The Major Core Protein of Messenger Ribonucleoprotein Particles (p50) Promotes Initiation of Protein Biosynthesis in Vitro*

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The major core protein of cytoplasmic messenger ribonucleoprotein particles (p50) has been shown previously to inhibit protein synthesis in vitro and in vivo. Furthermore, p50 is highly homologous to the Y-box-binding transcription factor family of proteins, binds DNA containing the Y-box motif, and thus may have a dual function in cells as a regulator of both transcription and translation. Here we show that binding or removal of p50 from rabbit reticulocyte lysate by monoclonal antibodies to p50 strongly inhibits translation of endogenous and exogenous globin mRNAs as well as prokaryotic β-galactosidase mRNA in a rabbit reticulocyte cell-free system. This, depending on the conditions, p50 not only may act as a translational repressor, but may also be required for protein synthesis. Translation inhibition with anti-p50 antibodies is not a result of mRNA degradation or its functional inactivation. The inhibition does not change the ribosome transit time, and therefore, it does not affect elongation/termination of polypeptide chains. The inhibition with anti-p50 antibodies is followed by a decay of polysomes and accumulation of the 48 S preinitiation complex. These results suggest that p50 participates in initiation of protein biosynthesis. Although uninvolved in the formation of the 48 S preinitiation complex, p50 is necessary either for attachment of the 60 S ribosomal subunit or for previous 5'-untranslated region scanning by the 43 S preinitiation complex.

Regulation of gene expression in eukaryotes often involves modulating the rate of initiation of protein synthesis (for reviews, see Refs. 1–3). Translation is controlled by proteins that are associated with mRNA and/or ribosomes and that either promote or repress the protein synthesis rate. One type of mechanism involves modulation of initiation factor activities (4) that usually affect global translation, whereas the second type involves specific protein repressors that bind to one or a small set of mRNAs (5), leading to specific control. The third type of mechanism may involve proteins associated with many or even all mRNA species, such as proteins present in messenger ribonucleoprotein particles (mRNPs)1 (6, 7). These proteins also may contribute to global control of protein synthesis.

mRNPs isolated from the cytoplasm of different cells and tissues contain two major proteins with molecular masses of 70 and 50 kDa (8). The 50-kDa protein (p50) is the most abundant and the most strongly bound protein within free nontranslatable mRNPs (9) and therefore can be regarded as the core mRNP protein of these particles in mammalian somatic cells. It is also present in mRNPs derived from polysomes, but in lower amounts (9, 10). Regardless of the fact that p50 displayed little or no specificity for RNA in in vitro binding experiments (10, 11), in cell extracts, p50 was found only in association with mRNA (12). This protein is located mostly in the cytoplasm, and its amount is ~0.1% of the total protein, which corresponds to ~5–10 molecules of p50/molecule of mRNA (12). Among all mRNP proteins, p50 is the most basic, with a pI of ~9.5 (10). In the absence of RNA, p50 forms large multimeric complexes with a molecular mass of ~800 kDa (11). When binding to mRNA, p50 melts up to 60% of the RNA secondary structure (11). We have shown earlier that p50 is responsible for the repressed nonactive state of globin mRNA within free mRNPs (13) and that it strongly inhibits translation of exogenous mRNA in cell-free translation systems (9, 14) as well as in vivo translation of mRNA expressed from a reporter gene (12).

According to its amino acid sequence and its affinity for DNA, p50 was identified as a member of the most evolutionarily conserved family of Y-box-binding proteins from bacteria to man (11). Some proteins of this family are known as transcription factors affecting transcription of genes containing Y-box sequence elements in their promoters (15–17). The prokaryotic Y-box-binding protein is also known as a major cold shock protein stimulating gene expression under cold shock conditions (18). Two homologous core mRNP proteins (p54/p56) from Xenopus oocytes have also identified as members of the Y-box-binding protein family and are closely related, if not identical, to FRG Y2, the germ cell-specific form of the transcription factor (19). They were reported to be responsible for the masked state of mRNAs in Xenopus oocytes (20–24). One can therefore conclude that the somatic mRNP protein p50 and the germ cell mRNP proteins p54/p56 have a similar function in the cytoplasm, namely that of preventing mRNA translation. Thus, the Y-box-binding proteins can influence protein synthesis both by affecting transcription of Y-box-containing genes and by inhibiting translation of a wide variety of mRNAs. By interacting with DNA in the nucleus and with mRNA in the cytoplasm, these proteins may establish a balance between gene activity and mRNA content in cells.

