YB-1 Autoregulates Translation of Its Own mRNA at or prior to the Step of 40S Ribosomal Subunit Joining

Olga V. Skabkina,† Dmitry N. Lyabin,† Maxim A. Skabkin, and Lev P. Ovchinnikov*

Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region, Russia

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YB-1 is a member of the numerous families of proteins with an evolutionary ancient cold-shock domain. It is involved in many DNA- and RNA-dependent events and regulates gene expression at different levels. Previously, we found a regulatory element within the 3′ untranslated region (UTR) of YB-1 mRNA that specifically interacted with YB-1 and poly(A)-binding protein (PABP); we also showed that PABP positively affected YB-1 mRNA translation in a poly(A) tail-independent manner (O. V. Skabkina, M. A. Skabkin, N. V. Popova, D. N. Lyabin, L. O. Penalva, and L. P. Ovchinnikov, J. Biol. Chem. 278:18191–18198, 2003). Here, YB-1 is shown to strongly and specifically inhibit its own synthesis at the stage of initiation, with accumulation of its mRNA in the form of free mRNPs. YB-1 and PABP binding sites have been mapped on the YB-1 mRNA regulatory element. These were UCCAG/ACAA for YB-1 and a ~50-nucleotide A-rich sequence for PABP that overlapped each other. PABP competes with YB-1 for binding to the YB-1 mRNA regulatory element and restores translational activity of YB-1 mRNA that has been inhibited by YB-1. Thus, YB-1 negatively regulates its own synthesis, presumably by specific interaction with the 3′UTR regulatory element, whereas PABP restores translational activity of YB-1 mRNA by displacing YB-1 from this element.

The mammalian Y-box-binding protein 1 (YB-1), also known as p50, dbpB, MSY-1, and EF1A, is a member of the multifunctional family of DNA/RNA binding proteins with an evolutionarily conservative cold-shock domain (26). Some prokaryotic members of this protein family were identified as major cold-shock proteins, because their synthesis is strongly enhanced by decreasing temperature, and they serve for bacterial adaptation to growth at low temperature (15).

Upon binding to DNA, YB-1 functions as a transcription factor and regulates expression of genes with a Y box in their promoters (34). In addition, YB-1 is involved in reparation (17, 21) and, probably, in replication of DNA (16). Through interaction with mRNA nuclear precursors, this protein participates in alternative splicing of mRNA (5, 33). In the cytoplasm, YB-1 serves as the main mRNA packaging protein (31) and regulates the life time (11) and mRNA template activity in protein synthesis (13, 23).

YB-1 has a dual effect on protein synthesis depending on the YB-1/mRNA ratio. At a low ratio typical for polysomal mRNPs, YB-1 stimulates translation at the stage of initiation (12, 23, 29). At an increased ratio corresponding to free mRNPs, YB-1 inhibits the protein synthesis both in vitro (23, 24) and in vivo (7) at the very beginning of translation initiation by displacing the translation initiation factor eIF4G from mRNP (24).

At the cellular level, YB-1 increases cell resistance to ionizing radiation and DNA-damaging chemicals; it can serve as an early marker of multiple drug resistance and induces resistance to oncogenic transformation by the phosphatidylinositol 3-kinase/Akt-kinase pathway (1, 19). The above data suggest that the nucleocytoplasmic distribution of YB-1, as well as its content within the cell, should be under strict control.

Recently, we have found a regulatory element within 3′ untranslated region (UTR) of YB-1 mRNA that specifically interacts with two major proteins of cytoplasmic mRNPs, the poly(A)-binding protein (PABP) and YB-1. As a result, PABP selectively activates translation of YB-1 mRNA, irrespective of whether or not it has a 3′ poly(A) tail (32).

