Autoregulation of phage μ transposase at the level of translation

Ronald L. Parsons and Rasika Harshey*

Research Institute of Scripps Clinic, Department of Molecular Biology, La Jolla, CA 92037, USA

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ABSTRACT

The bacteriophage Mu A and B genes, which lie adjacent to each other and are colinear on the phage genome, encode proteins that catalyze efficient transposition of Mu DNA. We show that the molar ratio of A and B proteins is approximately 1:20 in extracts prepared after induction of cells containing a Mu lysogen or a plasmid carrying the Mu fragment that encompasses A and B. In cells harboring the cloned genes, the proteins are synthesized from a single transcript. Pulse-chase experiments demonstrate that the lower amounts of A protein are not from preferential turnover of this protein. This suggests the existence of a post-transcriptional mechanism to down-regulate A protein synthesis. From an analysis of the activity of several β -galactosidase fusions to A protein, we infer that A protein may repress its own translation. By an agarose gel mobility-shift assay, we demonstrate that purified A protein binds specifically in vitro to its mRNA.

INTRODUCTION

Transposable genetic elements are efficient mutagens that bring about numerous types of gene rearrangements (see 1). Most transposons have complex mechanisms to regulate their mobility and maintain a delicate balance between their mutagenic ability and the survival of their hosts. Invariably, these regulatory mechanisms are directed towards stringently controlling the expression of the transposase, the protein that catalyzes transposition (2-4).

The transposable element Mu is a temperate bacteriophage (reviewed in 5), whose transposition is regulated in a complex manner at many levels. In the lysogenic state, the Mu repressor c, binds to an operator region (6), and negatively regulates transcription from the early promoter P_e. The transposition genes A and B are transcribed from P_e (see Fig. 1). A protein is the transposase, which binds specifically to the two ends of Mu DNA (attL and attR; 7) to bring about DNA transposition. The repressor protein shows homology to the amino-terminus of the A protein (8), and the two proteins have been shown to bind each other's DNA sites in vitro (7), suggesting that the A protein may also regulate its own transcription. In an in vitro reaction, the

repressor inhibits Mu transposition (7). There is also indirect evidence that transcription of the repressor itself, which proceeds toward attL may interfere with the action of A on that site (9).

The start of the early transcript originating from promoter Pe, which the repressor c regulates (see Fig. 1), has been mapped to 1028 basepairs from the Mu genetic left end (10). Upon induction of temperature sensitive repressor mutants by thermal inactivation, the early mRNA is synthesized rightward (11; Fig. 1). R-loop analysis indicates that this transcript is probably longer than 6 kb (cited in 10). Proteins encoded by the early transcript would include, in addition to A and B, Ner, a protein that negatively regulates transcription from both Pc and Pe (see Fig. 1; 12). DNA sequence information from this region does not indicate the presence of any rightward promoter other than Pe (8,13). The ner and A genes are tightly linked with only one nucleotide separating the stop codon of ner from the start of A (14), suggesting coordinate or sequential translation of these two genes. A is separated from B by 38 nucleotides that contain the translation initiation signals for B (13). Although A is sandwiched between ner and B, the expression of A protein is considerably less than that of both the upstream Ner and the downstream B proteins (see 15-18). In the absence of B, mutations that increase the expression of A protein also result in a decrease in its activity (measured as frequency of precise excision of Mu sequences inserted in Lac, 19; see also 20). To add to this complex scenario, the A protein acts stoichiometrically and appears to be inactive after one round of transposition (21).

In this study we explore the reasons for the lower expression of A protein relative to B from a polycistronic message that encodes both proteins. First, we show that this difference in expression persists even when the two cloned genes are transcribed as a single message from a foreign promoter. Second, this apparent lower expression of A is not caused by its more rapid turnover than B. Third, the level of production of A and B proteins appears to be unrelated to the frequency of distribution of nonoptimal codons in their genes (13; unpublished data). Fourth, computer analysis of the DNA sequence of the A gene (8) reveals no significant potential for secondary structure formation for the A mRNA that could potentially impede its translation (unpublished data). Finally, from the activity of β -galactosidase fusions to varying lengths of the A protein, we infer that A protein may repress its own translation. The purified A protein binds specifically to its mRNA in vitro suggesting a mechanism for translational repression.

MATERIALS AND METHODS

Bacterial strains

Used here were HM8305 (F'pro+lacZ::Muets/Δ(pro-lac) his met Str^R Mu^R); OR1265 (22; F' his ilv galP 308::IS2 λN7 N53 cI857 cro27 cryptic Su⁻ Str^R); JM101 (F' traD36 proAB lacI^q lacZAM15/supE thi Δ(lac-proAB).