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‡ The abbreviations used are: mRNPs, messenger ribonucleoprotein particles; PAGE, polyacrylamide gel electrophoresis; FRG Y2, frog Y-box transcription factor 2.

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In a detailed study of the effect of p50 on exogenous mRNA translation in reticulocyte lysates, we showed that p50 inhibits mRNA translation when added at a high p50/mRNA ratio. However, with high inhibitory amounts of purified mRNA, addition of low amounts of p50 increases the efficiency of mRNA translation (9). This intriguing result suggests that p50 may also play a positive role in translation. Here we ask if depletion of p50 affects the rate of protein synthesis in reticulocyte lysates. Reduced p50 activity was accomplished by adding p50-specific antibodies. In this instance, translation is inhibited at the level of initiation, thereby providing evidence for a positive role of p50 in promoting protein synthesis.

EXPERIMENTAL PROCEDURES

Preparation of p50—p50 from free mRNPs of rabbit reticulocytes was obtained as described earlier (11), except that the step of protein precipitation with (NH₄)₂SO₄ was omitted. Recombinant p50 was expressed in *Escherichia coli* BL21(DE3) cells, and the protein was purified by chromatography on heparin-Sepharose 4B and Superose 12 HR 10/30 columns (Pharmacia Biotechnology, Inc.) as described (25). The p50 preparations were dialyzed against buffer containing 10 mM Hepes-KOH, pH 7.6, 100 mM KCl, 5 mM MgCl₂, and 0.1 mM cycloheximide (Sigma); therefore, the same buffer was added to parallel control incubations throughout the experiments described. For Western blot analysis, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 20 °C with 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 0.05% Tween 20 in 10 mM Tris-HCl, pH 7.6, and 150 mM NaCl and probed with anti-p50 antibodies at 1:500 dilution. Immunocomplexes were detected with alkaline phosphatase-coupled secondary antibodies (Cappel) according to the manufacturer’s recommendation.

RNA Isolation—Globin mRNA was obtained from polysomes of rabbit reticulocytes by phenol/chloroform extraction followed by oligo(dT)₆₃ cellulose chromatography as described (27). *E. coli* β-galactosidase mRNA was obtained by *in vitro* transcription with SP6 polymerase from pJCS-β-galactosidase linearized with HindIII according to standard procedures (28).

In *In Vitro* Translation Assays—Translation reactions were performed in rabbit reticulocyte lysates prepared as described (29). Mixtures (30 μl) contained 15 μl of reticulocyte lysate, 10 mM Hepes-KOH, pH 7.6, 100 mM KOAc, 1 mM Mg(OAc)₂, 8 mM creatine phosphate, 0.5 mM spermidine, 0.2 mM GTP, 0.8 mM ATP, 1 mM dithiothreitol, 25 μM each amino acid except for the labeled ones, and 6 μCi of [³⁵S]Met (>300 mCi/mmol; Radioisotop). When micrococcal nuclease-treated lysates were used, the mixture was supplemented with mRNA as indicated in the figure legends. Translations were carried out at 30 °C for 60 min unless stated otherwise; 5-μl aliquots were treated with deacetyl solution (0.3 M NaOH and 150 mM H₂O₂) and measured for trichloroacetic acid-precipitable radioactivity. When [³⁵S]Met was used, the samples were also subjected to 10–22% SDS-PAGE and autoradiographed.

For ribosome transit time measurements, the volume of the translation mixtures was increased to 150 μl, and translations were carried out as described above. At the indicated intervals, 25-μl aliquots were diluted with 125 μl of ice-cold stop buffer containing 10 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, and 0.1 mM cycloheximide (Sigma) and placed immediately on ice. The mixtures were layered onto a 30%
glycerol cushion prepared with 10 mM Tris-HCl, pH 7.6, 100 mM KCl, and 5 mM MgCl2 and centrifuged at 100,000 rpm in a TLA-100.3 rotor (Beckman Instruments) for 40 min. This procedure deposits polyribosomes and monomeric ribosomes in the pellet. The ribosomal pellet was dissolved in 1% SDS, and this fraction and the supernatant were assayed for radioactivity as described above.