Here, we have analyzed the YB-1 effect on YB-1 mRNA translation. It was found that YB-1 can completely suppress its own synthesis at low concentrations that have a stimulating effect on translation of other mRNAs. The inhibition occurs at or prior to joining of the 40S ribosomal subunit to YB-1 mRNA. We have mapped the YB-1 and PABP binding sites on the regulatory element of YB-1 mRNA and have shown that YB-1 binds to two sequences (nucleotides [nt] 1133 to 1145 and 1165 to 1172) containing the same 8-nt motif (UCCAG/ACAA), and PABP interacts with an A-rich sequence of about 50 nt in length (nt 1149 to 1196). The binding sites of YB-1 and PABP overlap, and these two proteins compete for binding to the regulatory element of YB-1 mRNA. In addition, PABP can restore the translation of YB-1 mRNA inhibited by YB-1.

MATERIALS AND METHODS

Plasmid construction. The pBluescript II SK YB-1 construct containing YB-1 cDNA, the pBluescript II SK YB-1 A50 construct containing YB-1 cDNA with the 50 nucleotide-long poly(A) tail, and the pSp36T-LucA50 construct encoding the 5′UTR of β-globin mRNA fused with the luciferase coding sequence were described earlier (32, 36).

The pSp73 Δ3 part subfr construct was prepared as follows. The 3′UTR fragment of YB-1 cDNA, containing the last part of subfragment I (C1127 to C1203), was amplified by PCR from pBluescript II SK 3′UTR YB-1 (32). The forward primer was 5′-CGAGCAGCTGCGGUUUAGUCA-3′, and the reverse primer was 5′-CCCCGGGATCCACGTCAATGTC-3′. The PCR product was digested with PvuII and BamHI and cloned into the corresponding sites in pSp73 (Promega).
In vitro transcription. Luciferase cap$^\bullet$ poly(A)$^\bullet$ mRNA was transcribed by SP6 RNA polymerase from pSP36T-LucA50 linearized with BglII. Transcription of YB-1 mRNA and of its fragment were carried out by T7 RNA polymerase. The DNA template for YB-1 cap$^\bullet$ poly(A)$^\bullet$ mRNA was pBlueScript II SK YB-1 linearized with BamHI; for YB-1 cap$^\bullet$ poly(A)$^\bullet$ mRNA, it was pBlueScript II SK YB-1 A50 linearized with Smal; for the last part of subfragment I (78 nt), pSP73 2part subfrI was linearized with BspTI.

The transcription was performed as described previously (30). Capped transcripts were obtained by a reaction where a mixture of 0.2 mM GTP and 0.8 mM m7GpppG (Amersham Biosciences) was used instead of 5 mM GTP. To generate the $^{32}$P-labeled fragment of YB-1 mRNA, [$^{32}$P]UTP (2,000 Ci/mM; Radioisotop, Obninsk, Russia) was added to the reaction mixture, and the concentration of unlabeled UTP was reduced to 0.05 mM.

YB-1 and PABP purification. Recombinant YB-1 and PABP were purified as described previously (18, 35).

