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Overexpression in COS cells of p50, the major core protein associated with mRNA, results in translation inhibition

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ABSTRACT

p50, the major core protein of messenger ribonucleoprotein particles (mRNPs) in the cytoplasm of somatic mammalian cells, has been characterized previously as a member of the Y-box binding transcription factor family of proteins (YB-protein) by both high structural homology and ability to bind specifically the Y-box sequence in double-stranded DNA. YB proteins are present in a whole range of cell types and some have been identified as germ-specific cytoplasmic proteins masking stored mRNA from translation. Western blot analysis of the distribution of p50 in subcellular fractions of COS-1 cells shows that p50 is a cytoplasmic protein quantitatively associated with mRNA, both in polyribosomes and in free mRNPs. The level of p50 in COS-1 cells determined by Western immunoblotting is 0.10% of total protein, which is nearly equimolar to that of ribosomes and is ~5–10-fold higher than the mRNA level. Transient transfection of COS-1 cells with a p50-expressing vector results in a dramatic inhibition of protein synthesis. A control transfection with a vector expressing a frameshift mutant of p50 does not cause translation inhibition. Therefore the increase in p50 protein level is responsible for the inhibitory effect in these cells.

INTRODUCTION

A protein with an apparent molecular weight of 50 000 was identified as one of the major proteins in messenger ribonucleoprotein particles (mRNP) in the cytoplasm of mammalian somatic cells (1). The protein, called p50, is present in both free mRNPs and in mRNPs isolated from polyribosomes, although free mRNPs contain a higher ratio of p50 to mRNA (2,3). p50 was purified from rabbit reticulocyte free mRNPs and was shown to inhibit mRNA translation when added to a cell-free translation system (4). On the other hand, *in vitro* translation in a p50-depleted lysate strictly depends on p50 addition (V.M.Evdokimova, E.K.Davydova, E.A.Archakova, D.M.Naschjokin, J.W.B.Hershey and L.P.Ovchinnikov, submitted). From these results it was proposed that a small amount of p50 is required for efficient mRNA initiation, whereas at higher concentrations p50 masks mRNAs and prevents their translation (4).

Sequencing of p50 cDNA cloned from a rabbit reticulocyte library revealed an open reading frame encoding a protein of 324 amino acid residues. The calculated mass (36 kDa) is much less than expected, due to anomalous migration during SDS–PAGE (5). p50 exhibits ~98% amino acid sequence identity to mammalian Y-box binding (YB) transcription factors and binds specifically to Y-box containing DNA (5). Two closely related YB proteins found in *Xenopus* oocytes mRNPs are called mRNP4 (also named pp60 and p56) and mRNP3 (also named pp56 and p54). Their cDNAs, named FRGY2 and p54, respectively, were cloned and identified as oocyte-specific frog transcription factors (6,7). The two closely related YB proteins were shown to be responsible for masking stored mRNA from translation in oocytes (8–10). Inhibition of translation also was observed in *Xenopus* somatic cells transfected with a vector expressing FRGY2 (11). FRGY2 is located predominantly in the cytoplasm both in frog oocytes (7,12) and in transfected somatic cells (11). Thus, p50 and FRGY2 appear to function similarly as translational repressors. However, FRGY2 has only ~25% sequence identity to p50 outside the so-called cold-shock domain of ~80 amino acid residues, a region that is highly conserved among all YB proteins (5). Another *Xenopus* YB-transcription factor, FRGY1, has much higher sequence identity with p50 (~80%) (5). However, it is localized in somatic cells predominantly in the nucleus (11), and a role in translation repression has not been demonstrated.

Earlier p50 was shown to be present in a number of mammalian somatic cells and tissues (5). Here we determine the concentration of p50 in COS-1 cells and its subcellular distribution by Western immunoblot analysis. To test for possible masking of mRNA *in vivo*, the effect of p50 overexpression on translation in transiently transfected COS-1 cells is described.