**Depletion of p50 Activity in Cell Lysates**—For neutralization of p50 activity, reticulocyte lysates (15 μl) were preincubated with the indicated amounts of antibodies for 10 min at 0 °C. For immunodepletion experiments, nuclease-treated reticulocyte lysates (200 μl) were preincubated with various amounts of antibodies for 10 min at 0 °C with gentle agitation and passed through a 200-μl column of protein A-Sepharose equilibrated with 10 mM Hepes-KOH, pH 7.6, 100 mM KCl, and 1 mM MgCl2. The first 250 μl of flow-through material from the column was collected and frozen in liquid nitrogen in 15-μl aliquots. Neutralized or immunodepleted reticulocyte lysates were reconstituted by adding the indicated amounts of p50 directly to the lysate and incubating for 3 min at 0 °C. The neutralized, depleted, and reconstituted lysates were then assayed for protein synthesis activity as described above.

**Sucrose Gradient Analyses and Dot Blot Assays**—Cell-free translation mixtures (30 μl) were cooled and immediately layered onto 15–30% (w/v) linear sucrose gradients in buffer containing 10 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1 mM MgCl2. Centrifugation was carried out at 45,000 rpm in an SW 60 rotor (Beckman Instruments) for the times indicated in the figure legends. All gradients were monitored for absorbance at 254 nm during their collection from the bottom. Fractions of 300 μl were diluted with 700 μl of ice-cold water and treated with deacylation solution, and [3H]Leu incorporation into trichloroacetic acid-precipitable material was measured as described above. For dot hybridization analysis of globin mRNA, total RNA was extracted from the fractions by an equal volume of phenol/chloroform, filtered onto a nitrocellulose Hybond-N membrane (Amersham Corp.), and probed with a full-length α-globin cDNA 32P-labeled by the multiprime DNA labeling system (Amersham Corp.). For quantitation of hybridized RNA, each dot was excised, and radioactivity was measured by Cherenkov counting in a Beckman LS-100C scintillation counter.

**RESULTS**

**Cell-free Translation Systems Deficient in p50 Do Not Synthesize Proteins Efficiently**—To verify the suggestion that p50 may play a positive role in protein biosynthesis, we sought to deplete p50 activity in reticulocyte lysates. For selective reduction of p50 activity in the rabbit reticulocyte cell-free translation system, two different polyclonal anti-p50 antibody preparations were used. The antibodies were raised either in mouse against p50 from free rabbit reticulocyte mRNPs or in rabbit against recombinant p50 expressed in E. coli. Both p50 preparations were purified to homogeneity; no other peptide bands were detected by Coomassie Blue R-250 staining of these proteins resolved by SDS-PAGE (Fig. 1A). The two anti-p50 antibodies were purified on protein A-Sepharose, and their specificities were tested by immunoblotting. The antibodies reacted strongly with purified p50 and recognized only a single antigen in reticulocyte lysates corresponding in electrophoretic mobility to p50 (Fig. 1B). Therefore, the antibodies are monospecific and may be suitable for neutralization of p50 activity due to their specific binding to p50 in the rabbit reticulocyte cell-free system and for p50 immunodepletion by affinity adsorption from the lysate. Both antibody preparations gave identical results, so further reported experimental data are on antibodies against rabbit reticulocyte p50, unless indicated otherwise.

The effect of anti-p50 IgG addition on translation of endogenous and exogenous globin mRNAs in the rabbit reticulocyte cell-free system is shown in Fig. 1 (C and D, respectively). In both systems, the antibodies caused a dramatic inhibition of globin synthesis, whereas the control immunoglobulins from a nonimmunized animal (preimmune IgG) affected the activity of the system only slightly. A straightforward interpretation of these results is that anti-p50 IgG binds to p50 and inhibits its putative function in promoting mRNA translation.

