Translational Coupling to an Upstream Gene Promotes Folding of the Mycobacterial Plasmid pAL5000 Replication Protein RepB and Thereby Its Origin Binding Activity

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In the mycobacterial plasmid pAL5000 replication region, the replication genes *repA* and *repB* are organized in an operon. Earlier, a RepB-dependent origin binding activity was detected in *Escherichia coli* cells expressing the *repA-repB* operon. This activity was maximal when expression of the two genes was coupled (A. Basu, M. Chawla-Sarkar, S. Chakrabarti, and S. K. Das Gupta, J. Bacteriol. 184:2204–2214, 2002). In this study we have shown that translational coupling makes a significant difference in the structure and function of RepB. When *repB* expression was coupled to *repA*, the polypeptide folded into an active structure (referred to as RepB*), which possessed higher helical content than RepB expressed independently. RepB* could also be distinguished from the less active RepB on the basis of sensitivity to OmpT, an outer membrane protease of *E. coli*: RepB* was sensitive to the protease, whereas RepB was resistant. Similar conformational differences between RepB* and RepB could be observed when *repA* was replaced with an unrelated gene, *malE* (encoding maltose binding protein). These results show that translational coupling of *repB* to an upstream gene is necessary for better folding and origin binding activity. It is speculated that in coupled systems where translation machinery is passed on from the upstream to the downstream open reading frame, cotranslational folding of the polypeptide expressed from the downstream open reading frame is enhanced due to increased folding competence of translationally primed ribosomes.

The replication region of plasmid pAL5000, which is frequently used to construct mycobacterial vectors (5, 6, 9, 19), consists of a 1.8-kb fragment spanning two open reading frames (ORFs), encoding the replication proteins RepA and RepB (Fig. 1). This region also encompasses a 600-bp *cis*-acting element, which has been demonstrated to function as a replication origin and promoter element for the expression of the *repA* and *repB* genes (20, 21, 22). Several lines of evidence indicate that RepB is the key protein involved in the formation of an origin complex. In previous studies, it has been demonstrated that RepB by itself can bind to the origin (21). However, investigations undertaken in this laboratory indicate that RepB binds poorly to the origin unless there is coexpression of RepA (2).

The *repA* and *repB* genes are organized in an operon (17, 19). When the *repA-repB* operon is fused in frame to an inducible *E. coli* promoter, both *repA* and the downstream gene *repB* are expressed. In many ways, the *repA-repB* gene organization is reminiscent of operons in which the translation termination region of one gene overlaps the translation initiation signal of the next gene (7, 15). In such translationally coupled systems, expression of the downstream gene is facilitated by translation of the upstream gene. This may be due either to opening up of an occluded ribosome binding site (RBS) (13) or to efficient reinitiation by translating ribosomes (1). While working with the *repA-repB* operon, we have come across a phenomenon of translational coupling where it is observed that, apart from

promoting translation, coupled expression could facilitate the correct folding of the downstream gene product RepB. Since the exact role of RepA in the replication of pAL5000 is not known, this study unveils a novel mechanism by which expression of *repA* indirectly influences the activity of RepB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* XL1-Blue and *E. coli* BL21(DE3) were used in this study. *E. coli* BL21(DE3) carries the T7 RNA polymerase gene in the lambda DE3 prophage. *E. coli* XL1-Blue carries *ompT*⁺, whereas *E. coli* BL21 carries *ompT*.

The *ompT* gene encodes an outer membrane protease known to cleave certain expressed proteins following lysis by sonication (23). Both cells were grown in Luria broth (LB) with vigorous aeration at 37°C with the addition of kanamycin (25 µg/ml) or ampicillin (100 µg/ml) as appropriate (2).

Plasmid constructs. The various fusion constructs used in this study are summarized in Table 1. The construction of pAB1, pAB2, and pTAB2 has been described previously (2). Four new constructs, pAB3, pAB4, pTAB1, and pTAB1.1, were used in this study. Construct pAB3 was derived from pAB1 by the deletion of a 650-bp *EcoRI-EcoRI* (see Fig. 6) fragment containing the 5' half of *repA* and the vector RBS. In the resulting construct, *repB* can be expressed only from its own RBS.