MATERIALS AND METHODS

Preparation of polyclonal antibody against recombinant-p50

p50 was overproduced from pET3-1-50 (5) in the *Escherichia coli* strain BL21(DE3)pLysS. Two hours after addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, cells were collected and

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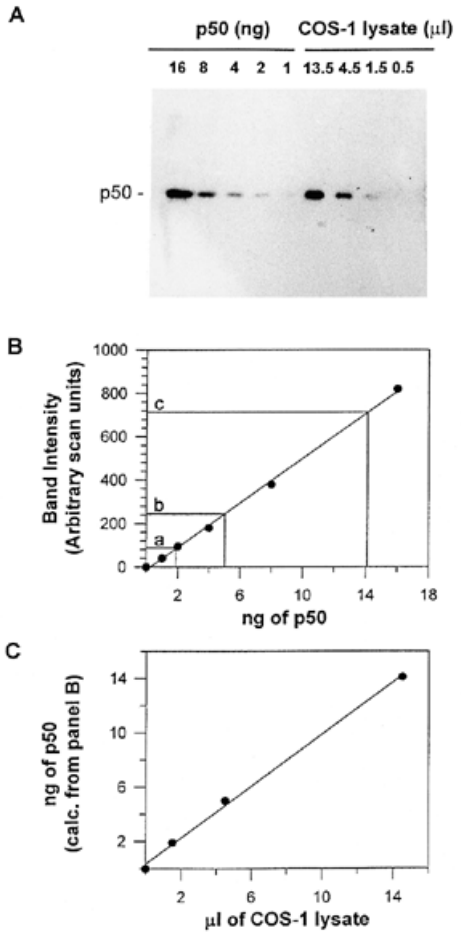

Figure 1. Quantitation of the p50 level in COS-1 cells. (**A**) A COS-1 lysate was prepared as described in Materials and Methods and protein concentration was determined by the Bradford assay (27). Different amounts of purified rabbit reticulocyte p50 and COS-1 lysate were subjected to 10% SDS–PAGE (13) and electrotransferred (1 h at 300 V) to an Immobilon^{TM} polyvinylidene difluoride membrane (Millipore) in 10 mM 3-(cyclohexylamino)propane sulfonic acid, 10% methanol, pH 11. Blots were blocked in phosphate buffered saline, 0.5% of non-fat dry milk solution for 2 h, incubated with affinity-purified anti-p50 antibodies (1:100 dilution) for 15 h and then with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (1:10 000 dilution) and developed with a chemi-luminescence detection system (Tropix Inc.). The figure shows a photograph of the developed film. (**B**) Band intensities for p50 in (A) were quantitated with a scanning densitometer (Molecular Dynamics 325 E) and are plotted against nanograms of p50 to generate a standard curve. The levels of p50 in the lysate samples were calculated from band intensities matched to the standard curve as shown. Boxes a–c represent 1.5, 4.5 and 13.5 µl of COS-1 lysate at 0.95 mg/ml protein. (**C**) The nanograms of p50 calculated for each of the lysate samples were plotted against the amount of lysate analysed. The resulting straight line was obtained by a least-squares fit of the data points. The slope of the line corresponds to a p50 level of 1.04 ng/µg of total protein, or to 2.6×10^6 molecules per cell, based on direct counting of COS-1 cells before lysis and the reported value of 154 pg of protein per cell (17) and a p50 mass of 35.7 kDa.

lysed by boiling for 10 min in SDS–gel sample buffer (13). After preparative SDS–PAGE and Coomassie R250 staining of the gel, the p50 band was excised and p50 was electroeluted. The primary immunization of rabbits with 150 µg p50 emulsified with Freund's complete adjuvant and four booster injections of 75 µg p50 each were performed at 2 week intervals. Ten days after the last injection, serum was prepared and stored at 4°C. Affinity-purified anti-p50 antibodies were obtained by binding to purified

