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Positive and Negative Effects of the Major Mammalian Messenger Ribonucleoprotein p50 on Binding of 40 S Ribosomal Subunits to the Initiation Codon of β-Globin mRNA*

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p50, the major core protein bound to mammalian mRNAs, has been reported to stimulate translation at low p50/mRNA ratios and inhibit translation at high p50/mRNA ratios. This study aims to address the molecular mechanisms underlying these phenomena using the *in vitro* assembly of 48 S preinitiation complexes from fully purified translational components in the presence or absence of p50 as analyzed by the toeprint assay. With limited concentrations of eIF2, eIF3, and eIF4F, p50 (but not pyrimidine tract-binding protein, which was taken for comparison) strongly stimulates formation of the 48 S preinitiation complexes with β-globin mRNA. This stimulation is observed when just a few molecules of p50 are bound per molecule of the mRNA. When the amount of p50 in solution is increased over some threshold p50/mRNA ratio, a remarkable repression is observed that can still be relieved by adding more eIF2 and eIF4F. At even higher concentrations of p50, the inhibitory effect becomes irreversible. The threshold ratio depends upon the extent of secondary structure of the 5′-untranslated region linked to the β-globin coding region. Chemical probing has confirmed that the binding of p50 to mRNA involves only the sugar-phosphate backbone of the mRNA leaving nucleotide bases free for interaction with other messenger ribonucleoprotein (mRNP) components. These data are best compatible with the functional role of p50 as a "manager" of mRNA-protein interactions in mammalian mRNPs.

mRNPs[1] isolated from the cytoplasm of different mammalian cells contain two major core proteins that migrate in the SDS-PAGE electrophoresis as 70- and 50-kDa proteins (1). The 70-kDa protein (poly(A)-binding protein) is bound to the poly(A) tail of mRNA and is the most widely studied mRNP to date, both structurally and functionally (2–5). The functional role of p50, which is tightly bound to other parts of mRNA molecules, is poorly understood.

According to its amino acid sequence and affinity for DNA, p50 was identified as a member of the family of cold shock domain-containing proteins that is evolutionarily conserved from bacteria to man (6). Its actual relative molecular mass was determined as 36 kDa (6). Some proteins of this family are known as transcription factors affecting expression of genes containing Y-box sequence elements in their promoters (7–9). The bulk of mammalian p50, however, is localized in the cytoplasm where it is tightly bound to mRNAs (10). Its ubiquitous occurrence in mammalian cells (1), the fact that the protein has a low specificity for RNA in *in vitro* binding experiments (6–11), and the fact that its content in mammalian mRNAs correlates with their translatability (12, 13) and stability (13) suggests that p50 may fulfil the role of a general modulator of mRNA translational activity.

In support of this hypothesis, it has been shown earlier that p50 strongly inhibits translation of exogenous mRNA in cell-free translation systems (12–15) as well as during translation *in vivo* of mRNA expressed from a reporter gene (10). In *Xenopus* oocytes, two proteins closely related to p50 were reported to be responsible for the masked state of mRNA (15–19). Therefore, it is conceivable that mammalian p50 plays a similar role thereby determining the level of translational repression of mRNAs. However, we have demonstrated that p50 not only inhibits but also stimulates translation depending on the p50/mRNA ratio in a cell-free system (12, 20). These experiments were performed with the use of a rabbit reticulocyte lysate depleted of p50 with specific antibodies. The p50-depleted lysate showed a low translational activity, whereas addition of p50 partially restored the level of polypeptide synthesis. We have also shown that p50 exerts its effect at the level of translation initiation rather than at the level of elongation or termination of polypeptide synthesis (20). To understand the mechanisms at play, additional experimentation in a more defined system is required. In addition, the depleted lysate system did not allow us to judge which step of translation initiation was stimulated or inhibited by the protein.

In this report, we present experiments designed to address the molecular mechanisms of the action of p50 on translation initiation using the *in vitro* assembly of 48 S preinitiation complexes from purified translational components in the presence of different amounts of p50. The yield of reconstituted complexes was monitored by the toeprint assay (21). It was found that p50 affects translation initiation at the level of 48 S preinitiation complex formation. At a low p50/mRNA ratio, the protein stimulates binding of 40 S ribosomal subunits to the initiation triplet of β-globin mRNA. This stimulation was spe-

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[1] The abbreviations used are: mRNP, messenger ribonucleoprotein; PTB, pyrimidine tract-binding protein; eIF, eukaryotic initiation factor; nt, nucleotide(s).

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cific for p50 rather than a common feature of cytoplasmic mRNA-binding proteins. At p50 concentrations exceeding some threshold p50/mRNA ratio, a remarkable repression is observed, the threshold ratio being dependent on the secondary structure of the 5′-untranslated region linked to the β-globin coding region.