Plasmids

The construction of pKC30 (23), pMC1871 (24), pUC8 and 9 (25), pRA-04 (19), pRA04-43 (26), pDB11 (19), and pRA-L29 (27) has been described. pT712 and 13 were purchased from Bethesda Research Laboratories (BRL), MD.

We made the following constructs (for easy comprehension, follow the restriction sites in Fig. 1): pRA121, by ligating a HindIII-HpaI fragment containing ner, A and most of the B gene into the HindIII-SmaI site of pT712; pRA122, by ligating the BalI-HpaI DNA fragment of Mu encoding A and most of B, into the SmaI site of pT713 such that the A gene was proximal to the T7 promoter; pRA123, by ligating the EcoRV-HpaI DNA fragment of Mu encoding the same genes as in pRA122, into the SmaI site of pT713 such that A was promoter-proximal; pUC9Z, by ligating the lacZ-containing PstI fragment from pMC1871 into the PstI site of pUC9, to create an in-frame lacZ gene; pDZ4, by ligating the lacZ-containing PstI fragment from pMC1871 into the PstI site of the A gene in pDB11, to generate an in-frame A-lacZ fusion; and pRA151, by ligating the BalI-PstI fragment of Mu containing part of the A gene, along with the PstI-lacZ fragment of pMC1871, into the SmaI-PstI site of pUC8.

pRA152 was constructed by first cloning a filled-in BalI-HinfI fragment, which includes the first 12 amino acids of Mu A, into the SmaI site of pUC9 to generate pRA-S10, such that the direction of A transcription was the same as that of the lacZ fragment on the pUC plasmid. A filled-in XmaI-ScaI lacZ-containing fragment of pMC1871 was then ligated to a filled-in EcoRI site of pRA-S10 to generate pRA152, which contained lacZ in-frame with Mu A. pRA152D was constructed in a manner similar to pRA152, except that the BalI-HinfI fragment of pRA-S10, was substituted with EcoRV-HinfI. To make pDZB2, a SmaI-ScaI lacZ fragment from pMC1871 was ligated to a filled-in BssHII site within A on plasmid pDB11, to generate an in-frame A-Z fusion. pDZB1 is similar to pDZB2 except that it has the upstream region of A from BalI to EcoRV intact.

pRA141 was constructed by ligating the EcoRV-DraI fragment containing A gene into the SmaI site of pCHL884 (a pACYC184 derived plasmid containing the lac OP and polylinker cloning site from M13mp8 and chloramphenicol resistance gene from pACYC184; 28). pRA158R encodes the first 269 residues of Mu A protein and was constructed by site-directed mutagenesis (to be described

elsewhere) to create an EcoRV site at position 2133 within the Mu A gene. An EcoRV fragment containing the NH₂-terminal A protein fragment was subsequently cloned into the SmaI site of pCHL884 to generate pRA158R.

The fusion joints in pRA-S10, pRA152 and pDZB2 were confirmed by DNA sequencing by the dideoxy chain-termination method of Sanger (29), using appropriate primers for chain extension.

Reagents

T7 RNA polymerase was from BRL. ³⁵S-methionine and Enlightning fluor were from New England Nuclear (NEN), Boston, MA. ¹²⁵I-Staphylococcus aureus protein A was kindly provided by H. Niman, Scripps Clinic, La Jolla, CA. Polyclonal antibodies to purified Mu proteins A and B were prepared by immunizing rabbits against these proteins. Anti β -galactosidase antibodies were purchased from Boehringer-Mannheim Biochemicals (BMB). E.coli IHF protein was kindly provided by A. Landy, Brown University, Providence, RI.

Measurement of A and B proteins

One ml of an overnight culture of the appropriate strain was inoculated into 20 ml of LB (supplemented with 100 μ g/ml of ampicillin for plasmid-containing strains) and grown with shaking until the Klett reached 40. Ten mls of the culture were withdrawn for 0' control. To the remaining culture were added 10 ml of 60° medium, and the culture was grown at 42°. Ten ml portions were removed at 0.5 and 1 hr, pelleted and resuspended in 1.8 ml of water. Proteins were precipitated by addition of 0.2 ml of 100% trichloroacetic acid (TCA). After 30 min incubation on ice, the precipitate was collected by a 30 min centrifugation at 20,000 x g, washed twice with acetone and resuspended in 0.2 ml of Laemmli sample buffer (30). Protein concentration was determined by the method of Lowry (31). Samples were electrophoresed on 10% SDS polyacrylamide gels (30). Proteins were electroblotted onto nitrocellulose membrane in 0.025M Tris base, 0.2M glycine, 20% methanol at 60 milliamps, overnight. Blots were developed as described (32) after incubation with a mixture of antibodies to both A and B proteins followed by ¹²⁵I-S. aureus protein A, subsequently washed and exposed to film. Various concentrations of purified A and B proteins were run as standards. Expression of A and B proteins in the experimental samples was determined by comparing the densitometric tracings of their autoradiograms to those of the standards.

