Autogenous Regulation of the *regA* Gene of Bacteriophage T4: Derepression of Translation

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ABSTRACT

The *regA* gene of phage T4 encodes a translational repressor that inhibits utilization of its own mRNA as well as the translation of a number of other phage-induced mRNAs. In recombinant plasmids, autogenous translational repression limits production of the RegA protein when the cloned structural gene is expressed under control of a strong, plasmid-borne promoter (lambda P_L). We have found that a genetic fusion which places the *regA* ribosome binding domain in proximity to active translation leads to partial derepression of wild-type RegA protein synthesis. The derepression is not due to increased synthesis of *regA* RNA, suggesting that it occurs at the translational level. Derepressed clones of the wild-type *regA* gene were used to overproduce and purify the repressor. In an *in vitro* assay the wild-type target was sensitive and a mutant target was resistant to inhibition by the added protein. The results suggest that the sensitivity of a *regA*-regulated cistron to translational repression may depend on the competition between ribosomes and RegA protein for overlapping recognition sequences in the translation initiation domain of the mRNA.

THE RegA protein of bacteriophage T4 is a translational repressor which regulates its own synthesis (autogenous control) as well as the synthesis of several other phage-induced proteins that are made during the early stages of T4 growth in infected *Escherichia coli* hosts [see WIBERG and KARAM (1983) for a review]. The protein binds to target regions in the translation initiation domains of regulated mRNAs and inhibits translation, apparently by blocking ribosome entry onto the message (KARAM *et al.* 1981; WINTER *et al.* 1987). Genetic and *in vitro* repression assays with T4 RegA protein and its regulated mRNAs have indicated that the repressor recognizes different target sequences which exhibit different sensitivities to repression, but the precise locations and nucleotide sequences of most of these targets are not yet known (KARAM, MCCULLEY and LEACH 1977; KARAM *et al.* 1981; WINTER *et al.* 1987). Recent developments in RNA footprinting techniques offer a direct means of identifying nucleotide residues that participate in the interaction between RegA protein and its mRNA targets (WINTER *et al.* 1987); however, the results of such assays are best interpreted in the light of physiological and mutational analyses on the putative regulatory sites. So far, only one *regA* target site, that of the nonessential T4 *rIIB* cistron, has been amenable to such biological analyses (KARAM *et al.* 1981; MILLER *et al.* 1987; WINTER *et al.* 1987). Also, *in vitro* repression and RNA binding assays require sources of wild-type RegA protein that can reliably distinguish the weakest *regA* binding sites (ADARI and SPICER 1986; WINTER *et al.* 1987). The recent *in vitro* studies with the T4 *rIIB* target (WINTER *et al.* 1987) suggest that two factors affect repressibility of a *regA*-regulated cistron: relative affinity of the RegA protein to its specific binding site and the efficiency of utilization by ribosomes of the translation initiation domain encompassing the site. We have been interested in how these two factors influence autogenous regulation of T4 RegA protein synthesis. Standard T4 infections produce low levels of RegA protein and, consequently, recombinant DNA techniques have been used to try to overproduce this repressor for biochemical work (MILLER *et al.* 1985; ADARI *et al.* 1985). Invariably, however, expression of the T4 *regA* gene under control of heterologous promoters in recombinant plasmids is accompanied by autogenous repression at the translational level, and this limits production of the protein (MILLER *et al.* 1987). Also, clones of the wild-type gene tend to select for *regA* mutations that alter the biological activity of the gene product (MILLER *et al.* 1985, 1987; ADARI *et al.* 1985). Some of these mutations can result in release of repression on weak targets (translational derepression), without obvious effects on more sensitive targets. In this report we show that the autogenous target, which is a weak target site for RegA protein (ADARI and SPICER 1986; WINTER *et al.* 1987), can be partially derepressed when it is placed downstream, and in close proximity, to translating ribosomes. The results suggest that, in a translation reinitiation context, ribosomes can compete more effectively against repression by RegA protein than in a translation initiation context. A derepressed $regA^+$ clone was used

