted lysate as in the parent load lysate (Fig. 9). This observation correlates with the lack of apparent labeling of PTB in UV-crosslinking assays with the HCV IRES (Fig. 10). Labeling is, in fact, detectable on prolonged exposure of the autoradiograms, but to achieve a comparable signal requires a 15-fold longer exposure for the HCV IRES assays than for the EMCV IRES, a difference that seems to be largely due to low affinity of PTB for the HCV IRES rather than inefficient crosslinking, as judged by results with unlabeled competitors (J.E. Reynolds & R.J. Jackson, unpubl. results).

At first sight, these conclusions would seem totally in conflict with those of Ali and Siddiqui (1995), published after this manuscript was submitted. However, with respect to the UV-crosslinking assays, the disagreement may not be as great as it first seems, even though an exact comparison is difficult because conditions were different: Ali and Siddiqui (1995) used HCV IRES probes substituted with 4-thio-U in crosslinking assays that were done only with purified GST-PTB or quite extensively purified HeLa PTB, but not with the cell-free extracts actually used for translation assays, as we have done. Nevertheless, two of their observations suggest that the affinity of PTB for the HCV IRES or its crosslinking efficiency was low in comparison with, for example, the EMCV IRES: (1) the labeling of GST-PTB in the crosslinking assays increased with increasing protein concentration up to at least 50 μg/mL (which is

also the case in our hands with the HCV IRES but not with EMCV IRES); and (2) the labeling of HeLa cell PTB was no greater with the HCV IRES than with the poliovirus IRES (Ali & Siddiqui, 1995), which, like the related rhinovirus IRES, is known to bind PTB with much lower affinity and crosslinks to it very much less efficiently than does the EMCV IRES (Pestova et al., 1991; Borman et al., 1993).

As for functional tests, the claim that PTB is required for HCV IRES function is based on an inhibition of in vitro translation of a monocistronic mRNA with the HCV 5′-UTR by added anti-PTB antibodies (Ali & Siddiqui, 1995). Although this inhibition was specific in that it was not seen with an irrelevant antiserum, no evidence was provided that it was specific for IRES-dependent translation as opposed to initiation by the scanning mechanism. Moreover, addition of recombinant PTB failed to rescue IRES-dependent translation in the immunodepleted extract. It is striking that a similar inability of recombinant protein to rescue IRES-dependent translation in immunodepleted extracts has been reported before, not just for PTB-depletion and poliovirus or EMCV IRES activity (Hellen et al., 1993), but also for depletion of the autoantigen La and poliovirus IRES function (Belsham et al., 1995). This type of result would certainly seem to eliminate the possibility that the active entity is singular PTB or La, respectively, and leaves considerable doubt as to whether the active factor might not be a protein that is co-precipitated, perhaps even fortuitously, by anti-PTB or anti-La antibodies.

The finding that internal initiation promoted by the TMEV IRES does not show any clear-cut requirement for PTB was rather surprising, for not only is PTB crosslinked efficiently to the TMEV IRES (if anything, the labeling of PTB is greater than with the EMCV IRES), but this virus is always considered to be closely related to EMCV. However, if PTB is not required for HCV IRES function in this system, as clearly seems to be the case, then PTB is unlikely to be fulfilling a central role as an essential catalyst of internal initiation, in much the same way as the canonical initiation factor proteins are certainly essential catalysts of initiation. This leaves open a possible role for PTB in promoting the correct folding of the IRES in order to present the critical primary nucleotide sequence motifs in the correct three-dimensional organization to allow internal ribosome entry. This interpretation would be consistent with the view proposed above that some of the four RNA-binding domains of PTB could contact segments of the IRES beyond the stem-loop structure that is the main binding site. A similar suggestion of an RNA-chaperone role has already been made with respect to the influence of La on the translation of various RNAs in rabbit reticulocyte lysates (Svitkin et al., 1994b). According to this view, EMCV IRES function has a greater dependency on PTB than TMEV IRES function because the TMEV IRES is more likely to adopt the correct structure spontaneously, without the need, shown by the EMCV IRES, for PTB binding to promote the appropriate folding.