EXPERIMENTAL PROCEDURES

Preparation of RNA—Native globin mRNAs were isolated from rabbit reticulocyte polysomes washed with 0.5 M KCl. Total RNA was phenol-extracted from 1000 A_{260} units of the salt-washed ribosomes, and poly(A^+) RNA was isolated with the use of a poly(A^+) RNA isolation kit (Amersham Biosciences). The mRNA was then layered on a 5–20% sucrose gradient in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% SDS and centrifuged at 4 °C in a SW41 rotor (Beckman) for 15 h at 35,000 rpm. The material from the 9 S region of the gradient was pooled, and the globin mRNA was precipitated with ethanol. Polyacrylamide gel electrophoresis showed no contamination of the globin mRNA with ribosomal RNA. The isolated material represented a mixture of α- and β-globin mRNAs. These RNAs behave similarly in reconstitution of 48 S preinitiation complexes and have a similar size (22). In this study, the 48 S preinitiation complex was analyzed only for formation of the 48 S preinitiation complex with β-globin mRNA. [^{32}P]β-Globin mRNA was synthesized *in vitro* by a T3 transcription reaction using the plasmid pβ-Glo linearized with *Sac*I. pβ-Glo represented a β-globin cDNA inserted between the *Hin*dIII and *Sac*I sites of the plasmid pBS (Stratagene). $(CAA)_n$-β-globin mRNA was obtained by T7 transcription of plasmid p$(CAA)_n$-βGlo linearized with *Sac*I. The plasmid was constructed by replacement of the β-glucuronidase (GUS) coding region in the p$(CAA)_n$-GUS vector at the *Nco*I site with the β-globin coding region produced using PCR. p$(CAA)_n$-GUS vector was a gift of Dr. I.V. Boni (Moscow, Russia).

Preparation of Factors, 40 S Ribosomal Subunits, mRNA-binding Proteins, and [^{35}S]Methionine-labeled Met-tRNA$_i^{Met}$—40 S ribosomal subunits and factors eIF2, eIF3, and eIF4F were prepared from rabbit reticulocyte lysate as described previously (21, 24). Recombinant eIF4A and eIF4B were purified as described elsewhere (21). Recombinant eIF1 was prepared from cells transfected with the expression plasmid pET-8c (Novagen) containing the coding sequence of eIF1 (25). The *Escherichia coli* cells with the recombinant plasmid were induced with isopropyl-1-thio-β-D-galactopyranoside and after 5 h at 23 °C were sonicated and clarified with a low speed centrifugation, and a 40–70% ammonium sulfate fraction was prepared from the supernatant. The dialyzed proteins were applied to a DEAE-cellulose column equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (buffer A100). The flow-through was applied to a Mono S HR 5/5 column (Amersham Biosciences), and eIF1 resolved at this step was concentrated and dialyzed against buffer A100. The eIF1A expression plasmid and factor eIF1A were prepared exactly as described by Pestova *et al.* (22), starting from the eIF1A cDNA-containing plasmid kindly provided by Dr. John Hershey. Purification of PTB and p50 was as described previously (20, 26). Both mRNA-binding proteins were purified to homogeneity using RNA affinity columns as the last step of purification. [^{35}S]Methionine-labeled Met-tRNA$_i^{Met}$ was prepared using calf liver total tRNA (Novagen, Madison, WI) and aminoacyl-tRNA synthetase isolated from *E. coli* MRE600 as described previously (21).

Assembly and Analysis of 48 S Preinitiation Ribosomal Complexes—Ribosomal 48 S preinitiation complexes were assembled as described earlier (21, 22), but the amounts of initiation factors eIF2, eIF3, and eIF4F were decreased. In this study, 0.2 μg (~1 pmol) of native globin mRNA was incubated with eIF1 (0.5 μg), eIF1A (0.5 μg), eIF2 (0.9 μg), eIF3 (1.2 μg), eIF4F (0.2 μg), eIF4A (0.5 μg), eIF4B (0.3 μg), Met-tRNA$_i^{Met}$ (1.5 pmol), and 40 S subunits (5 pmol) in a reaction volume of 20 μl for 10 min at 30 °C. p50 and PTB were included in some reaction mixtures as described in the text. 48 S complexes were analyzed by primer extension as described previously (21, 22) using primer 5′-TCACCACCAACTTCTTCCAC-3′ or 5′-CACATTCATTCACCTTGC-3′ complementary to nt 114–133 or nt 103–120 of the rabbit β-globin mRNA sequence, respectively. Electrophoresis of cDNAs was performed by denaturing 6% PAGE. Radioactive bands were visualized, and relative amounts of radioactivity in the bands were determined using a PhosphorImager (Molecular Dynamics).

Chemical Footprinting—The sugar-phosphate backbone of the RNA in the p50·β-globin mRNA binary complexes was probed with Fe(II)·EDTA according to the protocol described for the modification of ribosomal complexes (27). These complexes were formed in the buffer used for reconstitution of the 48 S preinitiation complexes.