Determination of stability of A and B proteins

One and a quarter ml of an overnight culture of plasmid-containing OR1265 cells (grown in 0.5% glucose M9 medium, supplemented with 20 μ g/ml thiamine and histidine, 40 μ g/ml valine and isoleucine and 100 μ g/ml

ampicillin) were added to 3.25 ml of fresh medium. Cells were grown at 32°C for 1 hr, then shifted to 42°C for 0.5 hr. The culture was divided into 1 ml fractions, to each of which were added 10 µl of a 1:10 dilution of ³⁵S-methionine (970 Ci/mmol; 10 mCi/ml). Incubation was continued for 2 min. Cells were chilled at 0° along with addition of 10 µl of cold methionine (1.5 mg/ml). Cells were pelleted and washed twice at 4° in M9 medium containing 15 µg/ml of methionine. They were resuspended in the same medium and incubated at 32° for various times. Proteins were precipitated by addition of TCA as described above. Approximately 50 µg of protein from each sample were electrophoresed on an 8% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, destained and soaked in Enlightning fluor for 30 min, dried at 65°C and fluorographed at -70°C with an enhancing screen.

Measurement of β-galactosidase levels

The lacZ-fusion plasmids were transformed into JM101, with or without pRA141 or pRA158R. The resulting strains were induced with 1mM IPTG for 1 hr and β-galactosidase activity was assayed in whole cells as described (33). Amounts of fusion proteins were determined as follows: One ml of an overnight culture of JM101 harboring the appropriate lacZ fusion plasmid was inoculated into 20 ml of LB (supplemented with 100 µg/ml of ampicillin) and grown with shaking at 37° until the Klett reached 30. Ten mls of culture were withdrawn for 0' control. To the remaining culture was added IPTG to a final concentration of 1mM and the shaking was continued for 1 hr. Proteins were precipitated with TCA, electrophoresed and transferred to nitrocellulose as described above, except that the blots were treated with anti-β-galactosidase antibody followed by ¹²⁵I- S. aureus protein A.

Northern blots

Cells were grown and induced as described above, to express A and B proteins. RNA was prepared as described by McKenney et al. (34). Samples were electrophoresed, transferred to nitrocellulose and hybridized by Hattman's method (35). Radiolabeled probes to A and B genes were prepared by nick translation (36) of EcoRV-PstI and DraI-HpaI fragments from these genes respectively (see Fig. 1), using α-³²P dCTP.

In vitro transcription of DNA

Transcription reactions were carried out with T7 RNA polymerase as described in the BRL catalog. Reactions contained 1 µg of plasmid DNA, 10 units of polymerase, 400 µM of all four ribonucleoside triphosphates and 10 µCi of α-³²P UTP (410 Ci/mmol; 1 mCi/ml) in 50 µl. Run-off transcripts were prepared from pRA121 digested with EcoRV, pRA122 digested with BssHII or DraI, and pRA123 digested with PstI, BssHII or DraI.

Gel assay for A protein-RNA complex formation

Approximately 10 nM RNA transcribed in vitro was incubated with varying amounts of Mu A protein in 0.15M KCl, 25mM Hepes (pH7.5), 10mM MgCl₂, and 1mM dithiothreitol (DTT) for 20 min at 37°. Samples were loaded on 1% agarose gels after addition of 5% (w/v) Ficoll 400. Gels were cast and run in 10mM sodium phosphate buffer, pH 6.8, at 50V. They were dried and autoradiographed. Essentially similar reactions were carried out with Mu attL containing plasmid pRAL29 (27), which was linearized with HindIII and labeled at the 5' ends with polynucleotide kinase and [γ -³²P] ATP.

RESULTS

Expression of A and B genes

We wished to determine whether subcloning the DNA fragment encoding the A and B genes away from the preceding regulatory gene ner and the early promoter Pe (see Fig. 1) would alter the pattern of expression of these two proteins. The ratio of the two proteins produced after prophage induction in a lysogen (HM8305) was compared with that produced upon induction of a strain containing the cloned genes under λP_L promoter control. Protein samples from these strains were fractionated on SDS-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Western blotting). The membrane was then incubated with antibodies to A and B proteins followed by incubation with ¹²⁵I-S. aureus protein A (Figure 2). The amounts of the two proteins A and B were estimated by comparing a densitometric tracing of the autoradiogram (not shown) to a standard tracing obtained by Western blotting several concentrations of purified A and B proteins and treating them with the same protocol (Fig. 2C). In Fig. 2A and B, the A protein amounts are in the linear range of estimation, but B protein amounts are beyond this range. The latter was therefore estimated at higher dilutions of the total protein. We estimate the molar ratio of A:B to be 1:20 in the induced Mu lysogen HM8305 and 1:24 in the pRA-04 containing strain. As can be seen, although the levels of these proteins are different in the two strains, their molar ratio is similar.

