The GA-binding Protein Can Serve as Both an Activator and Repressor of ribosomal protein Gene Transcription*

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The GA-binding protein (GABP), a heterodimeric transcription factor with widespread tissue distribution, has been found to be a strong positive regulator of several ribosomal protein (rp)-encoding genes. In such genes, e.g. the mouse rpL30 gene, the GABP-binding sites are located 40–80 base pairs upstream of the transcriptional start point. Potential GABP-binding sites are present in the promoters of numerous other rp genes, not only in upstream regions, but also in the immediate vicinity of the transcriptional start point. The mouse rpS16 gene is an example of the latter type. We demonstrate here that GABP binds to the rpS16 initiation region, and in so doing down-regulates rpS16 transcription both in vivo and in vitro. Supplementation of cell-free extracts with GABP inhibits transcription on rpS16 templates while concomitantly stimulating transcription on rpL30 templates. The repressive and stimulatory effects, which were proportional to the amount of GABP added, required both the GABPα subunit and either a β1 or β2 subunit. Mutations of the rpS16 GABP-binding sites that abolish binding increased rpS16 promoter activity in vivo and in vitro, whereas mutations that strengthen GABP binding caused a reduction in promoter activity. The binding of GABP to the rpS16 initiation region does not significantly affect the positioning of the transcriptional start points. Taken together with earlier studies, these new findings indicate that GABP can have a dual role as repressor or activator of rp gene transcription.

GA-binding protein (GABP) is also called nuclear respiratory factor 2 (NRF-2) or E4 transcription factor 1 (E4TF1). It is a heterodimeric transcription factor that has been shown to activate a number of genes, including those encoding the mouse ribosomal proteins L30 and L32 (1–7). One GABP subunit, termed α, is a member of the eIF4 family of DNA-binding proteins and binds weakly to DNA (1). The other subunit, β, contains ankyrin repeats and binds to DNA only in the presence of the α subunit. There are two isoforms of the β subunit, β1 and β2, which have different carboxyl termini; there are also two variants of each of these isoforms with differences in the internal portion of the molecule (2, 8, 9). The GABPα subunit, together with either the GABPβ1 or GABPβ2 subunit, can form dimeric complexes on a single GABP consensus motif (CG-GAAR), as occurs in the mouse ribosomal protein (rp) L32 promoter. A tetrameric complex, composed of two GABPα and two GABPβ1 subunits, which is stabilized by interactions through the β1 carboxyl termini, can form when two GABP binding sites are present (1). The binding sites can be contiguous, as in the mouse rpL30 promoter (7) and herpesvirus ICP4 enhancer (1), or separated, as in the genes encoding the cytochrome c oxidase subunits IV and Vb (CO4 and CO5b) (3, 5, 6). The ability to form tetrameric GABP complexes with noncontiguous binding sites is afforded by the flexibility of the β1 subunits. A previous study from our laboratory indicated that GABP is a strong positive regulator of the rpL30 and rpL32 genes (7). Subsequently, a search for CGGAAR motifs in the proximal promoter regions of other mammalian rp genes revealed many potential GABP-binding sites (7). While most of these sites are located upstream of the transcriptional-start point, as they are in rpL30 and rpL32, in five genes (mouse rpS16 and rpL7, human rpS6 and rpS14 and chicken rpS15) they are situated in the immediate vicinity of the transcriptional start point. It was previously observed that mutations in this region of the rpS16 promoter can influence the efficiency and precision of transcription both in vivo (10) and in vitro (11). However, these mutations were not suitable for assessing the role of GABP in promoter function because they also disrupted the integrity of the polyadenylylate/uridine-initiator, which is a common feature of all vertebrate rp genes. We were therefore interested in determining whether GABP binds to the rpS16 initiation region and if so, how it influences the efficiency and precision of transcription.

