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MOLECULAR MECHANISMS OF BIOLOGICAL PROCESSES

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Mutual Effects of Translation Initiation Factors in Binding to the IRES of the Encephalomyocarditis Virus RNA

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Three—classical cap-dependent, shunting, and IRES-dependent—major mechanisms are now known for mRNA association with the 40S ribosome subunit in eukaryotes [1]. In the IRES-dependent mechanism, the 40S subunit binds to a specific internal ribosome entry site (IRES) in the 5'-untranslated region of mRNA, rather than interacting with its 5' end. A classical example of IRES is provided by ribosome-binding RNA sites of picornaviruses, which include the mouse encephalomyocarditis virus (EMCV, cardiovirus group). Both picornavirus IRES and cap-dependent mRNAs utilize the same canonical translation initiation factors. Yet the key part is played by cap-binding subunit eIF4E of eIF4F in the latter case, whereas IRES is recognized by the large eIF4F subunit, eIF4G, which is also capable of RNA binding [2]. The third subunit of eIF4F is eIF4A, which occurs in free and bound continually exchanging forms [3]. Functionally, eIF4A unwinds the RNA secondary structure elements close to the initiation codon and, possibly, positions the initiation site of RNA into the proper channel of the 40S subunit. The large eIF4F subunit, eIF4G, has one eIF4E-binding site one-third its length away from the N end; eIF4A interacts with two eIF4G sites, which are in the central and in the C-terminal regions [4, 5]. The mRNA-binding site responsible for primary IRES recognition in picornavirus RNA is also in the central region of eIF4G [4]. Interacting with IRES, this site contacts domains J and K in the RNAs of the foot-and-mouse disease virus and EMCV [6, 7] (Fig. 1). The major motif of the corresponding recognition site of the IRES is 5'-AAAAA(G/A)-3' [6], which forms an internal loop at the junction of domains J and K. In addition, the central region of eIF4G contains a binding site for eIF3, which, in turn, binds with the 40S subunit [5]. Thus, the primary RNA binding is ensured by the following chain of interactions: IRES–eIF4G–eIF3–40S.

Other canonical initiation factors essential for the formation of the 48S preinitiation complex are eIF2, which brings the initiator tRNA to the 40S subunit, and eIF4B, which stimulates the helicase activity of eIF4A. The assembly of the 48S complex may be conveniently assayed by toe-printing, which has been described in detail elsewhere [8].

Besides the canonical translation initiation factors, specific accessory mRNA-binding proteins are essential for translation initiation on picornavirus IRES. The pyrimidine tract-binding protein (PTB) is necessary for the 48S complex assembly with the IRES of EMCV or the Theiler virus [9–11]. In addition to PTB, IRES-dependent translation initiation requires Unr and the poly(C)-binding protein (PCBP) in the case of the poliovirus and rhinoviruses [12, 13] or ITAF 45 in the case of the foot-and-mouse disease virus [11]. The exact roles of these proteins, PTB in particular, is unknown. There is no consensus as to whether PTB is indeed essential for the assembly of the 48S complex on the IRES of the EMCV RNA. We have found that PTB strongly stimulates translation initiation on the EMCV RNA [9]. More recently, we have observed that PTB only moderately (no more than twofold) affects the 48S complex assembly from purified components on this IRES [14]. It should be noted, however, that we have not performed systematic experiments with PTB, nor estimated the yield of the 48S complex as dependent on the PTB content. A strong PTB dependence of the 48S complex assembly on the EMCV IRES has been supported by Jackson and colleagues [10], who have more recently rejected this hypothesis and came to the conclusion that PTB is unnecessary for translation initiation on the EMCV IRES [15]. Here we convincingly demonstrate that PTB strongly affects the yield of the 48S complex assembled on the IRES of the wild-type EMCV, and provide important data on the function of PTB in 48S