To deplete p50 from the cell-free translation system, a mi-
immunodepleted with 12 mM system by antibodies due to their association with p50. These factors important for p50 activity were removed from the activity restoration. Another explanation is that other auxil- nontranslatable free mRNPs or recombinant p50 used for the can probably be explained by improper modification of p50 from protein biosynthesis by antibodies, incomplete restoration by p50/molecule of mRNA (12). After profound inhibition of prot- is near the p50/mRNA ratio in COS cells, globin mRNA (10–15 pmol of p50/pmol of globin mRNA), which which is in good agreement with our earlier observation that inhibition. Lysates were neutralized with 6 µg of anti-rabbit p50 IgG or immunodepleted with 12 µg of anti-rabbit p50 IgG. Data are expressed as the percent value of translational activity observed after identical treatment of the lysates in the absence of antibodies. The 100% values for [14C]Leu incorporation were 42,300 and 38,000 cpm for neutralized lysates with endogenous and exogenous globin RNAs, respectively, and 31,300 cpm for immunodepleted lysates with exogenous globin RNA. These data are representative of those obtained in at least five separate experiments.

inhibition (reduced to 10–20% activity), p50 stimulated translation 3–4-fold; the system’s activity increased linearly with increasing amounts of p50 and finally reached 45–65% of the initial lysate activity (Fig. 3A). A further increase of the p50 amount caused inhibition of translation (data not shown), which is in good agreement with our earlier observation that p50 acts as a translational repressor at a high p50/mRNA ratio (9). In reticulocyte lysate, after immunodepletion, biosynthesis was restored after addition of 2–3 µg of p50/1 µg of exogenous globin mRNA (10–15 pmol of p50/pmol of globin mRNA), which is near the p50/mRNA ratio in COS cells, i.e. 5–10 molecules of p50/molecule of mRNA (12). After profound inhibition of protein biosynthesis by antibodies, incomplete restoration by p50 can probably be explained by improper modification of p50 from nontranslatable free mRNPs or recombinant p50 used for the activity restoration. Another explanation is that other auxiliary factors important for p50 activity were removed from the system by antibodies due to their association with p50. These proteins are the subject of our study at the moment. Nevertheless, the fact that highly purified or recombinant p50 by itself is capable of restoring or stimulating protein-synthesizing activity strongly indicates that the inhibition by antibodies is mostly due to specific recognition and inactivation of p50 rather than some other proteins. These results suggest that p50 is required for efficient protein synthesis.

Inhibition of Protein Synthesis with Anti-p50 IgG Is Not Caused by Acceleration of mRNA Degradation—Antibody bind- ing to p50 may stimulate mRNA decay in the cell-free translation system, thereby causing translation inhibition. To verify this idea, RNAs were isolated from lysates incubated under cell-free translation conditions with and without anti-p50 IgG and tested for messenger activity. Both mRNA preparations possessed equal messenger activity in the cell-free translation system and produced full-length globin chains as determined by SDS-PAGE (Fig. 4). The messenger activity of these RNAs was the same as that of RNA from a nonincubated lysate (data not shown). Thus, anti-p50 IgG-induced protein synthesis inhibition is not caused by mRNA degradation.

Specific Anti-p50 Antibodies Inhibit Initiation but Not Elongation/Termination of Globin Synthesis in Reticulocyte Lysates—Fig. 5 shows the effect of anti-p50 IgG on the kinetics of protein synthesis in rabbit reticulocyte lysates with endogenous (panel A) and exogenous (panel B) globin mRNAs. With either type of mRNA, anti-p50 IgG inhibited strongly at high concentrations, whereas preimmune IgG had little or no effect. The inhibition of endogenous mRNA translation was a high anti-p50 IgG concentration resembled the inhibition caused by edeine, a specific inhibitor of the initiation phase of protein synthesis (30, 31). Thus, the shape of the curves suggests that the antibodies inhibit mainly translation initiation.