In vitro translation assays. Translation of exogenous mRNA in a rabbit reticulocyte cell-free system was performed as described previously (28). The incubation mixture (15 μl) contained a micrococcal nuclease (MN)-treated reticulocyte lysate (7.5 μl), 10 mM HEPES-KOH (pH 7.6), 100 mM potassium acetate, 1 mM magnesium acetate, 8 mM creatine phosphate, 0.5 mM spermidine, 0.2 mM GTP, 0.8 mM ATP, 1 mM dithiothreitol, 25 μM each of 20 amino acids, except for Met and $^{[35]}$Met ($\sim$1,000 Ci/ml; Amersham Biosciences). A volume of 0.075 or 0.15 pmol of appropriate mRNA was added to the mixture as indicated in figure legends. Translation was performed at 30°C for 1.5 h. $^{[35]}$Met- and polyadenylation-protected proteins were analyzed by sodium dodecyl sulfate–20% polyacrylamide gel electrophoresis (SDS–12% PAGE) followed by autoradiography. To determine the RNA sequence protected by YB-1, A nucleotides of RNA were modified with 2 μl of diethyl pyrocarbonate (Fliuka) in a final volume of 20 μl (10 mM HEPES-KOH [pH 7.6], 100 mM KCl, 0.15 μg of tRNA/μl) at 20°C for 25 min. RNA was precipitated twice with ethanol, dissolved in 20 μl of 1 M amine, pH 4.5, and incubated at 60°C for 20 min in the dark. It was then precipitated twice with ethanol, dissolved in 8 M urea, and analyzed by 12% PAGE with 8 M urea followed by autoradiography. To determine the RNA sequence protected by YB-1, chemical sequencing was used (27). After incubation with an increasing amount of YB-1, A nucleotides of RNA were modified with 2 μl of diethyl pyrocarbonate (Fliuka) in a final volume of 20 μl (10 mM HEPES-KOH [pH 7.6], 100 mM KCl, 0.15 μg of tRNA/μl) at 20°C for 25 min. RNA was precipitated twice with ethanol, dissolved in 20 μl of 1 M amine, pH 4.5, and incubated at 60°C for 20 min in the dark. It was then precipitated twice with ethanol, dissolved in 8 M urea, and analyzed by 12% PAGE with 8 M urea followed by autoradiography. Partial alkaline hydrolysis of RNA was performed in the presence of 40 mM NaHCO$_3$, or Na$_2$CO$_3$, pH 9.0, and 1 mM EDTA at 90°C for 15 min, followed by ethanol precipitation.

Electrophoretic mobility shift assay. The $^{32}$P-labeled last part of 3′ UTR subfragment I (0.06 pmol, 10,000 cpm) was incubated with YB-1 or PABP in a final volume of 10 μl (10 mM HEPES-KOH [pH 7.6], 100 mM KCl, 0.5 mM dithiothreitol, 1 mg of bovine serum albumin/μl, 100 μg of tRNA/ml) at 30°C for 10 min. Increasing amounts of PABP or YB-1 were added, and the incubation was continued for 10 min. RNA-protein complexes were analyzed by 6% PAGE in 0.5× Tris-borate-EDTA buffer followed by autoradiography. UV cross-linking assay. YB-1, PABP, or a mixture of the two was incubated with radiolabeled RNA (100,000 cpm) in a final volume of 18 μl (10 mM HEPES-KOH [pH 7.6], 100 mM KCl, 500 μg of casein/ml, 100 μg of tRNA/ml) at 30°C for 15 min. The reactions were irradiated at 0.4 J/cm$^2$ in a transilluminator–cross-linker (Vilber-Lourmat), incubated for 45 min at 37°C with 0.05 U of Mn$^{2+}$ and 0.5 μg of RNase A/μl, and analyzed by SDS–10 to 22% PAGE followed by autoradiography.

RESULTS

Exogenous YB-1 selectively inhibits translation of its own mRNA. The effect of YB-1 on translation of its own mRNA in comparison to that on luciferase mRNA was studied (Fig. 1). A mixture of equal amounts of the two mRNAs with or without the poly(A) tail was translated in a rabbit reticulocyte cell-free system with $^{[35]}$Met in the presence of increasing amounts of YB-1. The proteins were separated by SDS-PAGE, and...
[\textsuperscript{35}S]Met-labeled translation products were detected by autoradiography. We found that small amounts of YB-1 slightly stimulated translation of luciferase mRNA strongly inhibited translation of YB-1 mRNA. This inhibitory effect of YB-1 was independent of the presence or absence of the YB-1 mRNA poly(A) tail (Fig. 1). The used amounts of YB-1 did not inhibit translation of natural globin mRNA (data not shown). The above data allowed us to conclude that YB-1 mRNA translation is autoregulated and that at YB-1 concentrations optimal for translation of other cellular mRNAs, the synthesis of YB-1 mRNA association with the 43S preinitiation complex or at a step preceding its interaction with translation initiation factors.