Transcription of pRA-04 DNA

Upon prophage induction, transcription of early genes initiates at Pe (10). Unpublished data (cited in 10) indicate that the early transcript traverses genes ner, A and B. The polarity of insertion elements isolated within the early region, supports the idea of a single transcript spanning this region (37). To determine whether the translation of A and B proteins in

Fig. 1. Genetic organization near the left end of the Mu genome. The hatched rectangle and circle denote the attL and operator, which are primarily bound by A (transposase) and c (repressor) proteins, respectively. Pc and Pe are promoters at which repressor and early transcription (jagged line) is initiated. Relevant restriction sites used in various manipulations of this segment of DNA (see text) are indicated at the bottom (drawn approximately to scale). B-BalI, Bs-BssHIII, D-DraI, E-EcoRV, H-HindIII, Hf-HinfI, Hp-HpaI, P-PstI. Extent of Mu DNA present in various plasmids is indicated below the restriction map. Dotted line indicates a deletion. λ P_L, T7 and lac refer to the different promoters driving transcription of Mu DNA in the various plasmids.

a clone containing the A-B region fused to the λP_L promoter (pRA-04) occurred from a single transcript or from multiple transcripts, we hybridized probes specific to the A or B genes to Northern blots of RNA from cells harboring pRA-04. Figure 3 shows that, upon thermal induction of transcription from the λP_L promoter, both A and B gene probes hybridize to the same sized transcript (lanes 2 and 3 respectively). RNA prepared from a clone containing a deletion in B, pRA04-43, showed a reduced transcript size again hybridizing to both probes (lanes 4 and 5), ruling out the existence of two transcripts of the same length. These results suggest that differential synthesis of A vs B protein must be due to post-transcriptional controls.

Fig. 2. Western blot of A and B proteins present in induced cultures. One half hour after induction of expression of A and B proteins from the Mu lysogen HM8305(A) and the cloned DNA pRA-04 in OR1265 (B), proteins were prepared, electrophoresed and Western blotted as described in Methods. (A) 50, 100 and 200 μ g of total protein were loaded in lanes 1, 2 and 3, respectively. Lane 0 contains 50 μ g of protein 0' after induction. (B) 10, 20 and 40 μ g of total protein were loaded in lanes 1, 2 and 3, respectively. Lane 0 contains 40 μ g of protein 0' after induction. (C) Lanes 1-3 contain 50, 25 and 12.5 ng of purified B protein and lanes 4-6 contain 50, 75 and 100 ng of purified A protein. Arrows mark the position of A(75 kDa) and B(33 kDa) proteins. In (A), the bands below the top arrow are nonspecific, because they react even with protein from uninduced samples.

Stability of A and B proteins

To determine whether the difference in the ratios of A and B proteins results from more rapid degradation of A, we performed a pulse-chase experiment. Cells harboring pRA-04 DNA were induced for protein expression, pulsed for 2 minutes with labeled methionine, and then chased for various times with cold methionine. Figure 4 shows that both induced proteins are stable for up to 60 minutes after adding the chase element. Consequently, a difference in their rate of synthesis must cause the dissimilar ratios of the A and B proteins.

Activity of β -galactosidase fusions

We previously showed (19) that mutations upstream of the ribosome-binding site of A increase the synthesis of A protein, as measured by β -galactosidase levels of *lacZ* fusions to several such mutants. In this study, we fused *lacZ* to three different lengths of the A-coding sequence, with or without the upstream region between *Bal*I and *Eco*RV within the preceding gene

Fig. 3. Analysis of A and B transcripts from cloned DNA. pRA-04 or pRA04-43 containing OR1265 cells were thermally induced and RNA was prepared as described in Methods. (A) Ethidium bromide stained 1% agarose gel containing 20 μ g of RNA, 0 min (lane 1) and 30 min (lanes 2 and 3) after induction. The position of 23S and 16S RNA is indicated. (B) Northern blot of the same lanes. 1 and 2 were hybridized to a probe specific to the A gene, and 3 was hybridized to a probe specific to the B gene (see Methods). Lanes 4 and 5 contain 20 μ g of RNA from pRA04-43, prepared 30 min. after induction, hybridized as in lanes 2 and 3, respectively.

ner (we designate this area BE). Figure 5 tabulates the results. Consistent with our earlier results (19), the absence of the BE region increased β -galactosidase levels 1.5 to 3-fold in all three fusions. We observe that the lacZ fusion to the PstI site within A (pRA151; 116 amino acids of A protein) is 4-fold less active than the one to the HinfI site (pRA152; 12 amino acids of A protein). However, the fusion to the BssHII site within A (pDZB1; 350 amino acids of A protein) is 15-fold less active than the one in pRA152. A control lacZ gene inserted in the common pUC vector is slightly more active than the most active A-lacZ fusion (containing the BE region) in pRA152.