The phase of translation affected by an inhibitor can be determined more precisely by polysome profile analysis and measurement of the elongation rate. The run-off of ribosomes
from polysomes usually indicates inhibition of initiation, whereas maintenance of or an increase in polysome size suggests inhibition of elongation/termination. We have compared the polysome profiles of reticulocyte lysates incubated in the absence and presence of either anti-p50 IgG or preimmune IgG (Fig. 6). A short incubation (2 min) of the cell-free system without antibodies or with preimmune IgG did not produce any remarkable change in the polysome profiles (Fig. 6, compare A with B and C). However, incubation with anti-p50 IgG resulted in a complete polysomal decay (Fig. 6D). The process was accompanied by dissociation of the radiolabeled polypeptide chain from the ribosomes. This indicates that polysomal decay is not due to mRNA fragmentation by ribonucleases as such cleavage of polysomes produces ribosomes associated with the growing polypeptide. Rather, the run-off of ribosomes from polysomes implies that anti-p50 IgG inhibits mainly initiation and not elongation or termination.

The average time of elongation + termination of polypeptide chains (transit time) can be quantitatively determined by measuring the kinetics of radioactive amino acid incorporation into total protein and into completed polypeptides released from the ribosomes (32). We used this technique to determine the effect of anti-p50 IgG on the elongation + termination rate. From the results shown in Fig. 7, the transit time for globin synthesis in the control uninhibited lysate was 1.4 min. In the experimental system with anti-p50 IgG, the same transit time was obtained, although in this case, protein synthesis was suppressed 2-fold over the studied time range (6 min). Thus, anti-p50 antibodies do not affect the polypeptide elongation + termination rate and inhibit only initiation.

Specific Anti-p50 Antibodies Inhibit Initiation but Not Elongation of β-Galactosidase Synthesis in Reticulocyte Lysates—p50 is a universal mRNP protein associated with all or almost all mRNAs of mammalian somatic cells (11). The question arises as to whether p50 promotes only initiation of eukaryotic mRNAs having an m7G cap, a characteristic consensus sequence around the initiator AUG codon and 3’-poly(A) tail, or whether it would exert the same positive effect on translation of a bacterial mRNA lacking these features. To answer this question, an uncapped mRNA transcript encoding E. coli β-ga-

**Fig. 5. Effect of anti-p50 antibodies on the kinetics of globin synthesis in rabbit reticulocyte cell-free systems.** Rabbit reticulocyte lysates (15 μl) with endogenous (A) or exogenous (B) rabbit globin mRNA were preincubated with the indicated amounts of antibodies. Edeine was added at the beginning of the incubation. [14C]Leu incorporation into protein was measured as described under “Experimental Procedures.”
DISCUSSION

The results reported here indicate that p50, the major protein of cytoplasmic mRNPs, not only functions as a repressor of mRNA translation, but also is required for protein biosynthesis. Two independent preparations of monospecific anti-p50 antibodies raised against either rabbit or recombinant p50

FIG. 6. Anti-p50 IgG stimulates polysome decay in rabbit reticulocyte cell-free systems. Rabbit reticulocyte lysates (15 µl) with endogenous mRNA were preincubated for 10 min at 0 °C as indicated below. Following preincubation, translation assays with [14C]Leu were performed for 2 min at 30 °C as described under “Experimental Procedures.” Lysates were subjected to centrifugation through 15–30% linear sucrose gradients for 60 min at 45,000 rpm in an SW 60 rotor. UV absorption profiles at 254 nm (—), [14C]Leu incorporation into protein (●), and the sedimentation positions of 80 S monosomes are shown. A, the polysomal profile of the original rabbit reticulocyte lysate without any treatment or incubation (control); B, preincubation of lysate with no antibodies; C, preincubation of lysate with 8 µg of nonimmune IgG; D, preincubation of lysate with 8 µg of anti-p50 IgG.

FIG. 7. Anti-p50 antibodies do not affect the ribosome transit time. Rabbit reticulocyte lysates with endogenous RNA (75 µl) were preincubated without antibodies (A) or with 38 µg of anti-p50 IgG (B) for 10 min at 0 °C, and translations were carried out for the indicated times. [14C]Leu incorporation into trichloroacetic acid-precipitable material was measured as described under “Experimental Procedures.” Transit times were obtained by multiplying by 2 the distance along the time axis between the “total” and “post-ribosomal” lines. In B, the relevant portion of the curve is the early time points up to 6 min, where most of the [14C]Leu incorporation is due to elongation on already initiated mRNAs.