Taking these ideas one step further, one could imagine two different IRES conformations (which is undoubtedly an oversimplification, but serves to illustrate the point), that we could call the R and T forms: the R form would be active in promoting internal initiation and would be the form bound by PTB, and the T form would be inactive and would bind PTB only weakly, if at all. The presence of PTB would thus displace the R-T equilibrium in favor of the R form, and in this way could stimulate internal initiation. In this explanation, the main difference between the EMCV and TMEV IRESes is that the default conformation of the EMCV IRES would be mainly the T form, whereas the default TMEV IRES conformation would be mainly the R form, explaining why TMEV IRES function shows almost no requirement for PTB even though PTB binds and is efficiently crosslinked to the TMEV 5′-UTR. These considerations lead to the prediction, currently being tested, that there must be distinct, though probably subtle, differences between the secondary structure of the TMEV and EMCV IRESes.

MATERIALS AND METHODS

Plasmid constructs

All cDNA plasmid constructs were in pGEM-1 or pGEM-2 vectors (Promega) with the orientation of the insert such that transcription by bacteriophage T7 RNA polymerase would produce the sense RNA transcript.

Of the dicistronic constructs used to generate a dicistronic mRNA for assay of IRES-dependent translation of the downstream cistron, pXLJODA 1099 has been described previously (Hunt et al., 1993). It comprises the *X. laevis* cyclin B2 cDNA sequence as the upstream cistron, an intercistronic spacer consisting of the 5′-UTR sequence of TMEV (strain DA) from nt 395 up to the authentic initiation codon for viral polyprotein synthesis, followed by the first 40 nt of the TMEV coding sequence fused, via a 5-nt linker, to a slightly truncated derivative of the influenza virus NS cDNA-coding sequence and 3′-UTR (Hunt et al., 1993). pXL40-372.NS′ is similar except that the TMEV sequences and the 5-nt linker are replaced by nt 40–372 of the HCV genome: it thus has the *X. laevis* cyclin B2 cDNA as the upstream cistron, followed by the HCV 5′-UTR from nt 40 plus the first 10 codons of HCV coding sequence (the authentic initiation codon is at nt 342) fused in frame to the NS′ cDNA sequence. For the in vitro production of dicistronic mRNA for translation assays, both pXLJODA 1099 and pXL40-372.NS′ were linearized by digestion with *Eco*R I. Monocistronic derivatives of these plasmids, lacking the upstream *X. laevis* cyclin B2 cistron, were used for the production of probes for UV-crosslinking reactions. These monocistronic constructs were linearized with *Bam*H I prior to transcription; the transcripts thus include all the TMEV or HCV 5′-UTR and coding sequences and terminate at the 3′

end with the AUG triplet, which occurs at the start of the NS' coding sequences in the dicistronic constructs (Hunt et al., 1993).

pXL-EMCV-2A was generated from pSG1 (Kaminski et al., 1994a) by insertion of the *X. laevis* cyclin B2 cDNA in the *Eco*R I site. For generation of dicistronic mRNA, it was linearized with *Bam*H I prior to in vitro transcription. In order to generate a full-length IRES probe for UV-crosslinking assays, pSG1 was linearized with *Msc* I prior to in vitro transcription.

The individual domains of the EMCV 5'-UTR were cloned using PCR amplification of pSG1 described by Kaminski et al. (1994a), using primers that would create an *Eco*R I cleavage site at the 5' end of the domain, and, at the 3' end, a *Bam*H I site (or *Msc* I site, in the case of Domain 3). The primers were as follows, with the introduced restriction enzyme cleavage sites shown in bold: for Domain 1, the sense (forward) primer was GGG**GAATTC**CCCCTCTCCC and the antisense primer, GGG**GGATCC**GACCTTGCATTCC; for Domain 2, the sense primer was GGG**GAATTC**GGAAGCAGTTCC and the antisense primer, GGG**GGATCC**GGAGAGCCATTTGAC; and for Domain 3, the sense primer was GGG**GAATTC**GGGG CCGAAGGATG and the antisense primer, GGG**TGGCCA** TATTATCATCGTG. The PCR products were digested with *Eco*R I and *Bam*H I (or *Msc* I in the case of Domain 3), and subcloned in pGEM-1, using the *Eco*R I and *Bam*H I sites, or the *Sma* I site in the case of Domain 3. For the production of competitor RNAs or probes for UV-crosslinking reactions these plasmids were linearized with *Bam*H I prior to transcription.