Here we show that GABP binds to the initiation region of rpS16 by virtue of interactions with a weak (nonconsensus) binding motif that overlaps the start points and a stronger motif that is immediately downstream of the start points (Fig. 1). By constructing various mutated versions of these motifs and studying their effects on both GABP binding and rpS16 promoter function in vitro and in vivo, we further demonstrate that occupation of the binding site by either tetrameric or dimeric GABP causes a significant reduction in rpS16 promoter activity, apparently by interfering with the formation of the transcriptional initiation complex. Consistent with these observations, we found that supplementation of in vitro transcription reactions with GABP represses rpS16 transcription while concomitantly stimulating transcription of rpL30. Thus, it would appear that GABP can have a dual role as repressor or activator of ribosomal protein gene transcription. To our knowledge, this is the first report of a repressive role for GABP in transcriptional regulation.

MATERIALS AND METHODS

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from Hela cells by the method of Shapiro et al. (12). Escherichia coli expression vectors for GABP sub-
units and anti-GABP antibodies were generously provided by Catherine Thompson and Steve McKnight. Individual GABP subunits were expressed in E. coli and purified as described in Thompson et al. (1) with one modification: GABPβ was additionally purified on a FPLC Superose 12 column. GABP subunits were stored in 20% glycerol, 12.5 mM Hepes-K+ (pH 7.9), 0.2 mM EDTA, and 50 mM KCl. GABP subunits were judged to be >90% pure based on Coomassie Blue staining of a SDS-polyacrylamide gel. GABP binding was determined by EMSA in 25-μl volumes that contained 20 mM Hepes-K+ (pH 7.9), 50 mM NaCl, 0.2 mM EDTA, 10% glycerol (v/v), 0.1–0.5 ng of end-labeled double-stranded oligonucleotide probe, and 1 μg of poly(dI-dC)·poly(dI-dC). Binding reactions were performed at room temperature for 15 min with naive GABP as indicated or recombinant GABP subunits (approximately 3 ng each). The reactions were analyzed by electrophoresis in non-denaturing gels in 0.5 × Tris borate-EDTA (TBE). Antibody supershift experiments were carried out as described previously (7). The stability of GABP-DNA complexes was determined essentially as described in Thompson et al. (1).

Cell Culture and DNA Transfection—HeLa S3 cells (ATCC CCL 2.2) were maintained in spinner culture at a density of approximately 5 × 10^6/ml in spinner-modified Eagle’s medium supplemented with 5% calf serum and antibiotics. S194 mouse plasmacytoma cells (ATCC TIB19) were maintained in suspension culture in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and antibiotics. Approximately 3 × 10^6 S194 cells were transfected with 10 μg of wild-type or mutant rps16 plasmid and 5 μg of rpl30 plasmid by the DEAE-dextran procedure (13). Cells were harvested 40 to 48 h after transfection. For isolation of cytoplasmic RNA, cell pellets were washed with phosphate buffered saline and suspended in 500 μl of a solution containing 0.15 M NaCl, 2 mM MgCl2, 20 mM Tris-HCl (pH 7.4), and 0.5% Nonidet P-40. Nuclei were immediately removed by a 5-min centrifugation at 2,000 rpm in an Eppendorf microcentrifuge. Supernatants were recovered, and SDS and EDTA were added to 0.5% and 5 mM, respectively. Protease K (10 μg) was then added, and after a 15-min incubation at 37°C, the reactants were extracted twice with phenol-chloroform; the RNA was then precipitated with ethanol.

Western Blot Analysis—Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to an immobilon-P filter using a semidry blotter. After transfer, the filter was washed extensively with TBS containing 0.1% Tween 20 (v/v) and then incubated with a 1/500 dilution of horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. The filter was then processed using an ECL Western blotting analysis system according to the manufacturer.