Exogenous YB-1 inhibits translation initiation at or prior to the step of 40S ribosomal subunit joining. To learn at which step the initiation of YB-1 mRNA translation is inhibited by YB-1, we had to know in what complexes YB-1 mRNA was accumulated. So YB-1 was added to the cell-free translation system with YB-1 mRNA (0.15 pmol) were incubated for 10 min at 30°C without any additions (A), with 2.8 pmol of YB-1 added (B), with 1 mM m\textsuperscript{7}GpppG (C), or with edeine at a final concentration of 2 \mu M (D). After incubation, the systems were subjected to centrifugation through 15 to 30% linear sucrose gradients for 3 h at 45,000 rpm in an SW-60 rotor. UV absorbance profiles at 254 nm (-) and \textsuperscript{32}P radioactivity (○) are shown.

![FIG. 2. YB-1 inhibits translation of YB-1 mRNA at the step of 40S ribosomal subunit joining to mRNA. Rabbit reticulocyte cell-free translation systems with YB-1 mRNA (0.15 pmol) were incubated for 10 min at 30°C without any additions (A), with 2.8 pmol of YB-1 added (B), with 1 mM m\textsuperscript{7}GpppG (C), or with edeine at a final concentration of 2 \mu M (D). After incubation, the systems were subjected to centrifugation through 15 to 30% linear sucrose gradients for 3 h at 45,000 rpm in an SW-60 rotor. UV absorbance profiles at 254 nm (-) and \textsuperscript{32}P radioactivity (○) are shown.](https://example.com/fig2.png)
synthesis of YB-1 through its specific interaction with the regulatory element within YB-1 mRNA (32). Here we have shown that YB-1, which specifically binds to the same regulatory element, inhibits translation of its own mRNA. The simplest assumption explaining the mechanism of this effect is that the translational activity of YB-1 mRNA is strongly dependent on which of these two proteins (YB-1 or PABP) interacts with its regulatory element. Therefore, we learned whether PABP restored YB-1 mRNA translation inhibited by YB-1 upon displacement of the latter from the 3'UTR regulatory element. A mixture of equal amounts of YB-1 and Luc mRNAs was preincubated with YB-1, and then increasing amounts of PABP were added and the mRNAs were translated in a rabbit cell-free system (Fig. 5). Because PABP can stimulate translation per se, we also studied its effect on translation without YB-1 addition (Fig. 5, lanes 8 to 10). We found that YB-1 mRNA translation, which had been up to 90% inhibited by YB-1 (Fig. 5, lane 4), could be effectively restored to its initial level by PABP.

**DISCUSSION**

YB-1 controls overall protein synthesis, depending on the amount of YB-1 associated with mRNA; it can either stimulate (at a relatively low YB-1/mRNA ratio) or completely suppress (upon mRNA saturation with YB-1) translation of various mRNAs.

Here we report that YB-1 inhibits its own synthesis in vitro at concentrations that are optimal for translation of other mRNAs, i.e., YB-1 autoregulates its own synthesis and keeps its own concentration optimal for overall protein synthesis.

To determine the translation stage at which YB-1 autoregulates its own synthesis, we considered its effect on sedimentation distribution of YB-1 mRNA. It was shown that the inhibition by YB-1 results in accumulation of YB-1 mRNA in the form of free mRNPs. This indicates that the inhibition occurs at the initiation stage at or prior to the step of the 40S ribosomal subunit joining to mRNA. The joining should be preceded by the formation of a 43S preinitiation complex and by mRNA interaction with some translation initiation factors. As we demonstrated earlier, YB-1 does not affect the assembly of the 43S preinitiation complex (24), and therefore it most likely can prevent mRNA binding to translation initiation factors. Translation of other mRNAs was inhibited by high concentrations of YB-1, first of all due to displacement of the translation initiation factor eIF4G from its complex with mRNA (24). However, the mechanism of YB-1 action on YB-1 mRNA can differ from that of its action on other mRNAs, because smaller amounts of YB-1 are required for this. At present, it can only
be stated that the inhibition is not a result of eIF4E displacement, because it is also observed in the case of uncapped YB-1 mRNA (data not shown). Interestingly, the 5'UTR of YB-1 mRNA is GC rich (72.1%) and can form a highly developed secondary structure. It was shown that increased amounts of eIF4A and eIF4B are required for normal translation of mRNAs with a highly structured 5'UTR (9). It is probable that the inhibiting action of YB-1 on translation of its own mRNA is underlain by preventing eIF4A or eIF4B binding to mRNA.