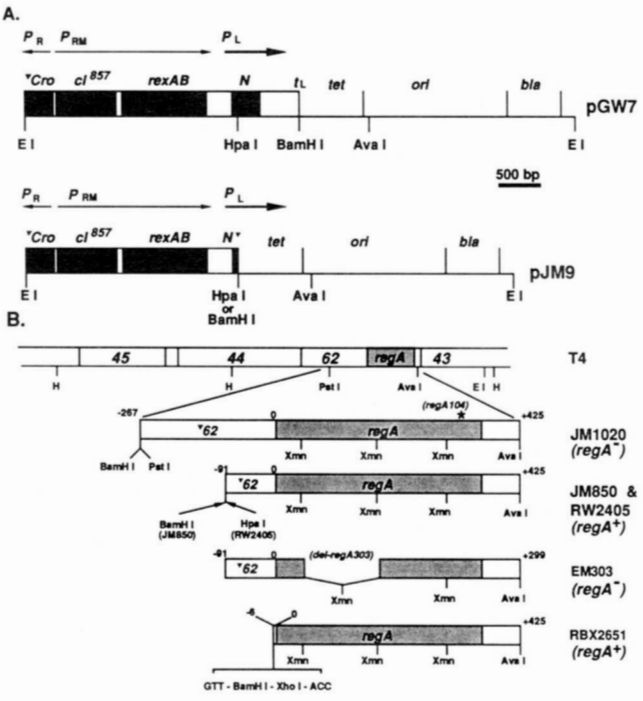

FIGURE 1.—The expression plasmids and T4 DNA fragments used. The plasmids are represented in linear form. The pGW7 vector was a gift from G. WILSON (New England Biolabs) and has been used and described in previous studies (TROJANOWSKA *et al.* 1984; MILLER *et al.* 1987; HSU *et al.* 1987). Its derivative pJM9 lacks the N function of the plasmid-borne lambda DNA. The T4 DNA fragments are drawn in the orientation of their insertion directly downstream of the lambda DNA, in the *Bam*HI (or *Hpa*I) to *Ava*I intervals of the expression vectors. In these clones, expression of the T4 *regA* gene could be brought about by growth of the plasmid-bearing cells at 42°. The $regA_{104}$ mutation was isolated and mapped by E. S. MILLER in cloned T4 DNA (MILLER *et al.* 1985 and personal communications).

to purify large quantities of the RegA protein, which was subsequently shown to be able to distinguish between wild-type and mutant autogenous targets *in vitro*.

MATERIALS AND METHODS

Bacterial strains and recombinant plasmids: The *E. coli* B strain NapIV (NELSON *et al.* 1982) was used as a host for recombinant plasmids in most of the studies described here. Some of the DNA preparations used originated from plasmids grown in *E. coli* K802, a K12 strain (WOOD 1966). In general, overproduction of plasmid-encoded T4 proteins proceeded more effectively in the *E. coli* B background.

The T4 DNA fragments and the expression plasmids used are diagrammed in Figure 1. The $regA^+$ fragment JM850, the $regA^-$ fragment JM1020 (carries mutation $regA_{104}$), the $regA^-$ fragment EM303 (carries an in-frame *Xmn*I internal deletion, $regA_{303}$), and the lambda $cI^{857}P_LN$-bearing, pBR322-derived expression plasmid pGW7 have been described (MILLER *et al.* 1987). The construction of plasmids carrying RBX2651 and other $regA^+$ fragments that bear alterations in the putative target for autogenous regulation will be described elsewhere. The RW2405 fragment is identical to JM850 except that the *Bam*HI site of the latter had been replaced by a *Hpa*I site. The pJM9 vector was derived from pGW7 by *in vitro* manipulations that involved removal by restriction enzyme digestions of the DNA interval bounded by the *Hpa*I and *Bam*HI sites of pGW7 and replacing it with either a single *Hpa*I (used for cloning RW2405) or a single *Bam*HI site (for cloning RBX2651). These manipulations allowed the expression of the cloned T4 DNAs under control of lambda P_L in the absence of the transcription antiterminator protein encoded by gene N. Also, in pJM9 clones T4 DNA sequences could be fused to the translated 5′ terminal segment of the N cistron. This seemed to be advantageous for overproduction of wild-type RegA protein at 42° (see RESULTS). No RegA protein synthesis was detected from either pGW7 or pJM9 clones when recombinants were grown at 30°. The EM303 clone was constructed by E. S. MILLER in pACYC184 (*ori* p15A background), which is compatible with plasmids carrying the pBR322 ($colE_1$) origin of replication and could be used in two-plasmid assays with pGW7 and pJM9 clones of other T4 DNA fragments. These assays were carried out as described recently by MILLER *et al.* (1987).