DNase I Footprinting—Probes for DNase I footprinting were generated by polymerase chain reaction (PCR) using oligonucleotide primers 5′ and 3′ to the GABP binding sites. Either the sense or antisense primer was 5′-end-labeled with [γ-32P]ATP and polynucleotide kinase prior to PCR. An equimolar amount of GABPα and GABPβ subunits were incubated with the 32P-labeled DNA fragment under conditions described for EMSA except reactions were scaled-up 5-fold. After a 15-min incubation at room temperature, MgCl2 and CaCl2 were added to 5 mM each and protein-bound DNA and free DNA were partially digested with DNase I. Reactions were terminated by the addition of EDTA to 50 mM and then subjected to electrophoresis on a 4% polyacrylamide gel containing 0.5 × TBE. Free DNA and protein-bound DNA were identified, recovered by electrodialysis, and subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Dideoxy sequencing markers generated with the same oligonucleotide primers used for PCR were run in parallel as markers.

Purification of GABP—GABP was purified from the S100 fraction of HeLa cells, which is a side product of nuclear extract preparation (12). S100 (approximately 1 g) derived from 50 liters of HeLa cells was pooled and loaded onto a 100-ml phenocellulose (P11) column equilibrated in buffer D (10% glycerol (v/v), 20 mM Hepes-K+ (pH 7.9), 0.2 mM EDTA) containing 0.1 M NaCl. The P11 column was washed with three column volumes of buffer D containing 0.1 M NaCl and bound protein was eluted by sequential elution with buffer D containing 0.5 M NaCl and 1 M NaCl. Column fractions were monitored for GABP binding by EMSA using an rps16 initiator oligonucleotide probe (~11 to +13). GABP was recovered in the P11 flow-through and 0.1 M NaCl wash, which were pooled and applied to a 50-ml Q-Sepharose column equilibrated in buffer D containing 0.1 M NaCl. The column was washed with three column volumes of buffer D containing 0.1 M NaCl and bound protein was eluted with a linear gradient of 0.1 to 1 M NaCl in buffer D. Fractions containing the bulk of GABP, eluting at approximately 0.8 M NaCl, were pooled and dialyzed against buffer D containing 0.1 NaCl.

This fraction was then subjected to two rounds of DNA affinity chromatography (14) using a catenated oligonucleotide containing rps16 sequences ~11 to +18. The purity of GABP was monitored by SDS-polyacrylamide gel electrophoresis and staining with silver. Using this procedure, we recovered approximately 20 μg of highly purified GABP.

In Vitro Transcription—Supercoiled plasmids (3 μg) were transcribed in 100-μl reactions containing 20 mM Hepes-K+ (pH 7.9), 6 mM MgCl2, 60 mM KCl, 8 mM (NH4)2SO4, 1.2% polyethylene glycol (20,000), 1 mM dithiothreitol, 6 mM rNTPs, and 20 μl of nuclear extract (5–8 S100 units). Reactions were performed at 37°C for 1 h. Whole reactions were supplemented with GABP, reaction conditions were adjusted accordingly. The reaction was terminated by the addition of an equal volume of stop buffer (10 mM EDTA, 100 mM sodium acetate, pH 5.5, 0.2% SDS, 10 μg/ml salmon sperm DNA), extracted twice with an equal volume of phenol/chloroform, and precipitated with ethanol. All experiments were performed with three different preparations of nuclear extract to verify reproducibility.

Primer Extension—In vitro synthesized RNA or 50 μg of cytoplasmic RNA was transcribed in 25 μl of hybridization buffer (10 mM Tris-HCl, pH 7.5, 0.25 mM KCl, 1 mM EDTA) containing approximately 0.5 ng (20,000 cpm) of a 5′-end-labeled primer (5′-CTCTATTTAGGTCTTCTTGGATCC-3′) complementary to cat vector sequences, heated to 65°C for 5 min, renatured, and then allowed to cool to 42°C. The extension reaction was extended by the addition of an equal volume of elongation mix (40 mM Tris-HCl, pH 8.3, 20 mM MgCl2, 0.5 mM EDTA, 4 mM dithiothreitol, and 2 mM dNTPs) containing 0.5 unit of avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. The extended products (86 and 224 nucleotides, respectively, for rps16 and rpl30 templates) were recovered by ethanol precipitation and analyzed on a 5% sequencing gel. Gels were dried and analyzed with a Fuji PhosphorImager and by autoradiography. For quantitation of the transfection data, the rps16 signals were normalized to the corresponding signal from a cotransfected rpl30 construct in order to correct for variations in transfection efficiency and RNA yield. The normalization equation was