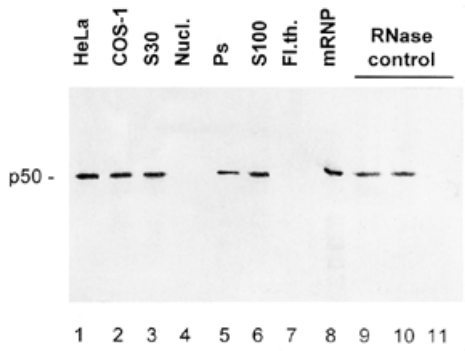

Figure 2. Distribution of p50 in COS-1 subcellular fractions. HeLa and COS-1 cell lysates and different COS-1 sub-cellular fractions were obtained as described in Materials and Methods, analysed by 10% SDS–PAGE and immunoblotted as described in the legend to Figure 1. Each lane contains an amount corresponding to 5×10^4 cells. Lane 1, HeLa lysate; lanes 2–6, total cell lysate (COS-1), post-nuclear (S30), nuclear (Nucl), polysomal (Ps) and post-ribosomal (S100) fractions of COS-1 cells; lanes 7 and 8, flow through and eluate obtained by oligo(dT)-cellulose chromatography of the post-ribosomal COS-1 S100 fraction; lanes 9–11, post-ribosomal COS-1 lysate, treated with 50 mg/ml RNase A (10 min at 37°C), and subsequent flow-through and eluate fractions obtained by oligo(dT)-cellulose chromatography of the nuclease treated lysate, respectively. Oligo(dT)-cellulose chromatography was performed as described in Materials and Methods. The figure shows a photograph of the immunoblot developed by the colorimetric method with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The migration position of p50 is indicated on the left.

rabbit reticulocyte p50 following SDS–PAGE as described (14). The affinity-purified antibodies were stored at 4°C and used at 1:100 dilution.

Fractionation of COS-1 cells

Freshly fed COS-1 cells were trypsinized, washed twice with PBS containing 1 mM phenylmethylsulfonylfluoride, counted, and lysed with 20 mM HEPES–KOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.25% Nonidet P-40, 2 µg/ml leupeptin, 2 µg/ml pepstatin and 10 µg/ml cycloheximide with 10 strokes of a syringe with a 23 gauge needle. The homogenate was clarified and nuclei were separated by a 15 min centrifugation at 14 000 *g*. Nuclei were purified from the resuspended pellet by spinning through a 50% glycerol cushion. The post-ribosomal supernatant was obtained by a further 15 min centrifugation of the post-nuclear supernatant at 100 000 r.p.m. in a TLA-100 centrifuge (Beckman). The pellet was used as the polysome fraction. Isolation of poly(A)$^+$ mRNPs from the post-ribosomal supernatant by oligo(dT)-cellulose chromatography was done as described (5); in particular, adsorption of poly(A)$^+$ mRNP to the column was with 500 mM NaCl at 4°C and elution was with no-salt buffer at 37°C.

Transient transfection and labeling of COS-1 cells

To construct a p50 expression vector for transient transfection, the 1.4 kb *Pst*I–*Xho*I fragment containing rabbit p50 cDNA lacking the 3′ untranslated region (UTR) was excised from pBSK-p50-2 (5) and cloned into the same sites of pMT3 (15). The resulting pMT3-p50 contains the SV40 origin of replication and utilizes the adenovirus major late promoter and SV40 enhancer to produce a dicistronic p50 dihydrofolate reductase (DHFR) transcript that begins with the adenovirus tripartite leader and a small intron upstream of the p50 coding region. A control plasmid, pMT3-fsp50,