Construct pAB4 was made by ligating a 600-bp PCR-amplified *repB* fragment including the coupling sequence (Fig. 1 and 6A) into the *EcoRI* and *HindIII* sites of vector pMalC2 (New England Biolabs) after necessary manipulations. The constructs pTAB1 and pTAB1.1 are pT7-7 (25)-based vectors derived from their pQE (Qiagen) counterparts, pAB1 and pAB1.1, respectively (2). The pT7-7-based RepA expression plasmid pTAB1 was constructed by cloning the 1.4-kb *XhoI-HindIII* fragment (containing the *repA-repB* region) from pMC2 into the *SalI* and *HindIII* sites of pT7-7. For constructing pTAB1.1, a 1.4-kb *BamHI-HindIII* fragment was excised from pAB1 and ligated into the *BamHI* and *HindIII* sites of pT7-7. As a result, RepA fusion is out of frame by 1 nucleotide in pTAB1.1 relative to pAB1. In pTAB1, pTAB1.1, and pTAB2 (Table 1), expression is T7 RNA polymerase dependent, and therefore it is obligatory to use *E. coli* BL21(DE3) for expression.

Preparation of cell extracts. For checking induction levels, lysis was done with sodium dodecyl sulfate (SDS) as mentioned previously (2). Cell extracts were

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FIG. 1. Map of pAL5000 origin of replication. The replication genes *repA* and *repB* are shown by light and dark shaded boxes, respectively. Arrows indicate the direction in which they are expressed. The RepB binding sites (21), L (low affinity) and H (high affinity), within the origin of replication (*ori*) are indicated. The DNA sequence at the junction of *repA* and *repB* (coupling sequence) is shown. Translation of *repA* stops at the stop codon TGA, and translation of *repB* starts from the overlapping ATG start codon. The RBS for *repB* is shown (sequence within a box).

prepared from *E. coli* XL1-Blue and BL21(DE3) cells transformed with the appropriate expression plasmids. Induction of expression was done by adding isopropyl- β -D-thiogalactopyranoside (IPTG) at a concentration of 1 mM (or as mentioned) at an A_{600} of 0.5 and incubated for 3 h at 37°C. Cells were harvested by centrifugation at $8,000 \times g$ for 10 min at 15°C, washed with 0.9% NaCl, and stored frozen at -80°C overnight. Frozen cells were thawed and lysed by sonication in buffer A which contained 20 mM phosphate buffer, pH 7, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. The resulting suspension was clarified by centrifugation at $12,000 \times g$ for 30 min at 4°C. This supernatant is referred to as the extract.

Purification of RepB. Recombinant RepB polypeptides were overproduced in *E. coli* BL21(DE3) cells. The cells were grown at 37°C in 500 ml of Luria broth containing the appropriate antibiotic to an A_{600} of 0.5. Expression was induced by adding 1 mM IPTG, and the cells were allowed to grow for 3 h. The RepB protein was purified by cation exchange chromatography. About 50 mg of pTAB2 extract was loaded onto a 3-ml carboxymethyl-Sepharose CL-6B column (Amersham Pharmacia Biotech) equilibrated with buffer A. After washing with 10 column volumes of buffer A, fractions (1 ml) were eluted with a 50-ml, 50 mM to 600 mM KCl gradient in buffer A at a flow rate of 1 ml/min. Eluates were checked by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining, and fractions containing about 99% RepB were dialyzed against buffer A.

Electrophoretic mobility shift assays and DNase I footprinting. Electrophoretic mobility shift assays were performed with an end-labeled 200-bp PCR-amplified origin fragment (nucleotides 4459 to 4663) as described earlier (2). DNase I footprinting (18) was done with the same probe. DNA-protein complexes were formed in a 200 μ l final volume, with 2 to 10 μ g of extract and 50,000

cpm of end-labeled origin probe in $1 \times$ binding buffer consisting of 10 mM Tris, pH 8.0, 60 mM NaCl, 3 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA, 1 mM CaCl₂ and 2% glycerol. DNase I (100 ng/ml) was added to the binding reaction and incubated at room temperature for 2 min. The reaction was stopped by adding 100 μ l of DNase I stop solution (50 mM Tris, pH 8, 2% SDS, 100 mM EDTA, 0.4 mg of proteinase K per ml) and incubated at 37°C for 30 min, followed by heat treatment at 90°C for 2 min. The resultant DNA was extracted with phenol-chloroform, precipitated with ethanol, and analyzed on a 6% DNA sequencing gel, followed by autoradiography (18).