Previously, we found a regulatory element within the 3'UTR of YB-1 mRNA that interacts specifically, with a high affinity, with both major mRNP proteins (YB-1 and PABP) and demonstrated that PABP stimulates translation of YB-1 mRNA in a poly(A) tail-independent manner (32). In this study we have shown that, in contrast, YB-1 inhibits its own synthesis.

We proposed that the specific binding sites of YB-1 and PABP at the regulatory element of YB-1 mRNA overlap, and that the two proteins can compete for the binding site on mRNA. Such a competition can underlie regulation of YB-1 synthesis.

Examples of such a regulation of protein synthesis can be illustrated by a specific binding site of YB-1 and PABP at the regulatory element of YB-1 mRNA overlap, and that the two proteins can compete for the binding site on mRNA. Such a competition can underlie regulation of YB-1 synthesis.

FIG. 4. PABP and YB-1 can displace each other from the last part of 3'UTR subfragment I of YB-1 mRNA. (A and B) Mobility shift assays; (C) UV cross-linking. (A) The 32P-labeled last part of subfragment I (0.06 pmol, 10,000 cpm) was preincubated for 10 min at 30°C with 2.2 pmol of YB-1 (lanes 2 to 7) or without YB-1 (lanes 1, 8, and 9) and for an additional 10 min in the presence of increasing amounts of PABP (0.12 pmol, lanes 3 and 8; 0.42 pmol, lane 4; 1.28 pmol, lane 5; 2.57 pmol, lane 6; 7.7 pmol, lanes 7 and 9). RNA-protein complexes were analyzed by PAGE under nondenaturing conditions and visualized by autoradiography. (B) The 32P-labeled last part of subfragment I (0.06 pmol, 10,000 cpm) was preincubated for 10 min at 30°C with 0.28 pmol (lanes 2 to 7) or 2.57 pmol (lane 8) of PABP or without PABP (lanes 1 and 9) and for an additional 10 min in the presence of increasing amounts of YB-1 (3 pmol, lanes 3 and 9; 6 pmol, lane 4; 9 pmol, lane 5; 12 pmol, lane 6; 15 pmol, lane 7). RNA-protein complexes were analyzed by PAGE under nondenaturing conditions and visualized by autoradiography. (C) The 32P-labeled last part of subfragment I (100,000 cpm) was preincubated for 10 min at 30°C with 0.14 pmol of PABP (lanes 2 to 7) and for an additional 10 min in the presence of increasing amounts of YB-1 (0.14 pmol, lanes 1 and 3; 0.28 pmol, lane 4; 0.56 pmol, lane 5; 1.12 pmol, lane 6; 2.24 pmol, lane 7). RNA-protein complexes were UV cross-linked, treated with RNase A and MN, analyzed by SDS-PAGE, and visualized by autoradiography.