Quantitative Western Blotting of p50 Bound to mRNA—β-Globin mRNA was synthesized *in vitro* with T3 polymerase in the presence of [α-^{32}P]UTP as described previously (21). The RNA was freed of unincorporated [α-^{32}P]UTP by gel filtration, and its specific radioactivity was determined by A_{260} and Cerenkov counting. Finally the integrity of the RNA was confirmed by denaturing 6% PAGE. The ^{32}P-labeled β-globin mRNA (5 pmol) was incubated with the corresponding amounts of all other translation initiation components under conditions of the 48 S complex formation (see above) with addition of 150 pmol of p50 (p50/mRNA ratio = 30). The mixture was incubated for 5 min at 30 °C and layered on a 5–20% sucrose gradient prepared in the reconstitution buffer. After centrifugation at 4 °C for 19 h at 33,000 rpm, the mRNP peak was isolated. The RNA content of the mRNP peak was determined by Cerenkov counting, and 60-μl aliquots along with different amounts of p50 were subjected to 10% PAGE. Proteins were transferred to nitrocellulose membranes under semidry conditions (SemiPhor, Hoefer Scientific Instruments), and then membranes were processed as described earlier (21) using polyclonal rabbit p50 antiserum diluted 5000-fold (20). Bands were revealed using an ECL system. After scanning the bands, the amount of p50 in the aliquots of the mRNA peak was determined using the calibration plot obtained from control bands of p50 (run on the same gel, see above).

RESULTS

p50 Affects the Binding of 40 S Ribosomal Subunits to the Initiation Codon in the in Vitro Assembly of 48 S Preinitiation Complexes from Purified Components—For the reconstitution assay we used a set of purified canonical initiation factors (eIF1, eIF1A, eIF2, eIF3, eIF4F, eIF4A, and eIF4B), reticulocyte 40 S ribosomal subunits, total calf liver tRNA where only the initiator tRNA was charged with methionine, native globin mRNA, and p50 (added to this system in varying amounts). After a short incubation, a primer and reverse transcriptase were added to the mixture, and the length of cDNAs resulting from primer extension were determined. The influence of p50 on assembly of the 48 S preinitiation complex was quantitatively estimated by the intensity of toeprint bands at positions +16 to +18 downstream from the AUG initiation triplet. It has been well established that the arrest of reverse transcriptase occurs at positions +16 to +18 (toeprint bands) only when the initiator tRNA forms a codon-anticodon interaction with the mRNA initiation triplet in the P-site of the 40 S ribosomal subunit or 80 S ribosome (21, 28, 29). No arrest of primer extension at these positions occurs when the initiator tRNA is absent from the 40 S ribosomal subunit or 80 S ribosome.

No effect of p50 was found using published protocols (21, 22) for assembly of 48 S preinitiation complexes (data not shown). However, when the concentration of eIF2, eIF3, and eIF4F was reduced about 3-fold resulting in 7, 2, and 0.8 pmol/1 pmol of mRNA, up to a 4-fold stimulation by p50 was observed on the yield of 48 S preinitiation complexes (Fig. 1). (It should be noted that the yield of 48 S complexes was quantitated as the percentage of toeprint radioactivity to the total radioactivity in the same lane. In this way, any variation caused by positive or negative effects of p50 on the transcription reaction itself were excluded.) Similar toeprint results were obtained when using the primer 5′-CACATTCATTCACCTTCG-3′ (data not shown). Its annealing site is separated from the toeprint nucleotides by just 32 nucleotide residues.

Stimulation was observed before the p50/mRNA ratio exceeded 30 (Fig. 1, *lane 6*). When the amount of p50 was further increased 2-fold, a repression of 48 S complex formation was evident (Fig. 1, *lane 7*). This inhibitory effect could be relieved by adding more of initiation factors eIF4F and eIF2 (Fig. 2, *lanes 4*, *5*, and *8*). The strongest effect was demonstrated for initiation factor eIF2 (Fig. 2, *lane 8*), whereas excess eIF3 was slightly inhibitory (Fig. 2, *lanes 6* and *7*). A further increase of

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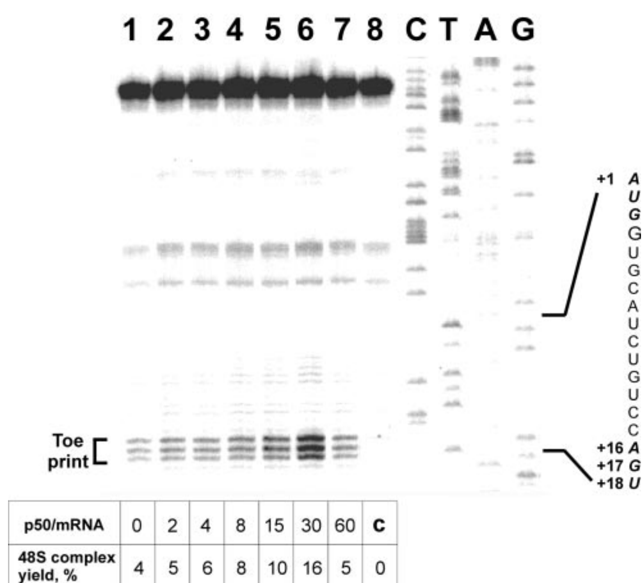


FIG. 1. **Effect of p50 on the reconstitution of the 48 S translation preinitiation complex with β-globin mRNA.** Primer extension inhibition (toeprinting) was performed in the presence or absence of p50. The p50/mRNA ratios indicated are molar ratios. "*c*" denotes a control sample where eIF2 was omitted to demonstrate the specificity of toeprint formation. A dideoxynucleotide sequence generated with the same primer (5′-TCACCACCAACTTCTTCCAC-3′) was run in parallel. The positions of the initiation AUG codon and toeprint are shown in *bold* on the *right* of the sequence. The yield of 48 S complexes was quantitated with a PhosphorImager as the ratio of radioactivity in toeprint bands relative to the total radioactivity of the respective lane.