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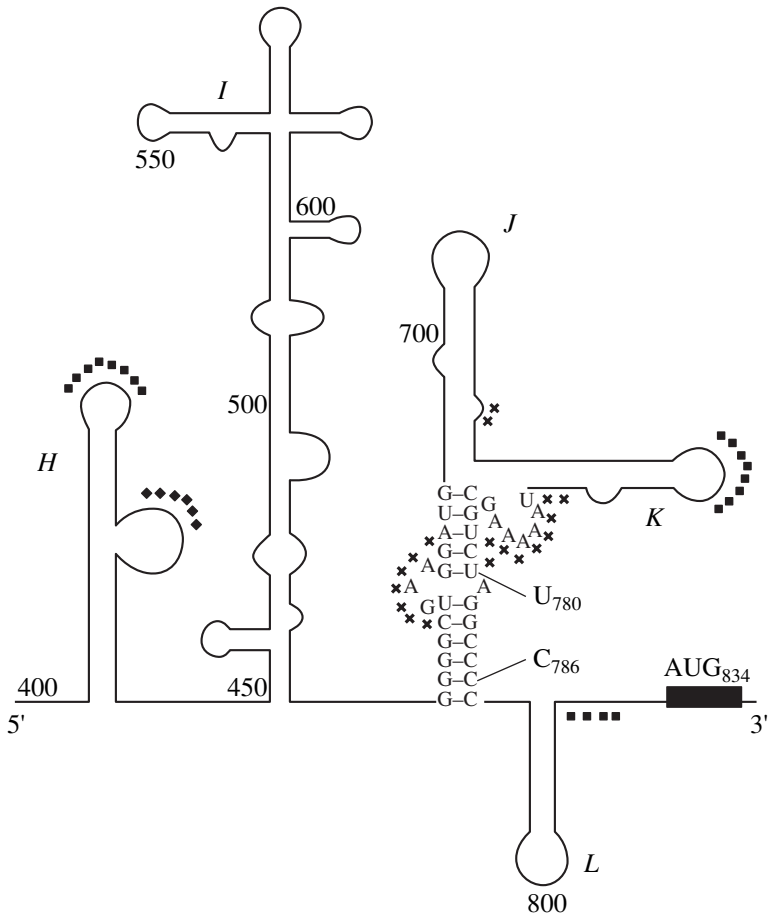

Fig. 1. IRES of the EMCV RNA. Secondary structure domains are indicated. Sites protected by eIF4F or PTB from chemical and enzymic modification are shown with crosses and squares, respectively. Termination sites of reverse transcription of IRES complexes with eIF4F alone (nucleotide 786) or combined with eIF4A (708 and 786) are indicated with arrows. The initiation codon is shown with a filled bar.

complex assembly on the RNA of EMCV and, possibly, other picornaviruses with structurally similar IRES.

The 48S complex was assembled from purified components as described previously [8], with a transcript synthesized with T7 polymerase. The transcript contained EMCV RNA region 315–846 fused with the 5' fragment of the firefly luciferase mRNA. Translation initiation factors and PTB were isolated as in [14]. Production of the 48S complex was inferred from the inhibition of primer extension in toe-printing [8]: when the codon–anticodon interaction between the initiator Met-tRNA and the initiation codon of the EMCV RNA was established in the 48S complex, reverse transcription stopped at positions +16 to +18, 3' of A of the initiation AUG (Fig. 2). Toe-printing confirmed that two scanning factors, eIF1 and eIF1A, are unnecessary for translation initiation on the EMCV RNA and that eIF4B stimulates the assembly of the 48S complex no more than twofold (data not shown). Thus, of all canonical translation initiation factors, the EMCV IRES strongly requires only eIF2, eIF3, eIF4F (which may be replaced by eIF4G + eIF4A), and an excess of free eIF4A.

The yield of the 48S complex progressively increased with increasing amount of recombinant PTB added to the 48S complex reconstruction mixture, which was evident from increasing intensity of toe-printing bands. As estimated with a phosphorimager, the intensity increased five- to sixfold at the most (Fig. 2a). We think it possible to put an end to the argument about the necessity of PTB for translation initiation on the EMCV RNA. PTB is necessary for translation initiation on all IRES examined in cardioviruses, aphthoviruses, rhinoviruses, and polioviruses, although its lack is not as fatal for EMCV as for other picornaviruses. Kaminski and Jackson [15] have not detected the stimulating effect of exogenous PTB possibly because of their experimental protocol. In their experiments, a reticulocyte lysate was depleted of endogenous PTB by affinity chromatography on Sepharose 4B modified with a short RNA correspond-