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\text{rps16}_{\text{corrected}} = \left( \frac{\text{rps16_{average}}}{\text{rps16_{measured}}} \right) \times \text{rps16_{measured}}
\]

Promoter Constructs—"Wild-type" constructs rps16 c-CAT (~179 to +29) and rpl30 c-GAT (~205 to +167) (16) were used in this study. Both of these constructs utilize the cat expression vector p106 and contain all the elements required for maximal promoter activity. Independently isolated PCR subclones of rps16 mutants were digested by PCR with Genuario et al. (7). Each site-directed mutant was confirmed by DNA sequencing. All plasmids were purified by two rounds of CsCl-ethidium bromide gradient centrifugation.

Oligonucleotides—The following oligonucleotides were used for binding assays in this study. Mutations are shown in lowercase. rps16 (~11 to +18) wild type: (5′-ATCCCCCGGGCCTTCCTTGGATCCGCG-3′) and (3′-GGGGCGCGAACAGGAAAGCGCCCGCTTAA-5′); rps16 cm8, (5′-CCCCCGGCTTCTTCTTTCCGTCGCCGGTGGTGGAGGACCT-3′) and (3′-GGGGCGCGAACAGGAAAGCGCCCGCTTAA-5′).
RESULTS

Demonstration That GABP Binds to the rpS16 Initiator Region—Several pieces of evidence demonstrated that GABP binds to the rpS16 promoter. First, recombinant GABP subunits were used in an electrophoretic mobility shift assay with a DNA probe containing the rpS16 promoter, which was supershifted by GABP-specific antibodies. EMSA of rpS16 oligonucleotide probe incubated with affinity purified native GABP (10 μl) in the presence or absence of preimmune serum or antibodies raised against recombinant GABP subunits. The positions of the tetrameric, dimeric, and monomeric complexes are indicated.

**Fig. 2.** Demonstration that GABP binds to the rpS16 promoter. A, recombinant α and β GABP subunits (rGABP) were incubated with 32P-labeled oligonucleotide probes derived from the rpS16 promoter (−11 to −18) or rpL30 promoter (−70 to −30) under conditions described for EMSA. Reactants were subjected to electrophoresis on a 4% polyacrylamide gel in 0.5 TBE. The complexes corresponding to αβ, tetramers and dimers and α monomers are indicated. B, various amounts (0.2, 2, 5, or 10 μl) of native GABP (nGABP) from the second round of DNA affinity chromatography were incubated with a 32P-labeled rpS16 oligonucleotide probe (−11 to +18) under conditions described for EMSA. The reactants were subjected to electrophoresis on a 4% polyacrylamide gel in 0.5 TBE. The slower and faster moving complexes are designated U and L, respectively. C, aliquots (50 μl) from the first (A1) and second (A2) rounds of DNA affinity chromatography were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by staining with silver. The relative molecular masses of the candidate GABP subunits are indicated on the right side of the panel. An aliquot (50 μl) of the nGABP A2 fraction was electrophoresed on a 10% SDS-polyacrylamide gel and visualized by staining with silver. The relative molecular masses of the candidate GABP subunits are indicated on the right side of the panel. The positions of the tetrameric, dimeric, and monomeric complexes are indicated.

**Fig. 3.** Electrophoretic mobility of rpS16-protein complexes is supershifted by GABP-specific antibodies. EMSA of rpS16 oligonucleotide probe incubated with affinity purified native GABP (10 μl) in the presence or absence of preimmune serum or antibodies raised against recombinant GABP subunits. The positions of the tetrameric, dimeric, and monomeric complexes are indicated.