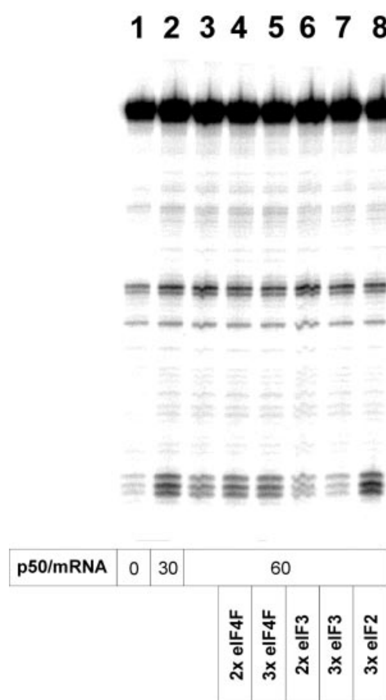
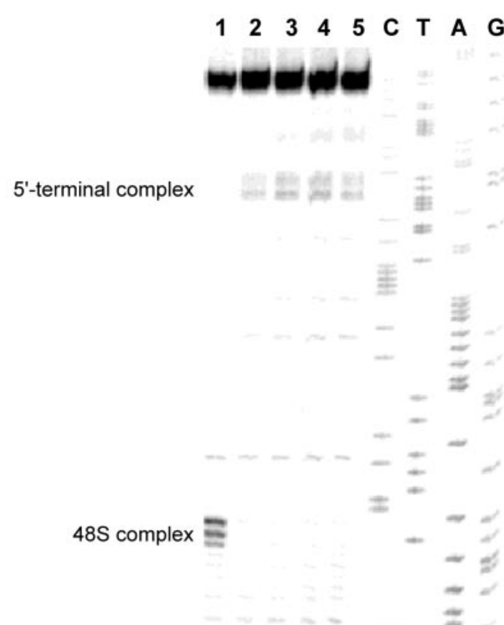


FIG. 2. **eIF2 and eIF4F relieve the inhibition of the 48 S complex formation at higher p50/mRNA ratios.** The formation of 48 S complexes was analyzed as indicated in the legend to Fig. 1, but dideoxynucleotide sequencing was omitted. *Lanes 1–3* contain standard amounts of the factors as indicated under "Experimental Procedures." The amount of eIF2, eIF3, and eIF4F added in *lanes 4–8* was 2- or 3-fold more than indicated under "Experimental Procedures."

the p50/mRNA ratio to 100 resulted in even greater suppression of 48 S complex formation, and addition of the same amounts of initiation factors (eIF2 or eIF4F) as indicated for Fig. 2 did not relieve this inhibition (data not shown).



p50/mRNA	0	0	10	30	60
eIF1 + eIF1a	+	−	−	−	−
5′-terminal complex yield, %	0	2,6	4,2	4,5	2,3

FIG. 3. **Effect of p50 on the reconstitution of the aberrant 5′-terminal 40 S·β-globin mRNA complex formed in the absence of initiation factors eIF1 and eIF1A.** The formation of 40 S ribosome·mRNA complexes was analyzed as indicated in the legend to Fig. 1.

p50 Is Able to Stimulate the Formation of Nonpositioned 48 S Complexes Bound near the 5′-End of mRNA—As has been shown by Pestova *et al.* (22), omission of initiation factors eIF1 and eIF1A from the reconstitution mixture results in formation of an aberrant 5′-terminal initiation complex. To be formed, this aberrant complex still requires all of the other translation initiation components including Met-$tRNA_i^{Met}$. Although this complex is not an intermediate of the true 48 S initiation complex, it was of interest to see whether p50 was able to stimulate ribosome binding *per se* or whether it acted only when all factors required for scanning to the start codon are present. In this way, one can get an idea whether p50, known to have prominent RNA unwinding characteristics (23), is involved in unwinding secondary structure of the 5′-leader of β-globin mRNA during the scanning process. As seen from Fig. 3 (*lanes 1* and *2*), omission of eIF1 and eIF1A resulted in appearance of the aberrant 5′-terminal initiation complex with concomitant disappearance of the authentic 48 S initiation complex. Addition of increasing amounts of p50 to this reconstitution mixture demonstrates stimulation of the 5′-terminal complex formation up to the same threshold p50/mRNA ratio = 30 as was found before for the authentic 48 S complex (Fig. 3, *lanes 3–5*). Thus, the stimulation effect is not based, at least entirely, on involvement of the protein in the scanning process of the 5′-leader of β-globin mRNA.