FIG. 8. Anti-p50 antibodies inhibit initiation but not the elongation phase of prokaryotic β-galactosidase synthesis. Translation reactions were carried out with 20 µg/ml E. coli β-galactosidase mRNA without antibodies (A) and in the presence of 8 µg of anti-p50 IgG added at zero time of incubation (B) or after 10 min of incubation (C). At the indicated intervals, 3-µl aliquots were removed, and [35S]Met-labeled translation products were resolved by SDS-PAGE and visualized by autoradiography. The protein bands migrating more rapidly than full-length β-galactosidase may represent either incomplete nascent chains or earlier quitters.
were used to reduce the activity of endogenous p50 in rabbit reticulocyte lysates or to prepare depleted lysates. Immunoneutralization or immunodepletion of p50 by both antibody preparations caused inhibition of protein synthesis. Addition of highly purified rabbit or recombinant p50 proteins to such systems restored the translational activity completely or nearly completely, which confirms the positive role of p50 in mRNA translation.

How can p50 function both to promote and to repress translation? The in vitro assays for p50 activity suggest that the level of p50 determines whether it stimulates or inhibits translation. With the p50/mRNA weight ratio increasing up to 2 (which is characteristic of polysomal mRNPs), p50 stimulates protein biosynthesis, whereas a further increase of the ratio up to 5–6 (close to the ratios characteristic of free mRNPs) causes a gradual inhibition of translation until it ceases completely (9). Thus, the inactive-to-active transition of mRNA may be connected with a decrease in the amount of p50 attached to an mRNA molecule.

The amount of p50 on mRNA can possibly be regulated by phosphorylation. It is known that p50 is a phosphoprotein (10, 34) and may be phosphorylated by a protein kinase present in mRNPs that resembles casein kinase II. Furthermore, the Xenopus proteins p54/p56 are phosphoproteins, and their binding to RNA is enhanced upon phosphorylation by casein kinase II (35). Phosphorylation of p54/p56 results in inhibition of protein synthesis, whereas inhibitors of casein kinase II activate translation (24, 35). Work is in progress to elucidate how phosphorylation of p50 may affect its binding to RNA and its activity in stimulating and inhibiting translation in vitro.

Immunoneutralization of p50 does not change the ribosomal transit time for globin synthesis and does not affect elongation of prokaryotic β-galactosidase. This means that p50 is not required for elongation and termination, and consequently, it is required only for initiation. The effect of anti-p50 IgG on the kinetics of protein synthesis in the cell-free translation system and on the decay of polysomes also points to inhibition of translation initiation.

Analysis of the distribution of globin mRNA in reticulocyte lysates inhibited by anti-p50 IgG shows that mRNA accumulates in 48 S preinitiation complexes. This means that p50 is not required for attachment of the small subunit of the ribosome to mRNA, although it is necessary for subsequent binding of the 60 S ribosomal subunit to the complex. We suggest that either p50 directly participates in attachment of the 60 S ribosomal subunit to the 48 S preinitiation complex or that it is involved in the previous step of 5′-untranslated region mRNA scanning by the 43 S preinitiation complex.

Several mechanisms of p50 participation in translation initiation can be proposed. (i) Nonspecific affinity of p50 for RNA and the presence of many copies of p50 on mRNA can protect mRNA against nonspecific binding of initiation factors along its entire length, thus contributing to their specific binding to the 5′-untranslated region (9, 36). (ii) Since p50 possesses RNA-unwinding activity (11), its direct participation in 5′-untranslated region scanning is quite possible. (iii) p50 provides the general mRNA structure favorable for translation initiation. (iv) Finally, we cannot rule out that p50 affects translation initiation by its direct interaction with translation initiation factors. These suggestions are being verified currently.

p50 is not the only mRNP protein implicated in the initiation phase of protein synthesis. Another major mRNP protein, p70 (or poly(A)-binding protein), appears to be involved in protein synthesis initiation, too (37–40). Whether poly(A)-binding protein and p50 interact directly on mRNAs has not yet been determined.

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