GAGACTTAA-5′; rpS16 cm9, (′-CCCCGGCT-TCCGTTTTCGCGCCCGCGGTGT-3′) and (′-GGGGCCGG-GAGGGAAAGCCAGCGCAGCGCTGGATT-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm10, (′-CCCCGGCTTCTTTCCGGTCCCGCCGTCGGGTGTTAA-5′). rpS16 cm11, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm12, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm13, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm14, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm15, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm16, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm17, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm18, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′). rpS16 cm19, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm20, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′) and (′-GGGGCCGG-ACACC-5′); rpS16 cm21, (′-AATTCCCCGCTCCGTTTCCGCTTGGTTAA-3′).
Most likely, these represent the variant GABP \( \beta \) isoforms characterized by other investigators (8, 9). Finally, the complexes formed with the purified native protein were specifically supershifted by antibodies raised against either recombinant GABP\( \alpha \) or GABP\( \beta \) subunits (Fig. 3). These results provide compelling evidence that the rpS16 promoter contains two GABP sites and can bind GABP as both a dimer and a tetramer.

Characteristics of GABP Binding to the rpS16 Initiator Region—DNase I footprinting with recombinant GABP subunits was used to further characterize the binding of GABP to the rpS16 initiator region. Analysis of a tetramer of GABP bound to the rpS16 DNA probe (GABP\( (\alpha \beta \beta) \); see Fig. 2A) revealed that only the site centered at +6 was protected from digestion (Fig. 4A). The lack of protection of the site centered at -2 suggested that the protein-DNA interaction in this region is relatively weak and unstable. To determine if this weak interaction is due to the presence of a nonconsensus G at +2, we changed the G to a C to match the consensus GABP motif (GGGAAG to CGGAAG), and characterized the footprint of the mutant construct. The footprint of this mutant (cm9; see Fig. 8) was much broader than that observed with the wild-type construct and encompassed both GABP sites (Fig. 4B), thus confirming that the G at +2 does indeed weaken the binding of GABP. These data indicate that the rpS16 promoter contains a weak (+2 to -4) and a relatively strong (+9 to +4) GABP-binding site, which together permit the formation of tetrameric complexes at the transcriptional start site. The dimeric complexes are exclusively located at the stronger site, as indicated by additional footprint analysis (data not shown) and mutational studies (see below).

A previous analysis of tetrameric GABP complexes formed on the rpL30 promoter (7) showed a broad footprint, similar to that seen with the cm9 mutant of rpS16. Thus, a comparison of footprint data indicates that the GABP tetrameric complexes formed on the rpS16 promoter are less stable than those formed on the rpL30 promoter. A difference in stability was also evident from the results of a kinetic competition experiment (Fig. 5). In this experiment, 84% of the rpS16 tetramers dissociated by 0.5 min, compared to 44% dissociation of the rpL30 tetramers. By 8 min, the fraction of residual rpS16 tetramer was about 0.1 that of the rpL30 tetramer. The decay of the tetramers in this experiment was not strictly first-order, as one might expect on theoretical grounds, possibly due to inadequate mixing at the later time points. For this reason, it is not possible to obtain a precise quantitative estimate of the difference in stability from these data. Nevertheless, it is clear that the GABP tetramers formed on the rpS16 initiator region are relatively unstable compared to tetramers formed on the upstream binding site of the rpL30 promoter. Indeed, the stability of the rpS16 tetramers is similar to that of dimeric complexes formed on either the rpS16 or rpL30 promoters (data not shown).