Ability of p50 to Promote Formation of 48 S Preinitiation Complexes from Purified Components at Low p50/mRNA Ratios Is Not a Common Property of mRNA-binding Proteins—One may suspect that any mRNA-binding protein with highly basic domains would promote assembly of the 48 S complex in the manner demonstrated above for p50. If so, the stimulation effect would be regarded as nonspecific. In this regard, one may recall an example of this kind: general RNA-binding proteins, as different as hnRNPA1, La autoantigen, PTB, and p50, all render translation in a rabbit reticulocyte lysate more cap-de-

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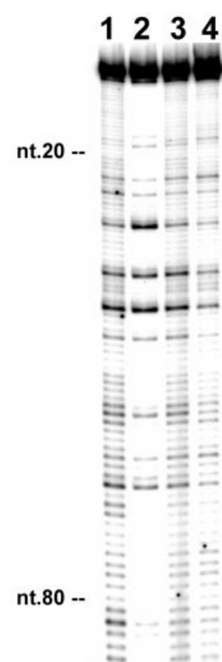
p50, µg	0	0.36	0.72	1.08	2.16	0	0	0	0	c
PTB, µg	0	0	0	0	0	0.36	0.72	1.08	2.16	

FIG. 4. **Comparative study of effects of p50 and PTB on the formation of 48 S preinitiation complexes with β-globin mRNA at different protein/mRNA ratios.** The amounts of p50 indicated under *lanes 2–5* correspond to p50/mRNA molar ratios of 10, 20, 30, and 60, respectively. "*c*" (*lane 10*) denotes a control where eIF2 was omitted from the incubation mixture.



p50/mRNA	0	0	0	30
eIF4A/mRNA	0	0	30	0
Fe(II)-EDTA	+	0	+	+

FIG. 5. **Interaction of p50 with the sugar-phosphate backbone of β-globin mRNA as revealed by Fe(II)·EDTA modification.** Binding characteristics of p50 are presented for the 5′-terminal part of β-globin mRNA (positions of nucleotides from the 5′ terminus of β-globin mRNA are shown to the *left* of the panel). Similar results were obtained for the next 100 nt of β-globin mRNA sequence (data not shown). *Vertical bars* to the *right* of *lane 4* show the mRNA sequences protected by p50 from chemical modification. *Lane 3* demonstrates the protection pattern for initiation factor eIF4A used as a control protein for unspecific uptake of the modifying reagent.

pendent irrespective of their cellular function and individual characteristics (30). To check this possibility, effects of the highly basic mRNA-binding protein PTB and p50 were compared in parallel assays of the 48 S preinitiation complex formation. As can be seen in Fig. 4, PTB does not stimulate the formation of the 48 S complex with β-globin mRNA; in fact, its addition is inhibitory. This is in agreement with our previous report that PTB did not stimulate translation of β-globin mRNA in PTB-depleted cell extracts (31).

At higher concentrations, PTB strongly inhibited not only formation of the 48 S complex but also the reverse transcription reaction as seen by the decrease of full-length product of primer extension (Fig. 4, *lanes 6–9*, *upper bands*). Presumably PTB interferes with either annealing of a primer to the mRNA chain or elongation of the cDNA product or both by virtue of its interaction with nucleotide bases (pyrimidines). In contrast, p50 does not inhibit the reverse transcription reaction (the yield of the full-length product) even at the p50/mRNA ratio of about 100 or more (when formation of 48 S complexes is strongly repressed; Fig. 2 and data not shown). Nucleotide bases appear to remain exposed in solution and available for interaction with RNA or protein components of translational machinery.

p50 Binds to the Sugar-Phosphate Backbone of mRNA and Reveals a Preference for Sequences with Completely Unpaired Nucleotide Bases—As follows from the results presented above, p50 does not inhibit reverse transcription even at high p50/mRNA ratios. These observations and the data reported earlier (6) suggest that p50 does not recognize nucleotide bases of mRNA. Chemical probing of p50·β-globin mRNA complexes confirmed this suggestion. No protection of nucleotide bases was found when p50·mRNA binary complexes were treated with dimethylsulfate (A and C residues) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (U residues). At low p50/mRNA ratios, protection of the sugar-phosphate backbone of mRNA from modification with Fe(II)·EDTA complexes was not seen either (data not shown).

At a p50/mRNA ratio of about 30, the sites of protection by p50 of the sugar-phosphate backbone are distributed along the whole length of β-globin mRNA with a preference for some sequences of the mRNA (Fig. 5, *lanes 1* and *4*). This preference may be accounted for by different potential for base pairing of different nucleotide sequences. In its turn, this may result in differential p50 binding to different parts of the mRNA. Indeed p50 is known to have a much higher affinity for single-stranded than double-stranded nucleic acids. Fig. 6 presents an obvious illustration of this feature. Here the effect of p50 on the 48 S complex assembly is shown for the $(CAA)_n$-β-globin mRNA. This mRNA differs from native β-globin mRNA in its 5′-untranslated region, $CAAGAA(CAA)_{19}CACCAUGG$. . . . , which makes up only ~1⁄10 of the entire molecule, and by the absence of a poly(A) tail. As has been shown by Tzareva *et al.* (32), the $(CAA)_n$-leader has a 100% single-stranded conformation. It has reduced requirements for some initiation factors and is extremely efficient in the 48 S complex formation.[2] With the $(CAA)_n$-β-globin hybrid mRNA, a remarkable inhibition of the toeprint is observed at the p50/mRNA ratio as low as 10 (Fig. 6, *lane 2*) that is 6-fold lower than the ratio with a similar inhibitory effect for β-globin mRNA (compare with Figs. 1 and 2). For the natural β-globin mRNA, a ratio of p50/mRNA of 10

[2] T. Pestova, personal communication.