Growth conditions and assays: Plasmid-bearing *E. coli* K802 and NapIV strains were grown and maintained in LB broth containing the appropriate antibiotic for the plasmid under study, *i.e.*, 20 μg/ml ampicillin for pBR322-derived recombinants and 10 μg/ml tetracycline for pACYC184-derived plasmids. Long-term storage of bacterial strains was at −20° in 50% glycerol. The medium for labeling bacterial and plasmid-derived proteins *in vivo* with [^{35}S]methionine (DuPont, NEN Division, Cat. No. NEG009A) was M9-19, which is M9 supplemented with all amino acids, except methionine (MILLER *et al.* 1987). The conditions for heat-inductions of recombinant clones, radiolabeling, preparation of extracts, and analysis of proteins by SDS-gel electrophoresis and autoradiography have been detailed in previous reports (*e.g.*, HSU *et al.* 1987; MILLER *et al.* 1987). Plasmid-encoded RNA was isolated, purified, and subjected to S1-mapping as described elsewhere (HSU *et al.* 1987; ANDRAKE *et al.* 1988).

The purified RegA protein that was used for *in vitro* repression assays was prepared from a pJM9 clone of RW2405 DNA (Figure 1) grown in *E. coli* NapIV cells. The cells were grown, heat-induced, harvested, and frozen in aliquots by K. CALLANAN of the fermentation facility at the Waksman Institute, Rutgers University, New Jersey. Cells were cultured in the 1000-liter fermenter at 30° in LB broth containing 20 μg/ml ampicillin until they reached a density of 5×10^8 cells/ml. At this point the growth temperature was rapidly increased to 42° and aeration was continued for 90 min after which the cells were harvested by centrifugation and frozen in dry-ice. Subsequent storage of the frozen cells was in a −80° freezer.

The *E. coli* cell-free S30 extracts and other reagents used for *in vitro* assays were either purchased from DuPont, NEN Division (Cat. No. NEK038) or prepared as described by PRATT (1984). Coupled transcription/translation assays were carried out in 6-μl mixtures containing 0.5 μl plasmid DNA (0.5 μg), 3 μl of S30 extract plus reagents including 5 μCi [^{35}S]methionine, and 0.75 μl (0–20 μM) RegA protein in Tris-acetate storage buffer (see below). Assay mixtures were incubated at 37° for 20 min before the addition of 100 μl SDS-extraction buffer. Samples were subsequently treated and analyzed similarly to samples from *in vivo* labelings with [^{35}S]methionine.

Purification of plasmid-generated wild-type RegA protein: The procedure used to purify the RegA protein included several modifications of methods reported by others (MILLER *et al.* 1985; ADARI and SPICER 1986). A summary of results from a typical purification that utilized 50 g of heat-induced NapIV cells carrying a pJM9 clone of RW2405

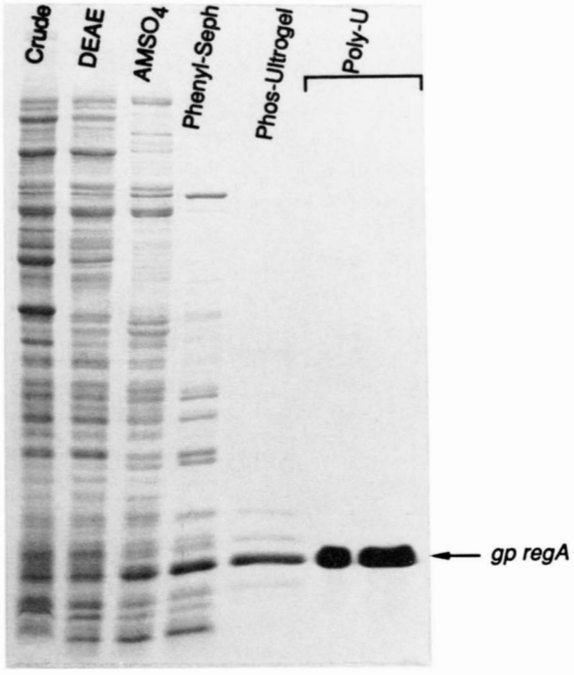

FIGURE 2.—Purification of plasmid-encoded T4 RegA protein. Details of the purification method used are included in the text. Samples from the different purification steps were subjected to SDS-gel electrophoretic analysis on a 1-mm-thick slab gel, which was subsequently stained for 30 min with 0.1% Coomassie-brilliant blue R250 in 50% trichloroacetic acid and destained with several changes of 7% acetic acid before it was dried on dialysis membrane and photographed. The identity of RegA protein was established by Western blot assays (not shown) that were performed on duplicate gels and by using *regA*-specific antibody.