To test the idea that the coding region of A in the fusion proteins was causing an inhibition of A translation, we provided either the A protein or an amino-terminal fragment (pRA141 or pRA158R, respectively) in trans, to strains harboring the A-lacZ fusions. With pRA158R (encoding 269 residues of A), we observed a 10% inhibition of β -galactosidase activity of pRA152, 35% inhibition of pRA151 and no inhibition of pDZB1 (Fig. 5). Similar inhibition patterns were obtained with the entire A protein provided in trans. The

Fig. 4. Pulse-chase experiment to determine the stability of A and B proteins. Protein expression was induced in appropriate strains as described in Methods. Thirty minutes after induction, they were given a 2 min pulse of S^{35} -methionine, washed, and then chased for various times with cold methionine. Protein samples were electrophoresed on 8% polyacrylamide gels and treated as described in Methods. Zero, 5, 15, 30 and 60 represent the time in minutes after addition of cold chase. (A) proteins expressed from plasmid pKC30 (23) without cloned Mu genes and (B) proteins from pRA-04, which contains Mu A and B cloned in pKC30. Arrows mark the positions of A (top) and B (bottom) proteins.

control plasmid pCHL884 (containing no A sequences) had no effect on any A-lacZ fusion plasmid.

To determine whether the drop in the activity of β -galactosidase with increasing length of the fusion proteins was due to a lower specific activity of the fusion proteins, we determined fusion protein levels by electrophoresing extracts prepared from the appropriate strain before and after induction with IPTG, Western blotting, and developing the blot using antibodies against β -galactosidase as described in Methods (Fig. 6). Visual examination of the blot shows that the differences in relative amounts of the fusion protein appear to closely correspond to the differences in β -

		β -galactosidase Units ($\times 100$)		
		-A	+A	% Inhibition
pRA152		136	122	10
pRA152D		204	179.5	12
pRA151		35	23	35
pDZ4		92	64	30
pDZB1		8.7	9.4	—
pDZB2		17	18	—
pUC9Z		170	161.5	5

Fig. 5. β -galactosidase levels in MuA-lacZ fusions. LacZ fusions to different regions of the A gene were constructed as described in Methods (see Fig. 1). B and E mark the BalI and EcoRV sites within ner. H, P and Bs mark the HinfI, PstI and BssHII sites within A. The ribosome binding signal for A translation, which lies within the preceding gene ner, is represented by a closed circle, and the arrowhead marks the start of A translation. Jagged lines represent the lacZ gene and the interrupted line marks the upstream DNA in the pUC plasmid. The lines are not drawn to scale. Cells containing these plasmids were induced with 1mM IPTG for 1 hr and assayed as described in Methods. β -galactosidase levels are expressed as Miller units (33). -A and +A refer to β -galactosidase units in the absence or presence of pRA158R encoding a 269-residue amino-terminal fragment of A protein. Similar inhibition profiles were observed with pRA141, encoding the entire A protein.

galactosidase activities measured for these fusions (see Fig. 5). This was confirmed by a densitometric tracing of the autoradiogram (not shown), in which pRA152 has 4-fold more protein than pRA151 and 12-fold more than pDZB1. These results show that lower activities of the fusion protein are due to their lower amounts.

The A protein binds to its mRNA

The above experiments suggested to us that A, a site-specific DNA-binding protein, may also bind RNA and in doing so regulate its own translation. We therefore developed an agarose gel-electrophoresis-based "mobility-shift" assay to observe A protein binding to its RNA. A gene RNA was transcribed in vitro using T7 RNA polymerase. Figure 7A shows that with increasing concentration of A protein, the RNA shifts to higher mobility, indicative of interaction with A (lanes 1-3). This interaction is sensitive to SDS (lane 4) and is not observed with tRNA (lanes 5,6). Three other

Fig. 6. β -galactosidase amounts in MuA-lacZ fusions. One hour after induction of β -galactosidase expression in various A-lacZ fusion plasmids, proteins were isolated and electrophoresed as described in Methods. All lanes contain 50 μ g of total protein. (A) Lane 0, uninduced sample of pUC9Z; lane 1-4 induced samples from pRA152, pRA151, pDZB1, and pUC9Z, respectively. The gel was stained with Coomassie Blue. A duplicate of these lanes was transferred to nitrocellulose. (B) Western blot of lanes in (A) prepared by incubating with anti- β -galactosidase antibody followed by 125 I-S. aureus protein A.