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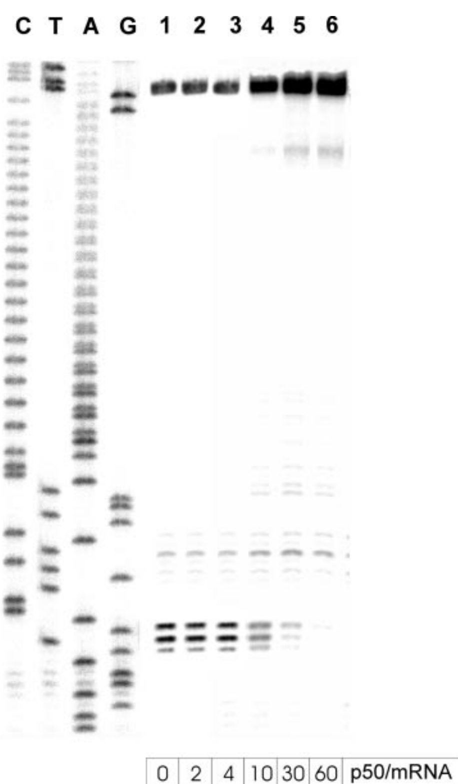

FIG. 6. **Effect of p50 on the assembly of the 48 S preinitiation complex with $(CAA)_n$-β-globin mRNA.** The formation of the complex was analyzed as indicated in Fig. 1. A dideoxynucleotide sequence generated with the same primer (5′-CACCACCAACTTCTTCCAC-3′) is shown at the *left*.

demonstrates a stimulatory rather than an inhibitory effect. These data present compelling evidence that p50 greatly prefers long stretches of unpaired nucleotides. The p50 molecules bound to the $(CAA)_n$-mRNA are concentrated mostly within its long unstructured 5′-leader and, as such, form a local inhibitory complex. In this connection, it is not surprising that no stimulation by p50 of the 48 S complex formation with the $(CAA)_n$-leader is seen at p50/mRNA ratios lower than 10. Apparently any stimulation in this case should be negated by the concomitant inhibitory binding of p50 within the $(CAA)_n$-leader.

Binding of Just a Few Molecules of p50 Per Molecule of β-Globin mRNA Is Sufficient to Stimulate the 48 S Preinitiation Complex Formation—To get an idea about the mechanism of action of p50 on the 48 S complex formation, it is useful to know how many molecules of the protein need to be bound to the mRNA to observe an effect. As follows from Figs. 1, 2, and 4, the stimulation is detected even at low p50/mRNA ratios and increases up to some threshold value of about 30 p50 per mRNA after which the yield of the 48 S complexes rapidly decreases. This does not mean, however, that these 30 molecules of p50 are all bound to the mRNA. The experimental conditions for the 48 S complex assembly involve the addition of a large excess of uncharged tRNA that is not participating in the reconstitution process. Although the tRNA has a very low affinity for p50, its high concentration sequesters some p50. This is evident from the sucrose gradient sedimentation of p50·β-globin mRNA complexes in the presence or absence of tRNA. In the latter case, a much heavier mRNP was formed that rapidly sedimented to the bottom of the tube (Fig. 7*A*). Therefore, the number p50 molecules bound to the β-globin mRNA in the presence of a large excess of tRNA was estimated. For this, ^{32}P-labeled mRNA with a known specific activity was incubated in the reconstitution buffer with p50 in the presence of all the translation initiation components needed to form the 48 S preinitiation complex. The mRNA·p50 complex was separated from unbound p50 and tRNA by sucrose gradient centrifugation, and the amount of mRNA in the mRNP peak was determined by Cerenkov counting. Western blot experiments performed in our laboratory showed that p50 does not dissociate from the mRNA during sucrose gradient centrifugation presumably because of its high affinity for single-stranded polynucleotides (data not shown). This allowed us to use sucrose gradient centrifugation as a technique to separate two pools of p50, the pool bound to mRNA and that sequestered by tRNA.

Aliquots from different fractions of the mRNP peak were then analyzed by SDS-PAGE along with a series of known amounts of p50. After transfer of the proteins onto a nitrocellulose membrane, the corresponding bands were developed with an ECL Western blotting system and quantified by densitometric scanning. The immunological response to increasing amounts of p50 was linear in the selected range of p50 used (Fig. 7*B*). In Fig. 7*C*, the same Western blotting experiment demonstrates a response of p50 antibodies to aliquots from the three different fractions of the p50/mRNA peak.

In a series of independent experiments, the p50/mRNA ratio in the mRNP peak varied from 6 to 8. This value represents the average number of p50 molecules bound to the β-globin mRNA at the threshold p50 concentration when stimulation of the translation initiation gives way to its inhibition.

DISCUSSION

We have recently shown that p50 plays not only a negative but also a positive role in translation and that this positive effect is exerted at the level of translational initiation (19). Here, using the reconstitution of the initiation complexes with purified components, we present strong evidence that p50 stimulates the binding of the 40 S ribosomal subunit at the initiation codon of mRNA. The stimulation reaches its maximum when less than 10 molecules of p50 are bound per one mRNA molecule of the size of rabbit β-globin mRNA (the length of the mRNA is 589 nt excluding the poly(A) tail that is normally bound to poly(A)-binding protein). On average, this results in a large separation between individual p50 molecules along the polynucleotide chain. It should be stressed that the stimulation can be observed at much lower p50/mRNA ratios (Fig. 1) and, hence, occurs with an average distance of more than 100 nt between bound p50 molecules.