DNA is presented in Figure 2. Purification was monitored by Coomassie blue staining of samples analyzed by SDS-gel electrophoresis, and identity of the RegA protein in the samples was established by Western blot assays (not shown) which utilized *regA*-specific antibody, a gift from E. S. MILLER (MILLER *et al.* 1985). The 50 g of frozen cell pellet were thawed in the cold while being stirred with 2.5 vol of a lysing buffer, which contained 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 mM NaCl, and 10 μg/ml DNase I (Grade I, Boehringer/Mannheim). The suspension was then passed twice through a French pressure cell at 16,000 psi and the lysate was made 2% in Triton X-100 and 0.5 M in NaCl. The Triton and salt treatments increased solubility of the RegA protein, which otherwise remained mostly bound to cell-membrane fractions (KARAM *et al.* 1982). After stirring in the cold for 3 hr, the lysate was centrifuged at 30,000 × *g* for 35 min at 4° in a Sorvall centrifuge and the supernatant was recentrifuged in a Beckman Model L8 ultracentrifuge at 100,000 × *g* for 35 min using a Ti45 rotor. About 60% of the RegA protein of the original extract was recovered in the soluble fraction following these treatments. The resulting supernatant was dialyzed against a buffer containing 50 mM Tris-Cl pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM NaCl, and 10% (v/v) glycerol and was then chromatographed on a 1.5 × 25 cm DEAE-Sephacel (Pharmacia) column that had been equilibrated with the same buffer. About half of the total protein and most of the nucleic acid in the sample bound to the column. Most of the RegA protein was found in the flow-through fraction. The amount of nucleic acid in the extract was further reduced by precipitation of extract proteins with 50% (w/v) ammonium sulfate. The ammonium sulfate precipitate was collected by centrifugation (15,000 × *g* at 30 min) and resuspended in 50 ml of DEAE chromatography buffer and dialyzed against buffer containing 0.8 M ammonium sulfate. The dialysate was then chromatographed over a 1.5 × 25 cm phenyl-Sepharose (Pharmacia) column that had been equilibrated in the same buffer. The RegA protein eluted in the flow-through (0.8 M ammonium sulfate) fraction along with about 15% of the total extract protein that was applied to the column. The flow-through fraction was dialyzed against a phosphate-based buffer (20 mM potassium phosphate buffer at pH 7.0, 1 mM EDTA, 1 mM β-mercaptoethanol) prior to chromatography on a 1.5 × 20 cm Phospho-Ultrogel (LKB) column equilibrated in the same buffer. Elution from the Phospho-Ultrogel utilized 350 ml of a linear 0–0.5 M KCl gradient, with 4-ml fractions collected. The RegA protein eluted at 0.1–0.2 M KCl in a sharp peak containing very few other extract proteins (Figure 2). The fractions containing the protein were pooled and subsequently dialyzed and chromatographed on a 3-ml poly(U)-Sepharose (Pharmacia) column in 50 mM sodium phosphate buffer at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1 M NaCl. The RegA protein binds to poly(U) and is eluted at 0.5–1 M NaCl concentrations (MILLER *et al.* 1985). The poly(U) chromatography yielded homogeneously pure RegA protein (Figure 2), which was subsequently concentrated by dialysis, first against solid polyethylene glycol 6000 (Baker) to reduce the volume by 2–3-fold and then against storage buffer consisting of 50 mM Tris-acetate pH 7.5, 0.1 mM EDTA, 1 mM DTT, and 50% (v/v) glycerol. The final dialysis led to another 2-fold increase in protein concentration. Typical yields were 10–15 mg RegA protein at about 5% recovery from 50 g cells. Most of the losses occurred prior to the phenyl-Sepharose chromatography and were due to aggregation with lipidous material and other extract proteins.

The protein was stored in aliquots at −20° and −80°. Phosphate was avoided in storage buffers because it interfered with *in vitro* transcription and translation assays (our unpublished observations), and all buffers used after the Phospho-Ultrogel chromatography were treated with 0.1% diethylpyrocarbonate (DEPC) in order to minimize RNase contamination of RegA protein stocks. The treatment consisted of autoclaving the DEPC-containing buffers and water (for dilutions) at 121° for 20–30 min.