proteins, MuB, E. coli IHF and BSA, show no binding under these conditions (not shown; Mu B and E. coli IHF are DNA-binding proteins). We then studied A protein binding to several RNAs synthesized in vitro. RNA made to the entire A gene bound A protein nearly as well as one made to the BssHII site within A (not shown), 4 to 5-fold more efficiently than one made to the PstI site (Fig. 7B), and at least 10-fold more efficiently than one to HindIII-EcoRV fragment encoding ner (Fig. 7C, lanes 1-4). No difference was detectable in binding RNA's with or without the BE region (Fig. 7C, compare top band in lanes 1-4 with lanes 5-7). Thus, A protein binds specifically to its mRNA in vitro. To compare the affinity of this binding to that of binding its known substrate, the ends of Mu DNA, Figure 7D examines via the same assay, the mobility shift observed with the left end (attL) of Mu DNA. We observe that

Fig. 7. Visualization of A protein binding to RNA. RNA was transcribed in vitro as described in Methods. TOP: (A) Approximately 10 nM ^{32}P -labeled RNA (synthesized up to the DraI site at the end of A in pRAL23) was incubated with 0, 0.1 and 0.5 μM A protein (lanes 1-3), run on 1% agarose gels and autoradiographed as described in Methods. Lane 4 is the same as 3 except that 0.1% SDS was added to the sample after the reaction. Lanes 5 and 6 contain approximately 10 nM ^{32}P -labeled tRNA incubated without and with 10 μM A protein, respectively. (B) 10 nM each of two different RNAs (larger one up to the BssHII within A in pRAL23 and smaller one up to the PstI site in pRAL23) incubated with 0, 0.1, 0.5 and 1 μM A protein in lanes 1-4 respectively, and treated as in (A). (C) 10 nM of two RNAs (larger one made up to BssHII site in A on pRAL23 and smaller one made up to EcoRV site within ner on pRAL21) were incubated with 0, 0.2, 1 and 2 μM A protein in lanes 1-4. Lanes 5-7 contain the same RNA as in lane 1 top, except the upstream BE region is included in the transcript (derived from pRAL22). The RNA was incubated with 0, 0.2 and 2 μM A protein. (D) 10 nM ^{32}P -labeled Mu attL containing DNA (pRAL-29) was incubated with 0, 0.05, 0.1 and 0.2 μM A protein and electrophoresed as above. BOTTOM: Schematic diagram of the genetic organization of the left end of the Mu genome and its relevant restriction map (as in Fig. 1). Thin lines below the restriction map indicate extent of Mu sequences in the transcripts used in various lanes in TOP figures A-D. Hatched line denotes extent of Mu DNA from pRAL-29.

similar amounts of A protein are required to bind the Mu left end as to bind A-mRNA, amounts shown earlier to be in the range required for specific binding to Mu DNA ends (27).

DISCUSSION

We have shown in this study that down-regulation of A protein synthesis occurs after transcription by a mechanism that involves lowering the rate of A protein synthesis, rather than increasing its rate of degradation. We surmise that hindrance to protein chain elongation resulting from non-optimal codon usage and mRNA secondary structure is unlikely to be the rate limiting step in A synthesis (unpublished data). Our previous results with β -galactosidase fusions indicate that sequences upstream of the ribosome binding site negatively regulate A protein translation, because mutation of these sequences consistently enhances the levels of A protein 2 to 3-fold. The importance of the 5' untranslated region in translational initiation has been documented for some proteins (38,39). It is thought that this region is involved in secondary structure formation, sometimes with sequences downstream in the coding region, so as to sterically hinder the binding of ribosomes to mRNA. Should such a mechanism operate for the Mu A gene, it would also affect the expression of the preceding ner gene, in whose coding region these regulatory sequences lie. Examination of levels of Ner protein relative to A from many published experiments (see 17) suggests that there is several fold more Ner protein than A protein. Therefore, although it is not clear how the upstream region might decrease A expression, reduced translation initiation may not be the sole reason for reduced A expression.

Comparison of β -galactosidase levels in the A-lacZ fusion in pRA152 with those in pUC9Z (which contains no A sequences, and presumably has good translation signals) shows that the two levels are similar. This suggests that translation signals in front of the A gene cannot solely account for the low levels of A protein synthesis. Also, the tight linkage of ner and A genes (14) in the early operon would serve to ensure co-translation of the well-translated ner gene (15-18) with that of the A gene.