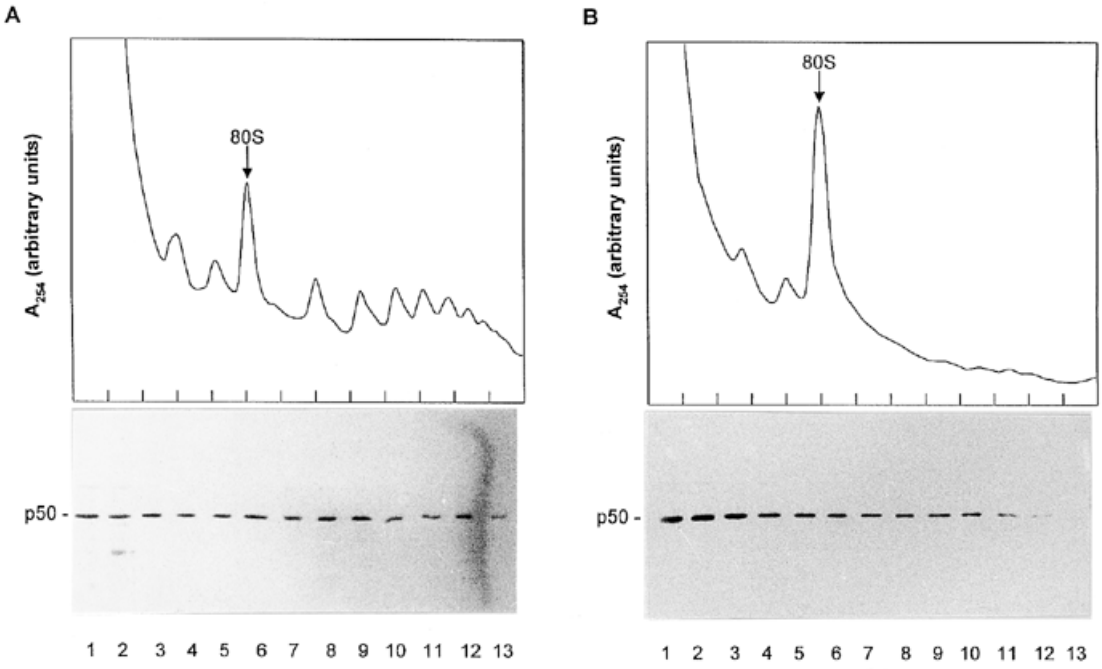

Figure 3. Distribution of p50 among cytoplasmic components of COS-1 cells. Post-nuclear COS-1 extracts (S30) were obtained as described in Materials and Methods. Extracts (10 A_{260} units) were layered on 15–45% (w/v) sucrose gradients in detergent-free lysis buffer and centrifuged at 4°C in a Beckman SW 60 rotor for 1 h at 50 000 r.p.m. Gradients were fractionated by bottom puncture and upward displacement using an Isco gradient fractionator and were scanned for absorbance at 254 nm with an Isco UV monitor. Thirteen fractions were collected, concentrated by TCA-precipitation and 1/10 of each fraction was analysed by 10% SDS–PAGE. Immunoblotting was done as described in the legend to Figure 1. (**A**) Standard extract of COS-1 cells was analysed. (**B**) COS-1 cells were incubated for 1 h on ice before lysis. The upper panels represent continuous UV scans of the gradients, with the 13 fractions indicated by vertical dashes at the bottom; sedimentation was towards the right. The lower panels are photographs of the Western blots developed by the colorimetric method (see legend to Fig. 2) with the gel lanes aligned beneath the corresponding gradient fractions. The migration position of p50 is shown on the left.

was constructed from pMT3-p50 by cleavage with *Xma*I (position 115 of the p50 open reading frame), filling in by Klenow enzyme and blunt-end ligation. The resulting 4 nt insertion causes +1 frameshift and generates a TGA stop codon at position 233 of the p50 coding region. Thus, the polypeptide encoded by pMT3-fsp50 contains 38 N-terminal p50 amino acid residues followed by 41 altered amino acids from the +1 reading frame.