RESULTS AND DISCUSSION

Autogenous repression of plasmid encoded wild-type T4 RegA protein: We previously demonstrated that pGW7 clones of the T4 $regA^+$ DNA fragment JM850 (Figure 1) encode a biologically active RegA protein that represses its own synthesis at the translational level (MILLER *et al.* 1987). In contrast, the JM1020 fragment (Figure 1), which carries a $regA^-$ mutation ($regA_{104}$) that abolishes *regA* activity, produces much larger amounts of its plasmid encoded protein due to translational derepression. Figure 3 shows the results of an experiment that demonstrated the autogenous repression of pGW7-encoded $regA^+$ (JM850, Figure 1) protein relative to $regA_{104}$ (JM1020, Figure 1) protein. In the pGW7 clones used for the experiment in Figure 3 (left panel), the translation initiation domain for *regA* was placed 600–800

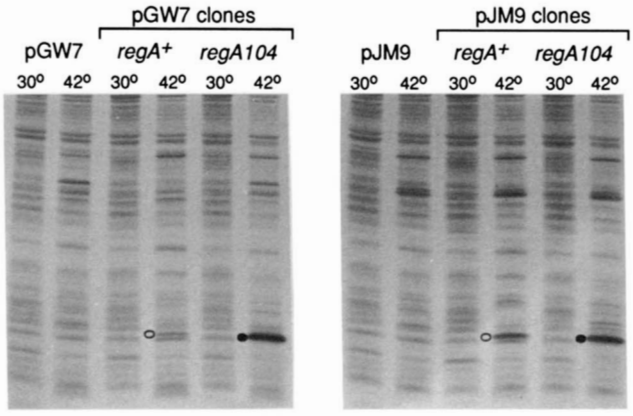

FIGURE 3.—Expression of cloned T4 *regA* genetic sequences under control of lambda P_L in pGW7 and pJM9. The autoradiograms show the different levels of T4 RegA protein that were obtained with heat-induced *E. coli* NapIV cultures that carried the pGW7 clones of JM850 (*regA*$^+$), and pJM1020 (*regA*$_{104}$) DNAs, and pJM9 clones of RW2405 (*regA*$^+$), and JM1020 (*regA*$_{104}$) DNAs. Note the difference in *regA*$^+$ protein production between JM850 (pGW7 clone) and RW2405 (pJM9 clone), which we attribute to the positioning of the *regA* translation initiation region in the genetic fusions. Plasmid-bearing cells were grown at 30° in M9-19 medium to a density of 3×10^8 and then were diluted 3-fold in media at 30° and 42°, respectively. The cultures were aerated for 30 min, 10 μCi [^{35}S]methionine/ml was added and aeration was continued for another 30 min. The labeled cultures were then chilled and the cells were harvested by centrifugation and extracted for analysis by SDS-gel electrophoresis and autoradiography.

nucleotides downstream of the termination codon for the nearest translated cistron, the plasmid-borne lambda N gene (Figure 1); (there are no other translated frames between N and *regA*). So, translation initiation at the *regA* cistrons of these clones was expected not to be coupled to the translation of the N cistron; however, the N protein is required to override the strong transcription terminator located near the junction of the lambda and T4 DNAs in the recombinant plasmids (t_L in Figure 1).

In a different type of construct we used the pJM9 vector (Figure 1) to fuse *regA*$^+$ DNA (RW2405, Figure 1) and *regA*$_{104}$ DNA (JM1020, Figure 1) to the reading frame of the N gene such that translation initiated at N could terminate within the cloned T4 sequence. Based on the nucleotide sequences of these genetic fusions, translation termination was expected to occur about 70 nucleotides upstream of the *regA* initiator AUG. Such constructs would be better poised for translation reinitiation at the *regA* ribosome binding site than in the pGW7 constructs. In Figure 3 it is shown that RegA protein production from the pJM9 clone of RW2405 DNA (Figure 3, right panel) was 5-fold higher than with the corresponding pGW7 clone of *regA*$^+$ DNA, JM850 (Figure 3, left panel). Since the level of RegA protein production from the RW2405-bearing pJM9 construct was lower than that from the constructs containing mutant (*regA*$_{104}$) JM1020 DNA (compare the left and right panels of Figure 3), it appears that only partial derepression of the *regA*$^+$ gene was achieved by these manipulations. In other work, we observed that fusing pJM9 to sites nearer to the *regA* AUG than with the pJM9 clone of RW2405 DNA resulted in decreased *regA* expression due to the loss of T4 sequences just downstream of the 5′-terminal end points of JM850 and RW2405 (about 80 bp upstream of the AUG) that enhance translation of the *regA* cistron (our unpublished data). In the RW2405 fusion with pJM9, translation termination occurs within this enhancing sequence.