β -galactosidase fusions to various lengths of the A sequence demonstrate that a fusion to the first 116 amino acids of A protein is 25% as active as one to the first 12 amino acids, whereas fusion to 350 amino acids is only 6% as active. Examination of the amounts of fusion proteins (using antibodies to β -galactosidase) shows that these are in good agreement with their observed activity levels. This excludes lower specific activity of a longer fusion protein as the cause of the low levels of β -galactosidase in these fusions. The results are compatible with the idea that the translated region of A in these fusions is repressing its own translation.

To test the role of amino-terminal A protein sequences in translation

repression, we provided the A protein or a fragment containing 269 amino acids of A in trans (Fig. 5). pRA152 and 152D showed only a 5% inhibition above the control pUC9Z, suggesting perhaps that A protein recognition sites on the RNA lie beyond these sequences. The best inhibition was seen with pRA151 and its derivative pDZ4. This inhibition (25-30% above the control pUC9Z) is not as high as expected (90-95%), perhaps because the fragment that is being provided in trans is already bound to RNA, acts poorly in trans, or as suggested above, the target sequences recognized by A protein may not all be present in these constructs. Noteworthy, however, is the observation that the pDZB fusions are not inhibited at all, a result consistent with the idea that they encode sufficient information to repress their own translation.

To visualize a direct interaction of A protein with its mRNA, we set up an RNA-binding gel assay, similar in principle to binding assays (40) that measure interactions of DNA and protein by the altered mobility of DNA-protein complexes relative to free DNA during gel electrophoresis. We observed that A protein has a high affinity for its own RNA synthesized in vitro. Although no difference in binding RNAs with or without the 5' BE region of A could be detected, a significant difference was observed in binding RNA made to the ner gene vs RNA made to the first 380 nucleotides of A or the entire A gene, suggesting that the binding region(s) may be within the A coding sequence. The affinity of A protein binding to its mRNA is comparable to that observed for its specific binding to the Mu DNA ends.

What features of the mRNA and A protein could participate in their interaction? As a DNA-binding protein, A uses a region in its amino terminus (27) to bind 6 specific sequences at the ends of Mu DNA (7). A consensus recognition sequence is TGNTTCANTNNAAPuPyPuCGAAAPu. Perhaps the target for translational repression could resemble this sequence. The best match to this sequence occurs towards the 3' end in the coding region of A, although sequences with lower homology occur elsewhere (8). Alternatively, since A protein also binds the Mu operator sequence, perhaps regions homologous to the operator sequence (consensus for the repressor binding site is CTTTTPyAPuA/TAANNA/T; 6), which also occur scattered throughout the A-coding region, may be the target for A-recognition. Results from the β -galactosidase fusion experiments argue that A protein sequences involved in RNA-binding lie within the first 350 amino acids at the amino-terminus of A, a region known to contain the site-specific DNA-binding domain of the A protein (27). These, as well as the RNA-binding experiments suggest that there may be multiple target sequences for the A protein, since RNA containing the first 300 nucleotides of

A binds A protein efficiently, but RNA to the entire A gene binds even better. Clearly, further experiments are needed to define the RNA and protein elements of this interaction.

There are several examples of translational or feedback repression of protein synthesis (see 41-43), including regulation of the phage T4 gene 32 and reg A proteins and of ribosomal proteins of E.coli. Campbell et al. (41) contend that proteins that bind to nucleic acids have the evolutionary potential of using their own nucleic acid-binding domain to repress self-synthesis; the mRNA target would evolve to look like the primary ligand. Since RNA can form stable secondary structures, double-strand DNA-binding proteins could potentially find their recognition sites in the RNA. The examples studied to date offer a good deal of evidence that translational regulatory proteins recognize both sequence and structure in the mRNA.

In summary, our results argue for a process that regulates Mu A protein synthesis at the level of translation as well as transcription. Our results are consistent with a direct interaction of the A protein and its mRNA to bring about this regulation.

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*To whom correspondence should be addressed