OmpT sensitivity assay. To test sensitivity to OmpT protease, a Triton X-100 extract was made from late-log-phase *E. coli* XL1-Blue cells essentially as described earlier (24). Cells from 25 ml of culture were harvested, washed with 0.9% NaCl, and then sonicated in a buffer (1 ml) containing 50 mM Tris, pH 7.5, 5 mM EDTA, and 0.1% Triton X-100. After removing cell debris by centrifugation, the supernatant (referred to as Tx-XL1) was used for OmpT cleavage assays. In some experiments a similar extract (Tx-BL21) made from *E. coli* BL21(DE3) (*ompT*) was used as a negative control. Approximately 10 μ g of extract was mixed with 1 μ l of 40-fold-diluted Tx-XL1 (or Tx-BL21) and incubated at 37°C for 2 h. After digestion, SDS-PAGE sample buffer was added and boiled for 15 min. The treated samples were analyzed by SDS-15% PAGE. RepB and its cleavage products were monitored by Western blotting (8) with anti-RepB serum.

Circular dichroism spectroscopy. Circular dichroism studies were done with a Jasco-600 spectropolarimeter, with 1-mm path-length quartz cuvettes and 0.25 ml of RepB protein (at a concentration of 10 μ M). Proteins were studied in buffer A at room temperature between 250 and 200 nm (0.2 nm steps, 20 nm/min scan speed, and 2-s time constant). Four spectra were averaged for each sample, and the spectrum for the buffer was subtracted as a blank. The raw ellipticity data (in millidegrees) were transformed to mean molar ellipticity per residue (θ MR) in degrees per square centimeter per decimole. Circular dichroism spectra (range, 240 to 200 nm) were analyzed into their secondary structure components with the k2d algorithm (<http://www.embl-heidelberg.de/~andrade/k2d/>).

RESULTS

RepB expressed from the *repA-repB* operon is truncated in *E. coli* XL1-Blue but not in *E. coli* BL21 cells. Sonicated extracts from pAB1- and pAB2-transformed *E. coli* XL1-Blue cells were resolved on SDS-15% PAGE. Upon Western blotting with anti-RepB serum, it was observed that, when expressed as a part of the *repA-repB* operon (RepB from pAB1, Fig. 2A, lane 2), there was significant truncation, while for direct expression (RepB from pAB2, Fig. 2A, lane 4), little or no truncation was observed. Moreover, the truncation was

TABLE 1. Vectors used in this study

Vector	Description	Reference
pMC2	Contains the 2.56-kb <i>EcoRV-HpaI</i> (nt 3895–1257) fragment derived from the pAL5000 replication region	3
pQE30, pQE31	Expression vectors for <i>E. coli</i> ; polypeptides expressed from these vectors contain an affinity tag of six histidine residues fused to the N terminus	
pT7-7	T7 promoter-based expression vector, expressed proteins carry no tags	25
pAB1	<i>repA-repB</i> operon expressed from pQE31; the <i>repA</i> part is fused in frame with the 6X His tag provided in the vector	2
pAB2	RepB expressed from pQE30 with a 6X His tag	
pAB3	Derived from pAB1; unlike the other constructs of the pAB series, in this case the RBS of the pQE vector is deleted; expression can take place only from the RBS of <i>repB</i>	
pAB4	In this construct <i>malE</i> , which encodes maltose binding protein, replaces <i>repA</i> ; as in the case of <i>repA-repB</i> , in the <i>malE-repB</i> fusion, <i>repB</i> expression is coupled to <i>malE</i>	
pTAB1	<i>repA-repB</i> operon expressed from pT7-7	
pTAB2	RepB expressed from pT7-7 without any tag	
pTAB1.1	Similar to pTAB1, but <i>repA</i> fused out of frame with the translation initiation signal provided in pT7-7; RepA is not produced due to out-of-frame fusion; another protein of about 12 kDa is expressed in the +1 reading frame which terminates at a fortuitous stop codon located 200 bp upstream of the RBS of RepB (Fig. 4B)