Differential Effects of GABP Supplementation on rpS16 and rpL30 Gene Transcription—GABP activates and represses rp gene transcription (4391).
GABP Activates and Represses rp Gene Transcription

rpl30 Transcription in Vitro—To evaluate the role of GABP in rpS16 transcription, we carried out a series of experiments in which cell-free transcription reactions were supplemented with bacterially synthesized GABP. As a positive control, we examined the effect of GABP supplementation on rpl30 transcription reactions. Consistent with the role of GABP as a positive activator, rpl30 transcription was strongly stimulated when GABP was added to the transcription reaction (Fig. 6A). The stimulation was proportional to the amount of GABP added (lanes 5–7), was dependent on both GABP subunits (lanes 1–3), and the repressive effect required both GABP subunits (lanes 2–4). The inverse effect of GABP supplementation on rpl30 and rpS16 transcription was dramatically illustrated by an experiment in which GABP was added to a single reaction containing both templates (Fig. 6C, lanes 1–3). In a parallel reaction, GABP had no effect on the transcription of a mutant rpS16 template, cm13 (see Fig. 8), which lacks GABP binding sites (Fig. 6C, lanes 4–6). These results indicate that the repressive effect of GABP requires binding to the template and that it is not due to a nonspecific squelching phenomenon.

We also observed inverse effects on rpS16 and rpl30 transcription when GABPα and β2 subunits were added to the reaction. Since β2 lacks the homodimerization domain of the β1 subunit, α and β2 do not form a stable tetrameric complex on the rpS16 initiator region. Nevertheless, they form a readily detectable dimeric complex (Fig. 7A). This binding occurs at the stronger downstream site and is completely eliminated when this site is destroyed in the cm8 mutant (see below). As shown in Fig. 7B, the GABPαβ2 dimers repressed rpS16 transcription (lanes 3–5), although not quite as effectively as the αβ2 complexes (lanes 2–4). As expected, rpl30 transcription was stimulated by the GABPαβ2 dimers under the same conditions. These results indicate that dimeric complexes at the downstream site are sufficient to elicit a repressive effect. The effect is apparently potentiated when αβ2 tetramers can be formed.

The Effects of Mutations on GABP Binding and rpS16 Promoter Activity—The foregoing results indicate that when GABP binds to the rpS16 initiator transcription is repressed.

This conclusion was further supported by the results of mutational experiments. In these experiments we produced a series of mutations in the GABP-binding sites and studied their effects on both GABP binding and rpS16 promoter activity. The mutations (Fig. 8, bottom) were designed to alter the characteristics of GABP binding while preserving, insofar as possible, the integrity of the polypurine tract that spans the start points (10). Single base pair substitutions in the core GGAA of the GABP motifs (cm8, cm11, cm12, and cm13) were used to abolish binding at one or both sites; such point mutations were previously shown to effectively eliminate GABP binding in the rpl30 and rpl32 promoters (7). In addition, a single base pair mutation, namely, substitution of a C for the nonconsensus G was used to strengthen the weak upstream site in cm9 and cm11. Finally in cm10, the sites were made contiguous by a single base pair deletion.

The effects of the various mutations on GABP binding were determined by an EMSA analysis (Fig. 8, top). As expected, no GABP binding was detected with cm13 (lane 7), and tetramer formation was eliminated in cm11 and cm12 (lanes 5 and 6). Interestingly, the cm8 mutation abolished dimer, as well as tetramer, formation (lane 2; also see Fig. 7A), indicating that
binding at the weak upstream site requires the presence of a functional downstream site. Conversion of the upstream site to the preferred consensus sequence not only increased the stability of the tetrameric complexes (cm9, lane 3), but also enabled dimeric complexes to be formed at the upstream site, even when the downstream site was nonfunctional (cm11, lane 5). Stronger GABP binding was also observed when the wild-type sites were made contiguous (cm10, lane 4), probably by enhancing the interaction between the β subunits. The stronger binding was confirmed by a DNase I footprinting analysis (data not shown).