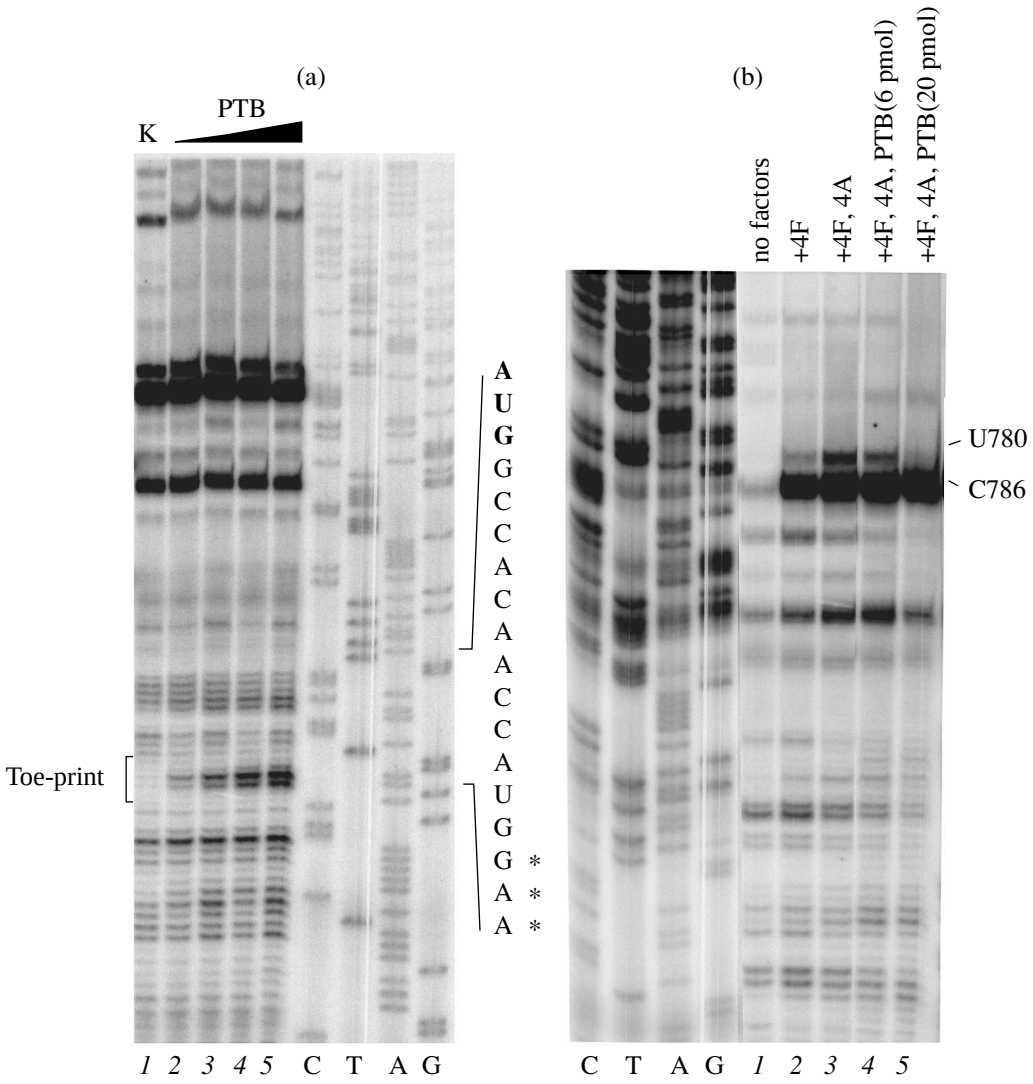

Fig. 2. (a) Effect of PTB on the formation of the 48S initiation complex on the EMCV IRES. In toe-printing, a control mixture (K, lane *1*) did not contain eIF2 and the initiator tRNA; PTB was (*2*) absent or present in (*3*) 2-, (*4*) 6-, or (*5*) 20-fold molar excess relative to RNA. Sequencing products obtained with the same primer and the corresponding cDNA are shown on the right. Reverse transcription termination sites are asterisked. (b) Effect of PTB on the termination of reverse transcription of IRES complexes with eIF4F or eIF4F + eIF4A. The PTB content per pmol RNA is indicated in parentheses. Termination sites U780 and C786, which are specific for the complexes, are indicated. The sequence of the corresponding EMCV RNA region is shown on the left.

ing to domain H of the EMCV RNA (see Fig. 1). Such chromatography inevitably removed a fraction of translation initiation factors along with PTB, so that it was impossible to completely remove PTB without a considerable loss of lysate activity. Hence exogenous PTB might fail to appreciably stimulate translation in this lysate. Moreover, the lysate was not tested for residual PTB.