FIG. 2. Cleavage of RepB expressed in the XL1-Blue and BL21 strains of *E. coli*. (A) SDS lysates (lanes 1 and 3) and sonicated extracts (lanes 2 and 4) of *E. coli* XL1-Blue cells expressing pQE-derived vectors pAB1 and pAB2 were analyzed on SDS–15% PAGE and Western blotted with anti-RepB serum. The bands corresponding to the intact and truncated forms of RepB are indicated. The size of RepB from pAB2 is about 0.6 kDa larger due to the presence of a six-His tag. (B) SDS lysates (lanes 1, 3, and 5) and sonicated extracts (lanes 2, 4, and 6) of pAB1 expressed in *E. coli* XL1-Blue and pTAB1 and pTAB2 expressed in *E. coli* BL21(DE3) were analyzed as in A. The constructs used are shown schematically below. The inducible promoters of the pQE and pT7-7 vectors are shown as QE and T7-7, respectively. A nonspecific band (NS) is indicated by a dot.

observed when sonicated extracts were used, but not if the lysis was done with SDS (Fig. 2A, lanes 1 and 3).

It is known that OmpT, the *E. coli* outer membrane-associated protease, is released upon sonication (23, 24). Thus, it appeared that RepB expressed in *E. coli* XL1-Blue was being cleaved by OmpT. It was further confirmed that the protease was sensitive to Cu^{2+} and Zn^{2+} , a characteristic of OmpT (24). Since *E. coli* BL21 lacks OmpT, it follows that if this strain is used to express RepB, only intact products would be obtained.

To test this, expression of pAB1 and pAB2, which are pQE-derived constructs (Table 1), was attempted in *E. coli* BL21, but this failed, as no transformants could be obtained. The inability to transform could be due to higher basal expression and toxicity of the expressed products. Expression was therefore attempted with the T7 RNA polymerase-dependent vector pT7-7 (Table 1), which is widely used for inducible expression in BL21-derived strains (25). Accordingly, two constructs, pTAB1 (pT7-7 equivalent of pAB1) and pTAB2 (pT7-7 equivalent of pAB2), were made. These were transformed successfully into *E. coli* BL21(DE3). Expression after IPTG induction was monitored by lysis with SDS or by sonication followed by SDS-PAGE and Western blot analysis with anti-RepB serum. For comparison, pAB1 expression was done in *E. coli* XL1-Blue and analyzed in the same gel (Fig. 2B, lanes 1 and 2). The results (Fig. 2B, lanes 3 to 6) show that there was no truncation of RepB when the expression was done in *E. coli* BL21(DE3) with the pT7-7-based constructs.

Differential susceptibility of RepB polypeptides to OmpT.

The results presented in the previous section indicate that RepB expressed from pAB1 in *E. coli* XL1-Blue is proteolytically cleaved in sonicated extracts. However, when expressed from pTAB1 in *E. coli* BL21(DE3), no such cleavage was observed. This could be a consequence of changing the expression vector or due to the shift to an *ompT* host. To test these possibilities, sonicated extracts of pTAB1- and pTAB2-expressing *E. coli* BL21(DE3) cells were treated with Triton X-100 extracts made from either *E. coli* XL1-Blue (*ompT*⁺) cells (Tx-XL1) or *E. coli* BL21(DE3) (*ompT*⁻) cells (Tx-BL21). The results show that RepB expressed as an untruncated polypeptide from pTAB1 (Fig. 3A, lane 1) was cleaved by Tx-XL1 (Fig. 3A, lanes 2 and 3) but not Tx-BL21 (lanes 4 and 5). RepB expressed from pTAB2, however, remained unaffected (Fig. 3A, lanes 6 to 10). The results indicate that irrespective of the expression system, RepB expressed from the operon construct pAB1 or pTAB1 is intrinsically susceptible to the OmpT protease present in *E. coli* XL1-Blue, whereas, when expressed independently, it is resistant.