All plasmids were propagated by standard procedures using *E. coli* TG1 and ampicillin selection (Sambrook et al., 1989).

Preparation of GST-PTB and PTB deletion mutant proteins

Human PTB cDNA was cloned in pGEX3X, as described previously (Patton et al., 1991, 1993). Overexpression of the GST-PTB fusion protein and its purification using glutathione-Sepharose were carried out exactly as described by Smith and Corcoran (1990). Because the eluate fractions contain glutathione, and thus may also contain oxidized glutathione, which can strongly inhibit translation in the reticulocyte lysate (Jackson et al., 1983), the eluted fractions containing GST-PTB were dialyzed against 20 mM HEPES-KOH, pH 7.4, 5% glycerol, 100 mM KCl, 7 mM 2-mercaptoethanol. To estimate the concentration of the GST-PTB, samples were subjected to gel electrophoresis and the Coomassie staining of the full-length GST-PTB protein was compared with the staining of bovine serum albumin standards run on the same gel.

PTB deletion mutants were made by digestion of the cDNA with various restriction enzymes, followed by subcloning the desired fragment in the pGEX3X vector. The overexpression, purification, and estimation of the final concentration of the GST-ptb mutant proteins was carried out exactly as for the full-length GST-PTB.

In vitro transcription and translation assays

Dicistronic mRNAs for use in translation assays were generated by transcription of the linearized plasmid DNAs with bacteriophage T7 RNA polymerase exactly as described previously (Dasso & Jackson, 1989; Kaminski et al., 1990), with a low concentration of [α-^{32}P]UTP (Amersham International; >400 Ci/mmol) added to the reaction to allow the concentration of the RNA product to be estimated as described in Dasso and Jackson (1989). Translation assays were carried out as described previously (Kaminski et al., 1990, 1994a), with a final concentration of 100 mM added KCl, and 0.5 mM added $MgCl_2$. Translation assays were routinely incubated at 30 °C for 90 min, and the translation products separated by gel electrophoresis using either a 20% polyacrylamide gel, or a 10–30% gradient gel similar to that described by Kaminski et al. (1990).

UV-crosslinking assays

High specific activity uncapped ^{32}P-labeled probes were prepared as described in Borman et al. (1993), except that the concentration of unlabeled UTP added to transcription reactions was 100 μM. In general, crosslinking reactions were performed in a final volume of 10 μL, containing 2 μL of reticulocyte lysate or affinity column flow-through fraction, as described by Borman et al. (1993), except that the heparin competitor was omitted, and reactions were irradiated using a Stratalinker XL-1500 (Stratagene) at an energy level of 2.4 J/cm^2.

Preparation and use of Domain 1 RNA affinity column

The procedure was based on that published by Berridge and Aronson (1973) and Arndt-Jovin et al. (1975). Domain 1 RNA for coupling to the column was prepared using a scaled-up transcription reaction under slightly modified conditions: 10 mM each rNTP, 5 μCi [α-^{32}P]UTP, 40 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.0, in a final volume of 0.2 mL. Approximately 300 μg EMCV Domain 1 RNA was covalently coupled to approximately 0.5 mL (packed bed volume) of pre-swollen CNBr-activated Sepharose 4B (Pharmacia) in approximately 1.5 mL 200-mM MES-KOH, pH 6.0, for 16 h at 2 °C on an end-over-end rotator. The product was first washed with water, and then with 0.1 M Tris-HCl, pH 7.8, for 2 h and finally equilibrated in 20 mM HEPES-KOH, pH 7.4, 5% glycerol, 100 mM KCl, 7 mM 2-mercaptoethanol before forming the column.

Rabbit reticulocyte lysate was prepared for in vitro translation (omitting the [^{35}S]methionine) before loading on the column. Either 2 mL or 3 mL of supplemented lysate was loaded onto a 0.5-mL column, which was run at 2 °C. The flow through was collected in 250-μL fractions.

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