COS-1 cells were transfected with the pMT3 constructs (5 µg of each/60 mm plate) by using the DEAE–dextran method (14). At 48 h post-transfection, cells were pulse-labeled for 30 min with 20 µCi/ml [^{35}S]methionine (>1000 Ci/mmol; DuPont NEN), trypsinized and lysed in SDS–gel sample buffer. Radioactively labeled proteins were analysed by autoradiography after 12% SDS–PAGE. For Northern blot analysis RNA was isolated from parallel transfection plates by the guanidinium isothiocyanate method (16), separated on a 1.2% formaldehyde–agarose gel, blotted onto a Hybond-N membrane by capillary action and probed with a 0.73 kb *Eco*RI fragment of DHFR cDNA ^{32}P-labeled by the multiprime DNA labeling system as described (15). The probe recognizes both the DHFR monocistronic transcript from pMT3 and p50-DHFR dicistronic transcripts.

RESULTS

Level of p50 in COS-1 cells

To determine the level of p50 in COS-1 cells by quantitative Western immunoblotting, monospecific anti-p50 antibodies were affinity purified from antiserum against rabbit recombinant p50 raised in rabbits as described in Materials and Methods. The cellular level of p50 was estimated by comparing the intensities of the band from crude lysates with the intensities obtained from known amounts of purified rabbit p50 (Fig. 1). Based on the slope of the line in Figure 1C, p50 represents ~0.10% of total protein in COS-1 cells which corresponds to 2.6×10^6 molecules/cell. The same results were obtained for HeLa cells (compare lanes 1 and 2 in Fig. 2). The value is nearly equal to the number of ribosomes (17) and is ~5–10-fold higher than the number of mRNAs in the cell. In terms of molar abundance, p50 lies between the most abundant initiation factors, eIF4A and eIF5A (~10×10^6 molecules/cell), and the other initiation factors (0.6–2.4×10^6 molecules/cell) (14,17,18).

Intracellular distribution of p50

Because p50 belongs to the YB-family of transcription factors, it was of interest to study the distribution of p50 between the nucleus and cytoplasm of COS-1 cells. Comparison of the amount of p50 by Western immunoblot analysis in different subcellular fractions shows that practically no p50 is found in the nucleus (Fig. 2, lane 4). Cytoplasmic p50 is present in both polysomal (Ps) and post-polysomal (S100) fractions (lanes 5 and 6). Practically all of the p50 in the post-polysomal fraction is associated with poly(A)$^+$ mRNA as shown by oligo(dT)-cellulose chromatography (lanes 7 and 8); control chromatography of an RNase-treated post-ribosomal fraction reveals no p50 adsorbed on oligo(dT)-cellulose under the conditions used (lanes 9–11). Thus, p50 is a cytoplasmic protein entirely associated with mRNA rather than present in a free, non-complexed state.

It was shown previously that p50 is a major polypeptide of both free and polysome-bound mRNPs isolated from rabbit reticulocytes (2), but a direct analysis of p50 distribution in polysomes was never made. COS-1 cells that were freshly fed serum were lysed and the post-nuclear supernatant was subjected to sucrose gradient centrifugation to generate a polysome profile. Gradient fractions were subjected to SDS–PAGE and immunoblotting to detect the presence of p50. Nearly half of the p50 is found throughout the polysome region (Fig. 3A). When COS-1 cells were incubated on ice for 60 min prior to lysing, initiation of protein synthesis was inhibited, polysomes were reduced and ribosomes appeared mostly as 80S monomers (Fig. 3B). In this case, the bulk of p50 appears near the top of the gradient, with <20% present in the polysome region. The results confirm that considerable amounts of p50 are associated with polysomes. p50 is found only in the subribosomal zone after RNase treatment of the cell extract (data not shown). The presence of p50 within polysomes indicates that the binding of p50 to mRNA does not necessarily prevent its translation.