According to our previous data [14], formation of a binary complex of 4F and EMCV IRES stabilizes the GC-rich stem at the base of domain J–K; this, in turn, made reverse transcription termination at position 786 more likely (Figs. 1, 2). The presence of eIF4F and an excess of eIF4A led to transcription termination at position 780 and generation of an additional band (Fig. 2b), which has not been noticed earlier. This agrees with the published data that subunit eIF4A plays an important part in the interaction of eIF4F with domain J–K of the EMCV IRES [4]. Interestingly, PTB dramatically reduced the intensity of this band, having virtually no effect on that of the band corresponding to reverse transcription termination at position 786. On this evidence, we assume that PTB somehow affects the interaction of eIF4F with domain J–K of the EMCV IRES.

The assumption was supported by the results of UV-induced crosslinking of EMCV RNA with complexes of translation initiation factors. RNA was

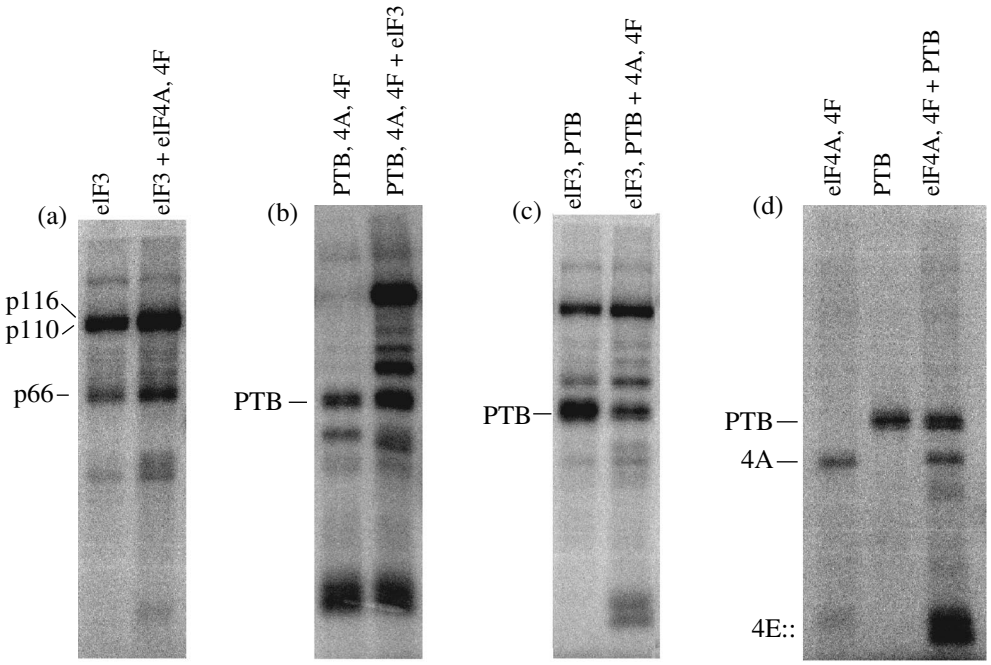

Fig. 3. UV-induced crosslinking of various combinations of eIF3, eIF4F + eIF4A, and PTB with the ^{32}P-labeled EMCV IRES. Subunits p116, p110, and p66 of eIF3 are shown on the left (panel a). Factor combinations are indicated at the top. See text for more detail.

labeled by T7 transcription with [^{32}P]UTP, incubated with various factors and PTB in a buffer allowing reconstruction of the 48S complex, and UV-irradiated at 257 nm. To prevent nonspecific RNA–protein interactions, the mixture was supplemented with a tenfold excess (by weight) of single-stranded RNA. After irradiation, the products were digested with RNases and resolved by PAGE according to Laemmli.

Mutual effects of translation initiation factors and PTB in interacting with the IRES are shown in Fig. 3. Crosslinking with eIF3 was increased by eIF4F (Fig. 3a), which is natural because the central region of eIF4G interacts with eIF3. In turn, eIF3 increased the crosslinking of PTB (Fig. 3b), while the effect of eIF4F + eIF4A was opposite (Fig. 3c). The extent of eIF4A crosslinking was much the same at various combinations of other proteins. A band of labeled eIF4G was absent from the patterns, which is not surprising. First, eIF4G is large (similar in mobility to a 220-kDa protein), and its crosslinking product hardly enters the gel. Second, a region interacting with eIF4G contains only a few nonpaired [^{32}P]U.