RW2405 and JM850 constructs produce similar amounts of total *regA* RNA: Since pJM9 is a deleted derivative of pGW7 (MATERIALS AND METHODS), the two cloning vectors may differ in their P_L-controlled RNA synthesizing capacities and/or in stability of the RNA fusion products with the RW2405 and JM850 DNAs. We used an S1-mapping assay to compare the patterns and levels of *regA* RNA made by the two types of clones (RW2405 in pJM9 and JM850 in pGW7) in heat-induced *E. coli* NapIV cultures. Results are shown in Figure 4. In preliminary studies that utilized T4-infected cells and a variety of expressible *regA* clones, we localized a transcription termination signal just downstream of the *regA* cistron in T4 DNA. In the experiment for Figure 4, the pGW7 clone of JM850 DNA showed low levels of termination at this signal: compare the level of readthrough (RT) *vs.* terminated (T) RNA species in Figure 4. In contrast, the pJM9 clone of RW2405 DNA, which lacks the transcription antitermination activity of N protein, synthesized both terminated and readthrough species in significant amounts (Figure 4). However, the total amount of RNA that hybridized to the *regA*-specific probe used in the experiment was about the same for the two types of clones. So, the difference in RegA-protein-synthesizing activities between the RW2405 and JM850 constructs (Figure 3) does not appear to be due to a difference in the amounts of *regA* RNA available for translation.

RW2405 construct overproduces wild-type RegA protein: Due to the previously observed genetic instability of some cloned *regA*$^+$ sequences (ADARI and SPICER 1986; MILLER *et al.* 1987), it was necessary to consider the possibility that overproduction of RegA protein with the JM9 clone of RW2405 was due to point mutations in the *regA* gene of RW2405 that lower the affinity of the protein product to the autogenous target. Alternatively, a mutation could have arisen in the nucleic acid target (the translational operator site) for repression. Nucleotide sequence determinations on the entire 516 bp T4 DNA fragment of RW2405 (results not shown) revealed no differences between it and the known wild-type *regA*$^+$ sequence, which has been determined for JM850