FIG. 5. PABP can recover translation of YB-1 mRNA inhibited by YB-1. cap-poly(A) YB-1 and Luc mRNAs (0.075 pmol each) were preincubated for 10 min at 30°C with 5.6 pmol (lane 3) or 11.2 pmol (lanes 4 to 7) of YB-1 or without YB-1 (lanes 8 to 10) and for an additional 10 min in the presence of increasing amounts of PABP: 0.7 pmol (lanes 5 and 8), 1.4 pmol (lanes 6 and 9), and 2.8 pmol (lanes 7 and 10). Translation reactions were carried out in 15-μl aliquots for 1.5 h at 30°C. [35S]Met-labeled translation products were resolved by SDS-PAGE and visualized by autoradiography.
found in the literature. Regulation of translation of nanos mRNA in Drosophila embryos was reported to occur due to competition between the Smaug repressor and an unknown activator for binding to overlapping nucleotide sequences within the 3′UTR of this mRNA (6). Sometimes the repressor and activator do not hamper binding of each other and do not compete directly, while the regulation is based on the ratio between the activator and repressor proteins associated with mRNA. gpl1 mRNA in C. elegans embryos can be taken as an example. The protein with zinc finger domains POS-1 suppresses translation of gpl1 mRNA by interacting with a regulatory element within the 3′UTR, and the SPN-4 protein activates gpl1 mRNA translation through binding to the other sequence of its 3′UTR and interacting with POS-1 (25).

To answer the question as to whether the binding sites of the two major mRNP proteins (YB-1 and PABP) overlap on YB-1 mRNA, we have mapped the binding sites of these proteins on the regulatory element of the YB-1 mRNA 3′UTR. It was found that PABP binds to an approximately 50-nt A-rich sequence (nt 1149 to 1196). This length could be enough for PABP to bind to poly(A) with a periodicity of 27 nucleotides (2). Suppression of translation of gpl1 mRNA by interacting with a regulatory element within the 3′UTR, and the SPN-4 protein activates gpl1 mRNA translation through binding to the other sequence of its 3′UTR and interacting with POS-1 (25).

Next we studied whether YB-1 and PABP can compete for binding to the regulatory element of YB-1 mRNA. Using the gel-shift and UV cross-linking assays, it was shown that, indeed, PABP and YB-1 can displace each other from the regulatory element. No complexes containing both YB-1 and PABP were observed.

Cell-free translation system experiments gave more evidence for competition between the two proteins. It was found that PABP can efficiently restore YB-1 mRNA translation inhibited by YB-1. So, we believe that YB-1 autoregulates its own synthesis through its specific interaction with a sequence within the 3′UTR of its own mRNA, and PABP can restore the YB-1 mRNA translational activity by displacing YB-1 from this sequence.

The above data allow us to conclude that YB-1 synthesis is controlled by two major proteins of cytoplasmic mRNP, YB-1 and PABP. Eukaryotic mRNAs always exist in the form of mRNP in association with mRNP proteins. The translational status of mRNAs depends on the amount of these two major cytoplasmic mRNP proteins as well as on their ratio. As a rule, actively translatable polysomal mRNP have both of these proteins, whereas free untranslatable mRNP have two times more YB-1 and no PABP at all (23). PABP is known to inhibit its own synthesis (3, 4, 8). At the same time, our previous results show that it can also be involved in regulation of YB-1 synthesis along with YB-1 per se. Taken together, the three regulatory processes can control the content and ratio of the two major mRNP proteins by maintaining their amounts at the level providing a maximal translational activity of other cellular mRNAs and thus controlling the overall protein synthesis (Fig. 6).

PABP mRNA is a member of the TOP mRNA family whose translation is activated by nutritional stimulation (22). The PABP synthesized is bound first of all to poly(A) tails of mRNAs and activates their translation. However, if the situation lasts for a long time, overproduction of the protein would occur in the cell. This does not happen, because after filling all vacant sites on poly(A), PABP can interact with a 61-nt A-rich element within the 5′UTR of its mRNA (4, 8) and with a sequence within the 3′UTR of YB-1 mRNA, for which it has a lower affinity than for poly(A) (32). Further synthesis of PABP will be inhibited, and YB-1 mRNA translation will be activated. The synthesized YB-1 will autoinhibit its own synthesis, thus preventing overproduction of YB-1 in the cell.

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