The possibility that the intrinsic protease resistance of RepB expressed from pTAB2 was due to rapid synthesis resulting in inefficient folding and hence aggregation was considered, and expression was done with reduced concentrations of IPTG to allow a slower rate of expression. The results (Fig. 3B, lanes 3 to 8) show that reduction of the IPTG concentration had no effect on protease sensitivity. However, induction at 20°C in-

FIG. 3. Protease susceptibilities of RepB expressed under different conditions. (A) Sonicated extracts of *E. coli* BL21(DE3) cells expressing RepB from pTAB1 (lanes 1 to 5) and pTAB2 (lanes 6 to 10) were treated with Triton X-100 extract Tx-XL1 or Tx-BL21 (as indicated) prepared from *E. coli* XL1-Blue (*ompT*⁺) and *E. coli* BL21(DE3) (*ompT*⁻), respectively, and subjected to Western blotting with anti-RepB serum. Twofold higher amounts of the indicated extract were used in lanes 3, 5, 8, and 10 than in lanes 2, 4, 7, and 9. (B) Relative OmpT susceptibility of RepB expressed from pTAB2, either uninduced or induced at low (0.1 mM) or normal (1 mM) IPTG concentrations. Tx-XL1 untreated (–) and treated (+) lanes alternate. Lanes 1 and 2, control cleavage pattern of RepB expressed from pTAB1. (C) OmpT susceptibility of RepB expressed from pTAB2 at 20°C (lanes 3 and 4) and 37°C (lanes 5 and 6). The control expression and OmpT cleavage of RepB in the pTAB1 extract is shown in lanes 1 and 2. Untruncated and truncated forms of RepB are indicated, and the nonspecific (NS) band is marked by a dot.

creased the protease sensitivity of RepB expressed from pTAB2 (Fig. 3C, lanes 3 and 4).

Synthesis of RepB is coupled to that of RepA. The *repA* and *repB* genes constitute an operon. In order to test the possibility of translational coupling, the expression of the two polypeptides from pTAB1 was monitored over a period of time after adding IPTG. The level of RepA was measured directly from the Coomassie blue-stained gel, while that of RepB was monitored by Western blot. It was observed that there was a lag period, and half-maximal levels were reached in the case of RepB after a delay of about 15 min compared to RepA (Fig. 4A). This shows that expression of *repB* follows that of *repA*. Yet another evidence for coupling was obtained from the expression of *repB* under conditions in which *repA* was not trans-

lated. In this construct (pTAB1.1), *repA* was fused out of frame (Fig. 4B). RepA was not expressed from this construct, but a smaller unrelated polypeptide of about 10 kDa was expressed in the +1 frame relative to RepA which terminated at a fortuitous stop codon. Ribosomes therefore begin translation from the RBS provided in pT7-7, but their progress is halted at a point far upstream of the RBS of *repB*. In this construct, RepB was produced at a much lower level relative to pTAB1 (Fig. 4B). This confirms that in the absence of *repA*, synthesis of RepB is adversely affected.

Synthesis of RepA modulates origin binding activity of RepB. In the previous study (2), it was found, by expression in *E. coli* XL1-Blue, that *repA-repB* extracts were more effective in origin binding than *repB* alone. Hence, it became necessary to test whether the same conclusion held in the case of expression from pT7-7 in the BL21 strain. An electrophoretic mobility shift assay revealed that given comparable levels of expression (Fig. 5A), extracts from *repA-repB*-expressing cells exhibited 10 times more activity than *repB* expressed without *repA* coupled expression (Fig. 5B). DNase I footprinting supported the results. The footprint obtained with pTAB1 extract was strong and was focused (Fig. 5C) on the high-affinity RepB binding site (H site) reported earlier (21), whereas pTAB2 extract showed a weak footprint in the same region (Fig. 5D). All these results taken together indicate that when coupled expression takes place, the origin binding activity of RepB becomes more efficient.

Translational coupling is necessary for OmpT sensitivity of RepB. To investigate this, a *repB*-only construct (pAB3) was first made in which an *EcoRI* fragment spanning the RBS of the pQE vector and a major part of *repA* was deleted, leaving behind the coupling sequence (Fig. 6A). In another construct, a *malE-repB* coupled expression system (pAB4) was made based on pMalc2, in which *malE* replaces *repA* as the upstream gene allowing *malE-repB* coupling through the *repA-repB* coupling sequence (Fig. 6A, inset). The constructs were transformed into *E. coli* BL21(DE3). After induction, sonicated extracts were prepared and treated with Tx-XL1 extract to test for OmpT sensitivity. It was observed that, as in the case of pTAB1 (Fig. 6B, lanes 1 and 2), RepB expressed from pAB4 is OmpT sensitive (lanes 5 and 6). In contrast, pAB3, in which no ORF is coupled, the product is OmpT resistant. This result shows that expression of *repA* per se is not important but that coupled expression is the deciding factor in the proper folding of RepB.