REFERENCES

1. Shapiro, J.A. (ed.) (1983) In Mobile Genetic Elements, Academic Press.
2. Kostriken, R., Morita, C. and Heffron, F. (1981) Proc. Natl. Acad. Sci. USA 78, 4041-4045.
3. Isberg, R.R., Lazaar, A.L. and Syvanen, M. (1982) Cell 30, 883-892.
4. Simons, R.Q., Kleckner, N. (1983) Cell 34, 683-691.
5. Symonds, N., Toussaint, A., van de Putte, P. and Howe, M.M., (eds) (1987) In Phage Mu, Cold Spring Harbor Laboratory, New York.
6. Krause, H.M. and Higgins, P.N. (1986) J. Biol. Chem. 261, 3744-3752.
7. Craigie, R., Mizuuchi, M. and Mizuuchi, K. (1984) Cell 39, 387-394.
8. Harshy, R.M., Getzoff, E.D., Baldwin, D.L., Miller, J.L. and Chaconas, G. (1985) Proc. Natl. Acad. Sci. USA. 82, 7676-7680.
9. Goosen, N. and van de Putte, P. (1986) J. Bacteriol. 167, 503-507.
10. Krause, H.M., Rothwell, M.R. and Higgins, N.P. (1983) Nuc. Acids Res. 11, 5483-5495.
11. Wijffelman, C. and van de Putte, P. (1974) Mol. Gen. Genet. 135, 327-337.
12. Goosen, N. and van de Putte, P. (1984) Gene 30, 41-46.
13. Miller, J.L., Anderson, S.K., Fujita, D.J., Chaconas, G., Baldwin, D. and

-
- Harshey, R.M. (1984) *Nuc. Acids Res.* 12, 8627-8638.
14. Priess, H., Kamp, D., Kahmann, R., Brauer, B. and Delius, H. (1982) *Mol. Gen. Genet.* 186, 315-321.
 15. Magazin, M., Reeve, J.N., Maynard-Smith, S., and Symonds, N. (1978) *FEMS Microbiology Lett.* 4, 5-9.
 16. Giphart-Gassler, M., Reeve, J. and van de Putte, P. (1981) *J. Mol. Biol.* 145, 165-191.
 17. Van Leerdam, E., Karreman, C. and van de Putte, P. (1982) *Virology* 123, 19-28.
 18. Tolias, P.P. and DuBow, M.S. (1986) *Virology* 148, 298-311.
 19. Harshey, R.M. and Cuneo, S. (1986) *J. Genet* 65, 159-174.
 20. Chaconas, G., Gloor, G., Miller, J.L., Kennedy, D.L., Giddens, E.B., and Nagainis, C.R. (1984) *Cold Spring Harbor Symp. Quant. Biol.* 49, 279-284.
 21. Pato, M.L. and Reich, C. (1984) *Cell* 36, 197-202.
 22. Reyes, O., Gottesman, M. and Adhya, S. (1979) *Virology* 94, 400-408.
 23. Rao, R.N. (1984) *Gene* 31, 247-250.
 24. Shapira, S.D., Chou, J., Richard, F.V. and Casadaban, M.J (1983) *Gene* 25, 71-83.
 25. Viera, J. and Messing, J. (1982) *Gene* 19, 259-268.
 26. Teplow, D.B., Nakayama, C., Leung, P.C. and Harshey, R.M. (1988) *J. Biol. Chem.* 263, 10851-10857.
 27. Nakayama, C., Teplow, D.B., and Harshey, R.M. (1987) *Proc. Natl. Acad. Sci.* 84, 1809-1813.
 28. Lee, C.H., Bhagwat, A. and Heffron, F. (1983) *Proc. Natl. Acad. Sci. USA* 80, 6765-6769.
 29. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
 30. Laemmli, U.K. (1970) *Nature* 227, 680-685.
 31. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem* 193, 265-275.
 32. Paruchuri, D.K. and Harshey, R.M. (1987) *J. Bact.* 169, 61-65
 33. Miller, J.H. (1972) In *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, NY, USA, pp.352-355.
 34. McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C. and Rosenberg, M. (1981) In *Chirikjian, J.C. and Papas, T.S. (eds), Gene Amplification and Analysis*, Elsevier-North Holland, Amsterdam, Vol. 2, pp.383-415.
 35. Hattman, S. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5518-5521
 36. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
 37. Giphart-Gassler, M., Goosen, T., Van Meeeteren, A., Wijffelman, C. and van de Putte, P. (1979) *Cold Spring Harbor Symp. Quant. Biol.* 43, 1179-1185.
 38. Stanssens, P., Renaut, E. and Fiers, W. (1985) *Gene* 36, 211-223.
 39. Coleman, T., Nakamura, K. and Inouye, M. (1985) *J. Mol. Biol.* 181, 139-143.
 40. Fried, M. and Crothers, D. (1981) *Nuc. Acids. Res.* 9, 6505-6525.
 41. Campbell, K.M., Stormo, G.D. and Gold, L. (1983) In *Beckwith, J., Davies, J., Gallant, J.A. (eds), Gene Function in Prokaryotes*, Cold Spring Harbor Laboratory, NY, USA, pp.185-210
 42. Winter, R.B., Morrissey, L., Gauss, P., Gold, L., Hsu, T. and Karam, J. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7822-7826.
 43. Nomura, M., Gourse, R. and Baghman, G. (1984) *Ann. Rev. Biochem.* 53, 75-117.
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