The most interesting finding was an intense band of the crosslinking product of eIF4E (which commonly forms two bands, most likely, as a result of partial phosphorylation), which was observed in the case of PTB binding to the IRES (Fig. 3d). The interaction of eIF4E with the EMCV RNA probably lacks any functional significance, because eIF4E is unnecessary for the interaction of eIF4F (more exactly, its large subunit eIF4G) with the IRES of the EMCV RNA (see above). We think that a dramatic increase in eIF4E crosslinking indicates that, on interaction with PTB, a eIF4G region adjacent to the eIF4E-binding site comes into contact with an IRES site. As a result of this conformational rearrangement of the IRES and/or eIF4G, the latter possibly becomes more competent for further events of translation initiation on the EMCV IRES. For instance, the rearrangement may correct the contacts between RNA and other translation initiation factors, which interact with eIF4G and participate in recognizing the initiation codon.

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REFERENCES

1. Hershey J.W.B., Merrick W.C. 2000. The pathway and mechanism of initiation of protein synthesis. In *Translational Control of Gene Expression* (Eds. Sonenberg N. *et al.*), Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 33–88.
2. Pestova T.V., Kolupaeva, V.G., Lomakin I.B., Pilipenko E.V., Shatsky I.N., Agol V.I., Hellen C.U.T. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci. USA.* **98**, 7029–7036.
3. Pause A., Methot N., Svitkin Y., Merrick W.C., Sonenberg N. 1994. Dominant negative mutants of mammalian translation initiation factor eIF4A define a crucial role

for eIF4F in cap-dependent and cap-independent initiation of translation. *EMBO J.* **13**, 1205–1215.

4. Lomakin I.B., Hellen C.U.T., Pestova T.V. 2000. Physical initiation of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G with to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. *Mol. Cell. Biol.* **20**, 6019–6029.
5. Lamphear B. J., Kirchweger R., Skern T., Rhoads R.E. 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translation initiation. *J. Biol. Chem.* **270**, 21975–21983.
6. Kolupaeva V.G., Pestova T.V., Hellen C.U.T., Shatsky I.N. 1998. Translation eukaryotic initiation factor 4G recognizes a specific structural element within the internal ribosome entry site of encephalomyocarditic virus RNA. *J. Biol. Chem.* **273**, 18599–18604.
7. Lopez de Quinto S., Lafuente E., Martinez-Salas E. 2001. IRES interaction with translation initiation factors: functional characterization of novel RNA contacts with eIF3, eIF4B, and eIF4GII. *RNA.* **7**, 1213–1226.
8. Shatsky I.N. 2001. Reconstruction of initiation complexes *in vitro* to study the molecular mechanisms of translation initiation in mammals. *Mol. Biol.* **35**, 628–637.
9. Borovjagin A., Pestova T., Shatsky I. 1994. Pyrimidine tract binding protein strongly stimulates *in vitro* encephalomyocarditis virus RNA translation at the level of preinitiation complex formation. *FEBS Lett.* **351**, 299–302.
10. Kaminski A., Hunt S.L., Patton J.G., Jackson R.J. 1995. Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. *RNA.* **1**, 924–938.
11. Pilipenko E.V., Pestova T.V., Kolupaeva V.G., Khitrina E.V., Poperechnaya E.V., Agol V.I., Hellen C.U.T. 2000. A cell cycle-dependent protein serves as a template-specific translation initiation factor. *Genes Dev.* **14**, 2028–2045.
12. Belsham G.J., Jackson R.J. 2000. Translation initiation on picornavirus RNA. In *Translational Control of Gene Expression* (Eds. Sonenberg N. *et al.*), Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 869–900.
13. Boussadia O., Niepmann M., Creancier L., Prats A-C., Dautry F., Jacquemin-Sablon H. 2003. Unr is required *in vivo* for efficient initiation of translation from the internal ribosome entry sites of both rhinovirus and poliovirus. *J. Virol.* **77**, 3353–3359.
14. Pestova T.V., Hellen C.U.T., Shatsky I.N. 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol. Cell. Biol.* **16**, 6859–6869.
15. Kaminski A., Jackson R.J. 1998. The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *RNA.* **4**, 626–638.