Structural features of RepB. The circular dichroism spectra of RepB purified from pTAB1 and pTAB2 were examined (Fig. 7). The results indicate significant differences in secondary structure. RepB derived from pTAB1 (RepB*) had a higher alpha helical content (31%) than RepB from pTAB2 (10%) (Table 2).

DISCUSSION

The present investigation was initiated following the observation that RepB, the pAL5000 replication protein, was proteolytically cleaved following cell lysis by sonication when expressed in *E. coli* XL1-Blue and the extent of susceptibility was greater when there was coupled expression of *repA*. In *E. coli*, expressed polypeptides are subjected to proteolytic cleavage

FIG. 4. (A) Kinetics of RepA and RepB accumulation. *E. coli* BL21(DE3) cells expressing pTAB1 were harvested at the indicated time points, lysed by SDS, and analyzed on duplicate gels. RepA expression was monitored directly by Coomassie blue staining whereas that of RepB was monitored by Western blot with anti-RepB serum. The values are expressed as percent of maximal expression. (B) Effect of out-of-frame fusion of *repA* (pTAB1.1) on the expression level of RepB. The levels of RepB expressed from the indicated constructs were observed by Western blotting with anti-RepB serum. The RepB-specific band is indicated by a dot. (B, lower panel) Schematic representation of *repA* fusion constructs (in frame in pTAB1 and out of frame in pTAB1.1). In pTAB1.1, any translation initiated from the vector sequences terminates at the stop codon TAA, as shown.

mainly by Lon and OmpT proteases (18). Neither of these proteases exists in *E. coli* BL21. Since the protease action occurred after sonication, it became apparent that it must have been caused by the outer membrane protease OmpT. Subsequently it was observed that the protease activity was sensitive to Cu^{2+} and Zn^{2+} but not to phenylmethylsulfonyl fluoride

(data not shown). These are characteristics of the OmpT protease (24).

The identity of the protease by itself is not a critical element in this study. Rather, the sensitivity to the protease, which could be correlated to higher activity, is the feature to be noted. The differential cleavage pattern gives clear indication

FIG. 5. Binding activities in extracts of RepB-expressing cells. (A) Western blot with anti-RepB serum of soluble extracts used for the electrophoretic mobility shift assay and DNase I footprinting experiments. The RepB-specific band is indicated. NS, nonspecific band. (B) Comparative electrophoretic mobility shift assay with extracts of pTAB1- and pTAB2-expressing *E. coli* BL21(DE3) cells. Lane F corresponds to labeled DNA without protein extract. (C and D) DNase I footprinting with pTAB1- and pTAB2-expressing *E. coli* BL21(DE3) cell extracts. The low (L)- and high (H)-affinity binding sites (21) for RepB are indicated.

FIG. 6. Coupled expression and OmpT sensitivity. (A) Construction of pAB3 and pAB4. The construct pAB3 was derived from pAB1 by deleting an internal *Eco*RI fragment spanning the vector RBS and a major part of *repA* but retaining the coupling sequence (boxed region; details given in inset). To construct pAB4, a PCR copy of *repB* and the flanking coupling sequence was inserted at the *Eco*RI and *Hind*III (E and H, respectively) sites of pMALc2. In this construct, the entire *repA* coding sequence except for the last seven codons, which constitute the coupling sequence, was replaced by *malE*. The promoter elements are indicated as P. (B) The effect of coupled (pTAB1 and pAB4) and uncoupled (pTAB2 and pAB3) synthesis on the OmpT sensitivity of RepB. The constructs were transformed into *E. coli* BL21(DE3). The sonicated extracts with (+) or without (–) Tx-XL1 treatment were resolved on SDS–15% PAGE, followed by Coomassie blue staining (top) or Western blotting (below) with anti-RepB serum. The nonspecific band (NS) and truncated RepB-specific bands are indicated.