With this low occupancy of mRNA, it is not immediately evident how this RNA-binding protein may affect the events that occur at the 5′-end of mRNA. There are two features, however, that distinguish p50 from the overwhelming majority of mRNA-binding proteins studied to date (33) and that may be a key to answer the question. As was reported previously (6) and confirmed here, the binding of p50 to mRNA does not engage the nucleotide bases. It binds the sugar-phosphate backbone and has no preferred sequence motifs on mRNAs. However, it has a great preference for the sequences with unpaired and probably weakly stacked nucleotide bases. We speculate that these properties primarily target p50 to the most unstructured regions within the mRNA molecules that are presumably also preferred sites for aberrant binding of initiation factors or other mRNA-binding proteins. As nonspecific RNA-protein interactions take place at the sugar-phosphate backbone of RNA, p50 efficiently displaces them from the irrelevant sites without interfering with specific interactions that normally involve nucleotide bases. In this way, one may easily imagine why just a few molecules of p50 are able to stimulate translation initiation, the process that is confined to

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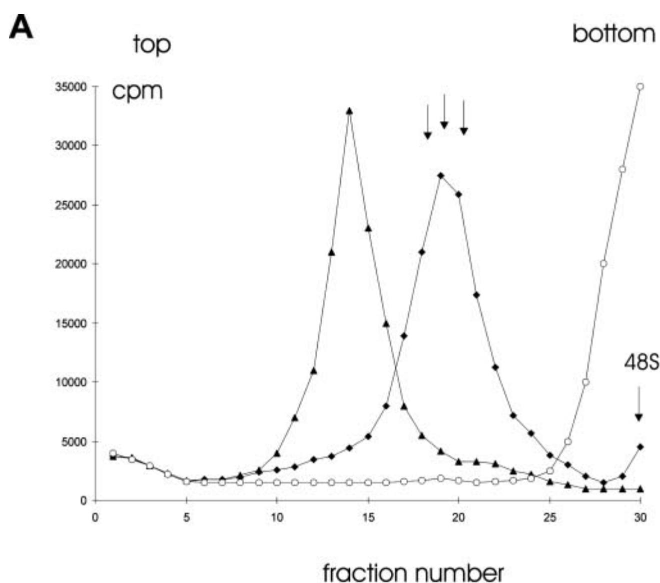

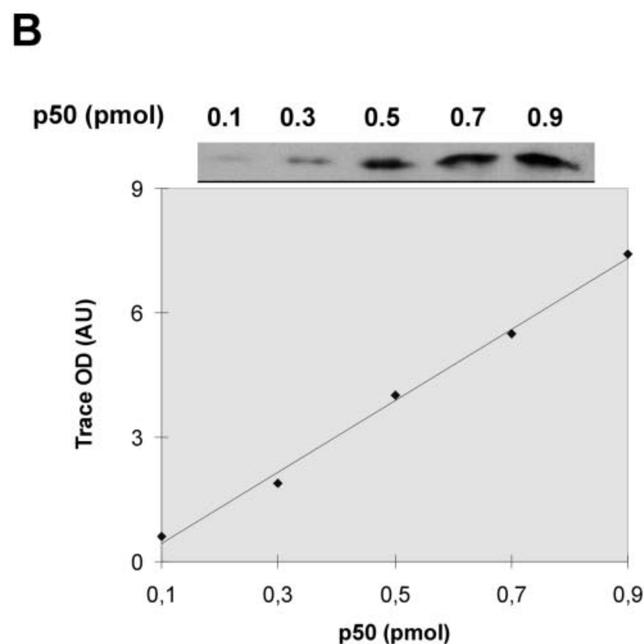

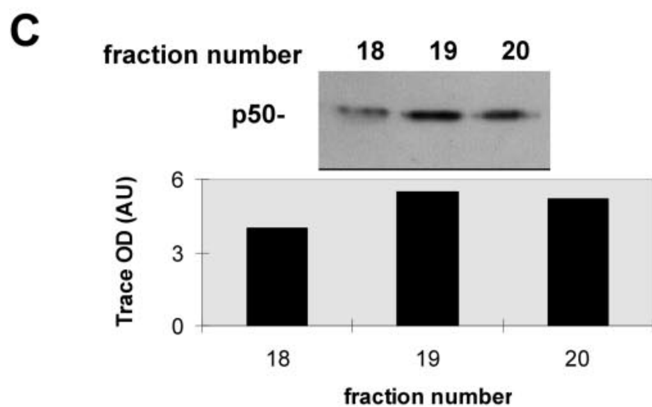

FIG. 7. **Quantitative Western blotting of p50 in the complex p50·β-globin mRNA.** The complex was formed at the p50/β-globin mRNA ratio of about 30 in the presence of all translational components needed for reconstitution of the 48 S complex and separated from unbound p50 by sucrose gradient centrifugation. *A* shows a sucrose gradient centrifugation of the p50·β-globin mRNA complex in the presence of a large excess of tRNA (*filled squares*) and in its absence (*open circles*). *Arrows* indicate fractions used to determine the p50/mRNA ratio in the mRNP peak. Sedimentation of free β-globin mRNA is shown with *filled triangles*. *B* demonstrates a dose response of different amounts of p50 to anti-p50 and the corresponding calibration plot. *C* demonstrates a response to anti-p50 of 60-μl aliquots from three different fractions of the mRNP peak shown in Fig. 5*A*. Each 60-μl aliquot from fractions 19–21 contained about 20 ng (~0.1 pmol) of β-globin mRNA. For other details see "Experimental Procedures." *AU*, arbitrary units.