Inhibition of protein synthesis in COS-1 cells transiently transfected with a p50 expression vector

A mammalian vector expressing p50 (pMT3-p50) was constructed with the rabbit p50 cDNA as described in Materials and Methods. p50 expression is driven by the adenovirus major late promoter and SV40 enhancer which generate a dicistronic mRNA containing the p50 open reading frame inserted downstream from the tripartite leader of adenovirus late mRNAs and upstream of the DHFR open reading frame. Western immunoblot analysis of cells transfected with pMT3-p50 reveals an ~20% increase in p50 level (Fig. 4). Since ~20% of the cells are usually transfected by the method used (14,15,19), this corresponds to a 2-fold increase of p50 in the population of cells expressing p50 from the plasmid. It is noteworthy that overexpression of p50 is much lower than that for most other cDNAs reported with the same vector (14,19). As a control, another vector, pMT3-fsp50, was constructed that is designed to fail to express full-length p50 due to a frameshift mutation. As described in Materials and Methods, the frameshift occurs at nucleotide 115, generating a severely truncated p50 protein. Transfections with pMT3-fsp50 results in no change in p50 levels (data not shown).

To study the effect of p50 expression on mRNA translation, pMT3-p50 was cotransfected with pMT3, which expresses the DHFR reporter gene. The rate of DHFR synthesis was measured by pulse-labeling cells with [^{35}S]methionine at 48 h post-transfection. As shown in Figure 5A, the DHFR band (~26 kDa) is readily detected in cells transfected with pMT3 alone (compare lane 1 with lane 6). When cells are cotransfected with pMT3-p50 and pMT3, the DHFR band is much weaker (lane 2). In contrast, cotransfection of pMT3 with pMT3-fsp50 does not result in inhibition of DHFR synthesis (lane 3). No band comigrating with DHFR is seen in cells transfected with pMT3-p50 or pMT3-fsp50 alone (lanes 4 and 5). Quantitation of these results by phosphor-imager scanning (Fig. 5A, bottom) shows an ~6-fold inhibition of DHFR synthesis from pMT3 cotransfected with pMT3-p50, and negligible inhibition with pMT3-fsp50. Northern blot analysis shows comparable amounts of DHFR mRNA transcribed from pMT3 in all three cases (Fig. 5B, lanes 1–3). It is unclear why dicistronic mRNA is detected in significantly lower amounts than monocistronic mRNA in the expression system (Fig. 5B and ref. 14). It is possible that these two RNAs have different stabilities. The results demonstrate that inhibition of DHFR synthesis in cells cotransfected with pMT3-p50 occurs at the translation level and that overexpression of p50 is responsible for this inhibition.

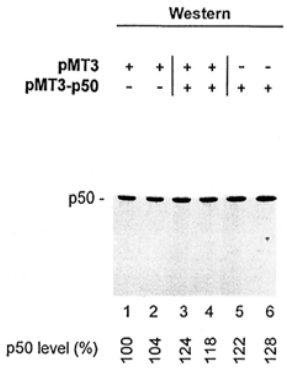

Figure 4. Analysis of p50 levels in transfected COS-1 cells. COS-1 cells transfected with pMT3 and/or pMT3-p50 as indicated at the top of the figure were trypsinized, counted and lysed as described in Materials and Methods. Lysate aliquots of 5×10^4 cells each from individual transfection plate were subjected to 12% SDS–PAGE and immunoblotted as described in the legend to Figure 1. A photograph is shown of the blot developed by the colorimetric method (see legend to Fig. 2). The migration position of p50 is indicated on the left. The results of densitometric scanning of the p50 bands are shown at the bottom.

DISCUSSION

Mammalian proteins with sequences nearly identical to that of rabbit p50 (up to 98% identity) have been characterized as transcription factors in rat (20) and human (21,22) somatic cells. The close relatedness of these proteins at the sequence level suggests that they are cognate proteins with similar functions. However, p50 was isolated from rabbit reticulocytes where it represents the most abundant and most strongly bound protein of free non-translated mRNPs (2–4). The presence of a significant amount of p50 in reticulocytes argues against the notion that it has primarily a nuclear localization and functions exclusively as a transcription factor. The physiological function(s) of p50 and related proteins in the cell is still unclear; they may participate in gene expression during both transcription and translation.