FIG. 7. Circular dichroism spectra of purified RepB* isolated from pTAB1 (open triangles) and RepB from pTAB2 (black circles). The k2d analysis results are shown in Table 2.

of structural differences between the two forms of RepB, expressed from either pTAB1 (RepB*) or pTAB2 (RepB). That indeed there are structural differences is also indicated by the circular dichroism spectra of the purified polypeptides. RepB* has more helical structure than RepB. The percent helicity as obtained experimentally is about 31%, whereas the overall predicted value is about 60%, as calculated with PSIPRED software (16). However, if the maximum confidence values are taken into consideration, the helicity would be about 35 to 40%, which is reasonably close to the observed helicity of RepB*. In contrast, RepB has a helical content of only about 9%. These results show that RepB can potentially exist in two alternative structures, as a properly folded polypeptide (RepB*) with higher helical content and origin binding activity and as a misfolded polypeptide with higher β -sheet structure and lower activity. The protease resistance of RepB appears to be caused by the increased β -sheet content (about 35% for RepB versus 10% for RepB*), which leads to aggregation (12).

In a coupled system, the facilitated translation of a downstream gene may be due to opening up of an occluded ribosome binding site which is inherently strong but cannot function properly due to impeding secondary structures (13), or efficient reinitiation by translating ribosomes can increase the activity of a weak downstream RBS (1). It has been found (data not shown) that when upstream sequences are removed and RepB expression is done with the minimal translation initiation signal of RepB, the expression level goes down about sixfold compared to when the same signal is part of a coupled system. Hence, the RepB translational initiation signal is inherently weak and requires translational coupling to increase its activity. This appears to be consistent with the second model involving reinitiation. However, the possibility that RNA secondary structures contribute to the translation of RepB cannot be ruled out altogether at this stage.

Translational coupling therefore provides two advantages

TABLE 2. Secondary-structure analysis^a of RepB* and RepB

Polypeptide	α -Helix	β -Sheet (%)	Random coil (%)
RepB*	31	10	59
RepB	9	35	55

^a The values were obtained from an analysis of the circular dichroism spectra (Fig. 7) with k2d software as mentioned in Materials and Methods.

for RepB synthesis. First, it increases the efficiency of translation of RepB, and second, it promotes folding. For this phenomenon to occur, it is not essential that the proximal gene be *repA*; the same effect was achieved by incorporating *malE*, which encodes an unrelated polypeptide. In other words, *repA* per se is not important, but its translation is the key factor. A possible mechanism could be that ribosomes initiating at the upstream RBS are more likely to be competent in cotranslational folding of the downstream gene product than ribosomes translating the downstream gene independently. This difference may involve the trigger factor, which is known to associate tightly with translating ribosomes (14).

It has been suggested that ribosome-tethered chaperones such as trigger factor in *E. coli* and Ssb in *Saccharomyces cerevisiae* constitute a first line of protection against misfolding (4). Also, it has been found that both association and dissociation of trigger factor from ribosomes are slow processes (14). Hence we propose that since the dissociation of trigger factor is a slow process, as the ribosomes are transferred from the upstream to the downstream gene they retain their association with the trigger factor. As a result the ribosomes remain primed to initiate folding. Hence folding of the downstream gene product takes place more efficiently in a translationally coupled system. The involvement of the trigger factor has not been proved directly, but we found that lowering the temperature increases OmpT sensitivity, which indicates that at lower temperatures, folding of RepB is more efficient. The activity of trigger factor has been found to be enhanced at lower temperatures (10), and hence this indirect evidence supports the possible involvement of ribosome-bound trigger factor in the RepB folding process.

However, an alternative model may be suggested in which RepA acts as a *cis*-chaperone, as in the case of fusions to maltose binding protein (11). It is not known whether RepA has any chaperone activity or not. A large in-frame deletion removing the N-terminal half of RepA was made, and the effect of this deletion on protease sensitivity and activity of RepB was tested (data not shown). The truncation of RepA did not have any effect either on the protease sensitivity or on the activity of RepB compared with a similar construct expressing full-length RepA. Therefore, it does not appear that RepA has any direct influence on the folding of RepB. Besides, *cis*-chaperone activity as reported in the case of maltose binding protein is applicable to fusion proteins (11). In this case, RepA and RepB are translated from independent open reading frames, and thus fusion proteins are not formed. Hence, the possibility of *cis*-chaperone activity seems remote.

Nevertheless one can imagine that the upstream and downstream gene products associate transiently during coupled translation, leading to better folding of either or both products, but this would again depend on coupling. The present study therefore unveils a novel phenomenon in which it is found that in translationally coupled systems, not only the expression level but also its biological activity of the downstream gene is regulated by the translation of the upstream gene.

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