The effects of these mutations on promoter strength and start point selection were then analyzed by both transient transfection experiments and in vitro transcription assays (Fig. 9, Table I). In contrast to the results of our previous mutational analysis of the rpl30 and rpl32 promoters (7), the activity of the rps16 promoter was not diminished when GABP binding was totally abolished (mutants cm8 and cm13). In fact, these mutants were about twice as active as the wild-type construct both in vivo and in vitro. Conversely, mutants cm9 and cm10, which bind GABP more tightly than wild-type, were even less active than the wild-type promoter. The in vivo activities of mutants cm11 and cm12, which form only dimeric GABP complexes, were similar to that of the wild-type rps16 promoter, consistent with the idea that tetramer formation is not essential for the repressive effect of GABP. As might be anticipated, when the in vitro reactions were supplemented with GABP, mutants cm9 and cm10 were strongly repressed, while mutants cm8 and cm13 were relatively unaffected (Fig. 9B). Although the activity of mutant cm12 was significantly reduced by GABP addition, the cm12 template was consistently more active than the wild-type template in unsupplemented reactions. The reason for the relatively high in vitro activity of cm12 is presently obscure. None of the mutations had a marked effect on the location of the transcriptional start points; at most, displacements of only one or two base pairs were observed.

DISCUSSION

The studies described above indicate that GABP can bind to the rps16 initiation region, and in so doing modulate the activity of the rps16 promoter. Based on the consensus recognition site for GABP, CGGAAR, the activity of the rps16 promoter is increased by GABP. Since the upstream site is considered to be suboptimal: the upstream site, which overlaps the transcriptional initiation sites. At most, displacements of only one or two base pairs were observed.

GABP Activates and Represses rp Gene Transcription
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GABP β can repress rps16 transcription. A, recombinant GABPα and GABPβ, or GABPβ2 were incubated with 32P-labeled oligonucleotide corresponding to wild-type rps16 sequences or mutant sequences under conditions described for EMSA. Reactants were subjected to electrophoresis on a 4% gel in 0.5 TBE. B, rps16 or rpl30 transcription reactions were supplemented with either rGABPα and rGABPβ (lanes 2-4) or rGABPα and rGABPβ2 (lanes 5-7). The amount of rGABP used to supplement the reactions was identical to that used in Fig. 6A. Lane 1 was not supplemented with GABP. Reaction products were characterized as described in Fig. 6.

FIG. 8. Effect of mutations on GABP binding. Upper panel, rGABP was incubated with 32P-labeled oligonucleotide probes containing wild-type sites or sites with point mutations. The reactants were subjected to electrophoresis on a 4% polyacrylamide gel containing 0.5 TBE. Complexes corresponding to tetramers (αβ), dimers (αβ), and monomers (α) are indicated. Lower panel, summary of GABP site mutants used in the study. For the ease of comparison, the sequence of the rps16 antisense strand is shown. The large and small arrows represent the major and minor rp16 transcriptional initiation sites. Lower case letters indicate mutations; D and U refer to the downstream and upstream sites, respectively; – and + represent mutations designed to abolish or increase GABP binding, respectively. The consensus binding motif for GABP (B) is shown at the bottom of the figure (R = purine).

As illustrated in Fig. 8, one of the rps16 recognition sites is suboptimal: the upstream site, which overlaps the transcriptional start points, has a nonconsensus G at position 1. DNase I footprinting analysis, kinetic competition experiments and mutational studies demonstrated that tetrameric GABP complexes formed on these sites are relatively unstable compared to tetrameric complexes formed on a tandem pair of consensus sites, e.g. those in rpl30. Since the upstream site is considerably weaker than the downstream site, the dimeric complexes that form on the rps16 promoter are located almost exclusively on the downstream site.

Given that GABP serves as a transcriptional activator of
several other genes, we were initially surprised to find that it has a repressive function for rpS16. Earlier mutational studies of the rpS16 initiation region (10, 11) were inconclusive on this point because the mutations that abolished GABP binding also obliterated the polypyrimidine tract that spans the start points. In the present study we have eliminated this ambiguity, and find that transcriptional activity is either increased or decreased relative to the wild-type gene depending on whether the mutations eliminate or strengthen GABP binding. Moreover, when GABP was added to a cell-free transcription system, transcription from the wild-type rpS16 promoter decreased in a dose-dependent manner while transcription from the rpl30 promoter concomitantly increased. The magnitude of the repressive effect of GABP, estimated by comparing the activities of mutants cm8 and cm13 with that of wild-type in vivo or in un-supplemented in vitro reactions, is about 2-fold. Although this is not a large effect in absolute terms, it is comparable to the contribution of any single transcriptional activator to the strength of an rp promoter (7, 11, 15–17), and is therefore likely to be biologically significant.