Knowledge of the cellular concentration of p50-like proteins may shed light on their function. The finding that p50 represents ~0.1% of total protein in both COS-1 and HeLa cells places it in the moderately abundant class of proteins. Its molar concentration approximates that of ribosomes and falls within the range found for the translation initiation factors (0.2–3 molecules per ribosome). On the other hand, the p50 level is significantly higher than those found for transcription factors and for mRNA species. These facts are more consistent with a role in translation than a role in transcription.

Localization of a protein in a cell and its association with precise cell components can tell a great deal about its intracellular function. p50 in fractionated COS-1 cell lysates is essentially absent in the nuclear pellet, but co-purifies with ribosomes in the cytoplasm. Immunofluorescence data confirming predominant cytoplasmic localization of p50 and showing only traces of p50 in the nuclei of 3T3 cells have been obtained recently (N.L.Korneeva, personal communication). Cytoplasmic p50 is found in association

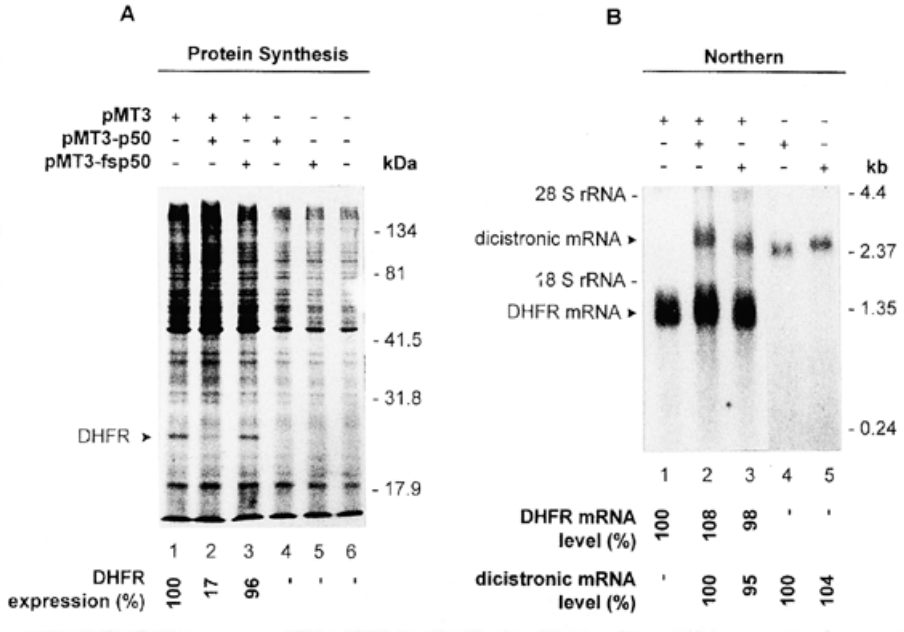

Figure 5. Analysis of DHFR synthesis and DHFR mRNA levels. (**A**) SDS–PAGE analysis of [^{35}S]methionine-labeled lysate proteins. COS-1 cells transiently transfected with pMT3, pMT3-p50, pMT3-fsp50 and their combinations as indicated at the top were pulse-labeled with [^{35}S]methionine, lysed as described under Materials and Methods and analysed by 12% SDS–PAGE. The figure shows a photograph of the autoradiogram. Molecular mass markers are shown on the right in kDa, the migration position of DHFR is indicated by an arrowhead on the left. The results of phosphorimager quantitation of DHFR bands shown at the bottom are normalized to the intensity of cellular proteins in each lane to account for loading variations. (**B**) Northern blot analysis. RNA was isolated from parallel transfection plates (indicated at the top) and analysed by Northern blot hybridization with a DHFR probe as described in Materials and Methods. A photograph of the autoradiogram is shown. Positions of DHFR mRNA, dicistronic mRNA that encodes p50, and 18 and 28 S rRNAs are indicated on the left. Positions of RNA size markers are shown on the right. Comparative DHFR mRNA and dicistronic mRNA levels measured by phosphorimager quantitation from the Northern blot are shown at the bottom. The values are corrected for the amounts of RNA loaded as determined by densitometric scans of the 18 and 28 S rRNA bands in a photographic negative of the ethidium bromide-stained gel.