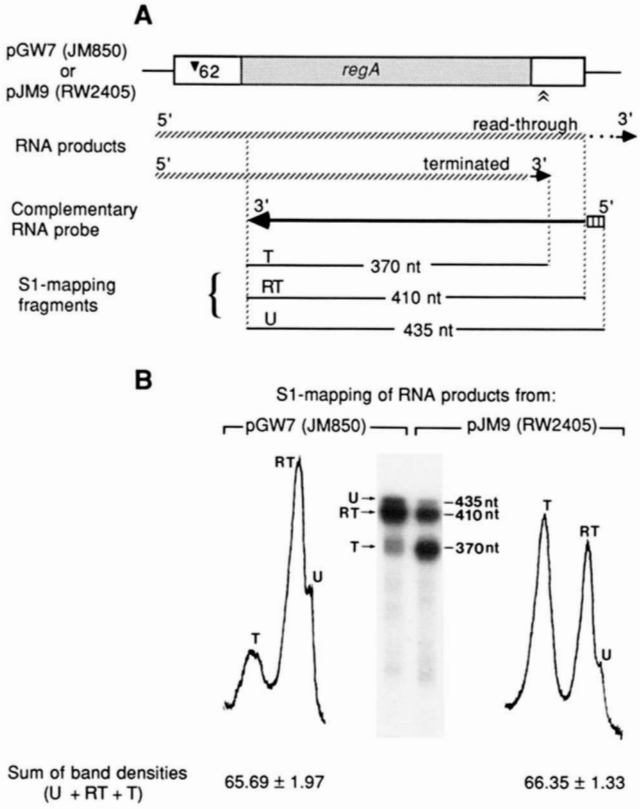

FIGURE 4.—Analysis of RNA from heat-induced pGW7 and pJM9 clones of the T4 regA$^+$ DNA fragments of JM850 [pGW7(JM850)] and RW2405 [pJM9(RW2405)]. The plasmids and T4 DNAs used are diagrammed in Figure 1. Plasmid-bearing *E. coli* NapIV cultures were grown and heat-induced as described for analysis of RegA protein synthesis in Figure 3 and the MATERIALS AND METHODS. In each case RNA was purified from about 10^{10} cells by using previously described methods (HSU *et al.* 1987). Subsequently, small samples of RNA were used in S1-mapping assays that utilized a ^{32}P-labeled riboprobe prepared from *in vitro* transcription by T7 RNA polymerase of a pGEM3 (Promega) clone of a T4 DNA fragment encompassing all the *regA* coding sequence minus the first 14 nucleotides and extending to the *Ava*I site downstream of *regA* (deletion 963 described by MILLER *et al.* 1987). Details of riboprobe preparation, annealings (RNA-RNA hybridizations), digestion with Nuclease S1, and analysis of S1-resistant products by electrophoresis and autoradiography using 6% acrylamide gels in Tris-borate-urea-based buffer are described elsewhere (ANDRAKE *et al.* 1988). The ≪ symbols designate the approximate locations of transcription termination sites on T4 DNA downstream of the *regA* gene (our unpublished data). The striped box at the 5′-side of the riboprobe refers to a 25-base sequence from the pGEM3 cloning vector. The designations used for the autoradiogram bands are T (terminated transcripts), RT (readthrough transcripts), and U (undigested annealing products). The presumed lengths (nt = nucleotides) of the separated RNAs are also shown. Densitometric scans of the autoradiogram bands were obtained on a Joyce-Loeble microdensitometer. Five scans per autoradiogram lane were used in the calculation of densities from the areas of band tracings.

(TROJANOWSKA *et al.* 1984) and for T4 DNA from other sources (ADARI *et al.* 1985); however, since small differences in sequence over long stretches are sometimes difficult to detect, we sought to utilize functional assays to confirm the wild-type character of the RegA protein encoded by the pJM9 clone of RW2405.

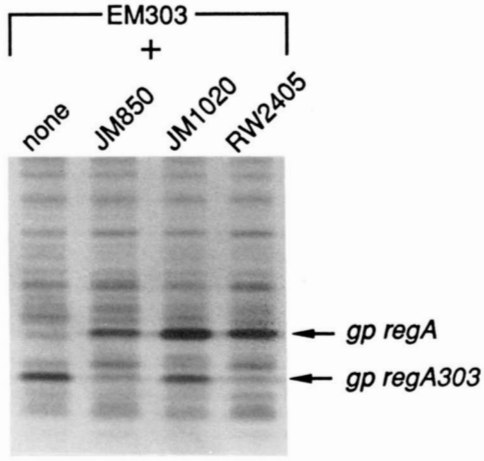

FIGURE 5.—Two-plasmid assays for the effects of RW2405-encoded RegA protein on the wild-type autogenous target. The controls JM850 and JM1020 are known to encode wild-type and mutant RegA proteins, respectively (MILLER *et al.* 1987). Plasmids carrying these inserts and RW2405 (all ampicillin-resistant) were each transformed into *E. coli* NapIV that was already carrying the tetracycline-resistant and compatible pACYC184 plasmid clone of EM303 (see Figure 1). Selection was done on LB agar containing ampicillin and tetracycline. The two-plasmid-containing cells were subsequently grown in M9-19 containing the two antibiotics and heat-induced and analyzed as described in Figure 3. Note that the JM1020-bearing recombinant plasmid (which encodes mutant $regA_{104}$ protein) fails to repress $regA_{303}$ protein synthesis, whereas the RW2405-bearing plasmid and the JM850-bearing regA$^+$ control both repress synthesis of the truncated mutant protein.

Figure 5 shows the results of two-plasmid complementation assays in which pGW7 or pJM9 clones of JM850, JM1020, and RW2405 were placed in NapIV hosts containing the pACYC184-derived clone of EM303, which encodes a truncated RegA protein ($regA_{303}$) that is easily distinguishable from wild-type-sized protein by SDS-gel electrophoresis. Expression of the two *regA* alleles in each combination of pairs could be brought about by a temperature shift (to 42°) of the two-plasmid-bearing cultures after growth at 30°. The results in Figure 5 show that $regA_{303}$ protein synthesis was inhibited in the presence of expressed clones carrying the JM850 and RW2405 DNAs, but not with the JM1020 ($regA_{104}$) control. So, protein encoded by the pJM9 clone of RW2405 is active against a wild-type *regA* autogenous target, that of EM303 DNA, *in vivo*.

In addition to two-plasmid assays, we transferred the putative regA$^+$ RW2405 fragment from the pJM9 clone to pGW7 and observed that the new construct was indistinguishable from the pGW7 clone of JM850 DNA in its induced levels of RegA protein synthesis (results not shown). That is, the RW2405 DNA harbors both a wild-type autogenous target and a wild-type structural gene.