Our studies suggest that occupation of the downstream GABP site would be sufficient to cause repression. This was indicated by the fact that repression could be elicited by αβ2 dimers, which form stable complexes only at the stronger downstream site. Thus, in the wild-type rpS16 promoter, the combination of relatively unstable tetramers and residual dimers on the downstream site effectively modulates the transcriptional activity. The binding of GABP does not significantly affect the selection of the transcriptional start points.

Although the mechanism by which GABP regulates transcriptional activity is not fully understood, there is general agreement that the DNA binding specificity resides mainly in the α subunit, while the activation function resides exclusively in the β subunit (1, 4, 9), apparently in a region that is common to the various β isoforms (8, 9). This region contains evenly spaced clusters of hydrophobic residues, which could mediate interactions with components of the basal transcriptional apparatus (18, 19). In agreement with this general concept, we also observed that both α and β subunits are necessary to elicit the regulatory function of GABP, both as an activator of rpl30 and as a repressor of rpS16 (Fig. 6). The particular components of the transcriptional apparatus that interact with GABP have not yet been identified, and it is presently unclear whether the activator and repressor functions involve the same or different constituents of the β subunit. It seems reasonable to suppose that similar interactions might be involved in both functions and that the distinction between activation and repression is determined by the context of the GABP sites within the promoter architecture. Thus, depending on precise spatial relationships, the GABP interactions could either facilitate or impede the assembly of a transcriptional initiation complex.

A relationship between GABP function and binding site context might be inferred for the four rp genes that have been analyzed to date: an activation function for rpl30, rpl32, and Xenopus rpL14 (7, 20), where the sites are located 50–75 base pairs upstream of the transcriptional start points, and a repressive function for rpS16, where the sites overlap the transcriptional start points. Yet, in the CO4 and CO5b promoters, GABP bound to sites that overlap with transcriptional start points apparently has an activation function (3, 5). Thus, other contextual features besides proximity to the start points may determine whether GABP will have a positive or negative effect on transcription.

The fact that a single transcription factor can serve as both an activator and repressor is not unprecedented (21–23). The use of this principle for the regulation of rp gene transcription would make sense if there is not a large variation in GABP content from cell type to cell type. In this case, the opposing effects could be part of a fine-tuning mechanism, which helps ensure relatively uniform rates of transcription among the many unlinked rp genes (16). However, if the level of GABP were to vary greatly among different cells, then the opposing effects could actually widen differences in rp gene transcription rates. Although GABP is known to have a widespread tissue distribution (8), virtually nothing is known about its relative abundance in different tissues. Without this information, it is presently difficult to judge whether the activator/repressor function of GABP is advantageous for the coordinate regulation of rp genes or whether it is simply a tolerated consequence of evolutionary tinkering.

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TABLE I

| Mutant | GABP complexes | Activity |
|--------|----------------|----------|
|        |                | In vivo  | In vitro |
| cm8    | None           | 213 ± 33 (3)* | 233 ± 8 (3) |
| cm9    | Strong tetramer and dimer | 59 ± 12 (3) | 26 ± 5 (3) |
| cm10   | Strong tetramer and dimer | 59 ± 22 (3) | 37 ± 13 (6) |
| cm11   | Dimer at upstream site | 106 ± 15 (3) | 67 ± 7 (6) |
| cm12   | Dimer at downstream site | 75 ± 8 (3) | 196 ± 35 (6) |
| cm13   | None           | 157 ± 7 (3) | 175 ± 13 (5) |

*The number of experiments is shown in parentheses.
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The GA-binding Protein Can Serve as Both an Activator and Repressor of ribosomal protein Gene Transcription

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