with mRNA. The failure to detect a form not bound to RNA is consistent with its exceptionally high affinity for RNA (2,3). Taken together with our findings that the amount of p50 is 5–10-fold higher than the amount of mRNA in the cell on a molar basis, these data indicate that the average mRNA molecule associates with 5–10 molecules of p50, which is consistent with evaluations published earlier (3). Analysis of p50 in polysome profiles shows that at least 50% of p50 is associated with polysomes from actively translating cells, whereas when ribosomes run off mRNA, 80% of the p50 is located in fractions lighter than polysomes. Along with the demonstration that all of the cytoplasmic p50 is bound to poly(A)$^+$ mRNA, the results indicate an association both with active polysomes and with non-active mRNP particles. Together, these findings support the view that p50-like proteins in COS-1 cells are primarily involved in translation and that they may both promote and repress translation. However, we cannot exclude the possibility that a low amount of p50 is present in nuclei and functions as a transcription factor.

Since p50 was first isolated from free mRNP particles, it was suspected that the protein may play a role in masking the activity of mRNAs. A repressing role in translation was demonstrated *in vitro* by adding p50 to reticulocyte lysates, thereby causing an inhibition of protein synthesis. In the transient transfection experiments with COS-1 cells reported here, we demonstrate that merely a modest overproduction of p50 results in substantial inhibition of the synthesis of a reporter protein, DHFR. No such inhibition is found in a control experiment with a severely truncated frameshift mutant form of p50. The production of p50 from the pMT3-p50 vector is much less efficient than that observed for other proteins using the same system (14,19). A likely explanation is that total protein synthesis in the transfected cells is inhibited as p50 accumulates, thereby reducing the yield of p50. This is reminiscent of the failure to accumulate high quantities of an inhibitory mutant form of eIF2α, where the site of phosphorylation, serine-51, was altered to aspartate to mimic phosphorylation of the protein (23). That a modest increase in the level of p50 results in an inhibition of protein synthesis provides a convincing demonstration that p50 is capable of repressing translation *in vivo*. The results are consistent with those obtained by FRGY2 overproduction in somatic cells (11), and reinforce the conclusion that these YB-binding proteins play a role in masking cytoplasmic mRNAs.

Paradoxically, p50 also appears to promote the translation of mRNAs. In work to be reported elsewhere (V.M.Evdokimova, E.K.Davydova, E.A.Archakova, D.M.Naschjokin, J.W.B.Hershey and L.P.Ovchinnikov, submitted), it was demonstrated that reticulocyte lysates depleted of p50 are less active in translation, but are stimulated by the addition of p50. A role for p50 in protein synthesis is reinforced by our finding that p50 is associated with active polysomes. How p50 enhances translation is not clear. However, it is known that p50 melts mRNA secondary structure. We postulate that the binding of one or a few p50 molecules 'opens' the mRNA structure for translation. Further binding of p50 at a higher p50:mRNA ratio 'closes' the structure and masks mRNA from translation. Thus the ratio of p50:mRNA may be a critical factor for protein synthesis. Unfortunately, such ratios are difficult to determine precisely for complex mixtures of mRNAs differing greatly in overall length and thus are not available.

There is another possible model for explaining the dual translational effect of p50. It was shown that p50 is phosphorylated *in vivo* (3,21). The contradictory effects of p50 on translation might be caused by different phosphorylated forms of p50. For example, phosphorylation of FRGY2 affects its binding to RNA (24–26). It is therefore possible that free and polysome-bound mRNPs contain p50 molecules that are phosphorylated to different extents or at different sites. A detailed analysis of p50 phosphorylation is in progress to establish whether or not this mechanism contributes to p50 function.

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