Sensitivity of the autogenous *regA* target *in vitro*: RegA protein was purified from a heat-induced culture of the pJM9 clone of RW2405 (MATERIALS AND

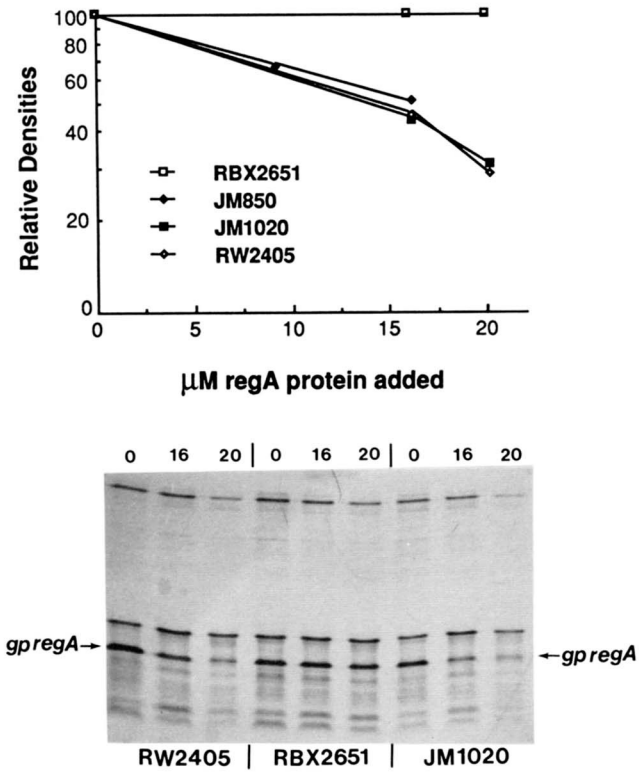

FIGURE 6.—*In vitro* repression of wild-type and mutant regA-autogenous targets by RegA protein purified from *E. coli* NapIV cells harboring a pJM9 clone of RW2405 DNA (see Figure 1) RW2405. The RegA protein was purified and used in *in vitro* transcription/translation assays as described in the MATERIALS AND METHODS. Note that added RegA protein inhibits synthesis of RegA protein from plasmid clones of JM850, JM1020, and RW2405 DNA but not from the clone of RBX2651 DNA. The autoradiogram does not include the results from the JM850 clone.

METHODS) and was used for *in vitro* assays that compared the autogenous-target sensitivities to repression in four constructs: JM850-bearing plasmid (reference normal target), JM1020-bearing plasmid (presumed normal target, but mutant structural gene), RBX2651-bearing plasmid (carries an altered putative target), and RW2405-bearing plasmid (suspected to have a normal target). The effects of added repressor on plasmid specified RegA protein synthesis in an *in vitro* transcription/translation coupled system are shown in Figure 6. We observed that the RegA protein purified from cultures harboring the RW2405-bearing pJM9 plasmid was able to inhibit *regA* gene expression equally from all three plasmid constructs bearing the putative normal targets, *i.e.*, 50% inhibition at about 12 μM protein (Figure 6). These levels of inhibition are similar to those that have been determined for RegA protein purified from T4-infected cells (WINTER *et al.* 1987). The experiment of Figure 6 also showed that *in vitro* expression of the *regA* gene in the RBX2651 clone was unaffected by the addition of purified RegA protein, thus confirming that RBX2651 lacks a target for autogenous repression. Other work (our unpublished data) localizes the autogenous *regA* target to nucleotide residues overlapping the initiator AUG and some nucleotides upstream to it.

In summary, the JM9 clone of RW2405 DNA exhibits a partial insensitivity to autogenous translational repression by the wild-type RegA protein that it encodes. This property may be related to the positioning of the repressor-binding site of *regA* mRNA relative to upstream translation. Other studies of *regA*-mediated translational repression *in vivo* (MILLER *et al.* 1987) and *in vitro* (WINTER *et al.* 1987) suggest that ribosomes and RegA protein compete for overlapping binding domains on repressible mRNAs and that RegA protein cannot dislodge ribosomes that are already bound to a potential target site for the translational repressor. In the pJM9 clone of RW2405 DNA, the placement of the *regA* translation initiation domain close to upstream translation may provide an advantage for ribosomes to reinitiate translation at the *regA* cistron and thus partially counteract RegA protein binding. We should point out, however, that long-range intramolecular interactions in the mRNA products of the genetic fusions described here cannot be ruled out as a cause for differential effects on translation initiation and repressibility of *regA* transcripts. The ability to derepress wild-type RegA protein synthesis circumvents difficulties involved in obtaining sufficient quantities of this protein for biochemical studies.

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