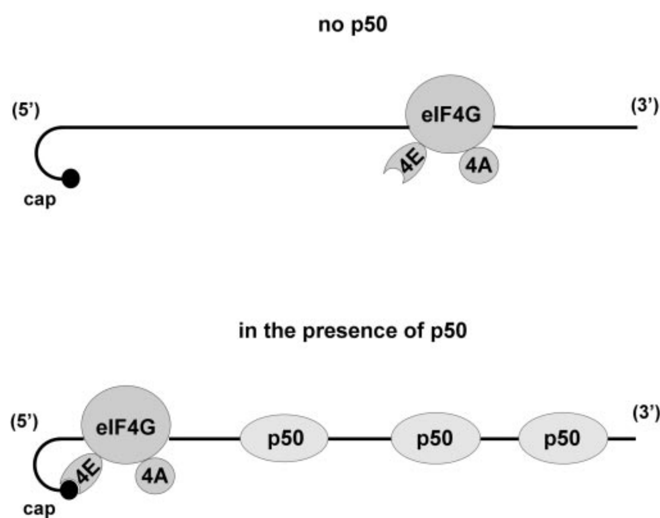

FIG. 8. **Model of the stimulation effect of p50 on the 48 S complex formation.** The model shows how p50 may displace eIF4F, and presumably other basic factors as well, from irrelevant sites on the mRNA, thereby allowing them to be free for initiation events at the 5′-end of the mRNA. For other explanations see the text. *4E*, eIF4E; *4A*, eIF4A.

a rather limited region of mRNA nucleotide sequence. The corresponding model that extends the model suggested earlier by Minich and Ovchinnikov (12) is presented in Fig. 8. The competition between key initiation factors eIF4F and eIF2 and p50 was clearly demonstrated in this study. The inability of p50 to stimulate the assembly of the 48 S complex at high molar excesses of eIF2 and eIF4F is also in line with this interpretation. The model ("competitive model") describes p50 as a general organizer of RNA-protein interactions in mRNPs.

In contrast, another mRNA-binding protein, PTB, fails to promote the binding of 40 S subunits to the start codon of β-globin mRNA. Unlike p50, PTB may be "retained" by pyrimidine-rich sequences of mRNA. It is logical, therefore, that PTB promotes translation initiation only in specific cases (some picornaviruses (34)), and its stimulation effect appears to be of a quite different nature (35).

One should bear in mind that p50 possesses prominent RNA unwinding properties (6, 23). This feature presumably also based on its strong affinity to single-stranded sequences may allow p50 to modulate mRNA secondary structure (see Ref. 36), thereby facilitating interaction of initiation factors and 40 S ribosomal subunit with the RNA polynucleotide chain. None of the experiments described above excludes this possibility. However, the bulk of evidence presented in this paper offers support for the competitive model.

It should be pointed out that the stimulatory effect of p50 on translation initiation at low p50/mRNA ratios and its repressive effect at high p50/mRNA ratios may be based on somewhat different features of p50. Whereas the stimulation is observed under conditions of a large separation between individual molecules of p50 on the polynucleotide chain, its repressive effect becomes prominent when p50 molecules seem to establish pro-

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tein-protein contacts. This is the most plausible explanation for a remarkable inhibition of the toeprint even at a low p50/mRNA ratio for the $(CAA)_n$-β-globin mRNA. In this case, several molecules of p50 appear to cooperatively bind within the 68-nt single-stranded leader of this artificial mRNA leaving the remaining part of the 5′-untranslated region essentially free of bound protein. This presumably results in transition of mRNP into a locked form (for model, see Ref. 37). In contrast, a natural RNA requires a more complete filling of the polynucleotide chain with p50 molecules to establish cooperative protein-protein interactions. At low p50/mRNA ratios, individual molecules of p50 are still separated by unoccupied structured RNA elements that prevent them from establishing protein-protein contacts. When these protein-protein interactions are finally achieved at the 5′ terminus of the mRNA, they start to compete with initiation factors for binding with the 5′-untranslated leader. In particular, they should interfere with interaction of mRNA-binding domain of eIF4G with the 5′-untranslated region. As a result (see Ref. 38), the overall affinity of eIF4F to the mRNA will drop with a concomitant easier dissociation of eIF4E from the cap structure. This model offers an alternative explanation of some results recently reported by Evdokimova *et al.* (13). An intriguing possibility is that some or all types of mammalian cells are able to regulate the general level of protein synthesis just by changing the intracellular concentration of p50. As suggested by the experiment presented in Fig. 6, this may differentially affect expression of mRNAs with unstructured and highly organized 5′-untranslated regions. Studies on the regulation of the synthesis of p50 itself that should clarify this interesting issue are now in progress.

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