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NOTES

Multiple Transcribed Elements Control Expression of the *Escherichia coli btuB* Gene

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Repression by vitamin B_{12} of the cobalamin transport protein BtuB in the outer membrane of *Escherichia coli* operates at both the transcriptional and translational levels and is controlled by transcribed sequences within the leader and proximal portion of the *btuB* coding sequence. The effects of deletions from either end of this region on repression and expression were determined with *lac* fusions. An element at the 5′ end of the transcript and the putative attenuator within the coding sequence were required for transcriptional repression. The presence of either element caused a marked reduction in *btuB-lacZ* expression which was reversed by the presence of a conserved sequence element in the leader, suggesting the importance of long-range interactions in the *btuB* leader for expression and regulation.

Most genes for biosynthetic and catabolic activities and for nutrient transport systems are regulated in response to the availability of specific substrates or products. Vitamin B_{12} (cyanocobalamin [CN-Cbl]) is known to repress expression of the *btuB* genes of *Escherichia coli* and *Salmonella typhimurium* (*Salmonella enterica* serovar Typhimurium) for the outer membrane Cbl transport protein BtuB (6, 16) and the *cob* operon for Cbl biosynthesis in *S. typhimurium* (2). (For a recent review of Cbl biosynthesis, transport, and regulation, see reference 14). Regulation of these genes exhibits several unusual features. Their expression occurs in response to adenosyl-Cbl (Ado-Cbl), not CN-Cbl (7, 14), and appears to operate after the stage of transcription initiation. Evidence has been presented for both attenuation control of transcript elongation and control of translation initiation by sequestration of the ribosome-binding site (8, 12, 13). The *btuB* and *cbiA* (the first gene of the *cob* operon) genes are transcribed with long leader segments (241 and 468 nucleotides [nt], respectively) which are the sites of numerous mutations that decrease repression (8, 12, 13). These leader segments have few regions of sequence similarity, other than a conserved 25-nt sequence called the B12 box (12). However, extensive portions of these leader sequences might form alternative secondary structures. An RNA secondary structure that can sequester the Shine-Dalgarno sequence is important for regulation, as shown by analysis of compensatory mutations that maintain this structure but not its sequence (11). A potential Rho-independent terminator structure that might serve as a transcriptional attenuator lies in the proximal portion of the coding sequence. Figure 1 shows a schematic representation of the locations of these possible regulatory elements.

Transcriptional and translational fusions to *btuB* or to *cbiA* show different levels of regulation depending on the position of the fusion junction. Fusions well within the coding regions show control of both types of reporters. Fusions early in the coding region show repression of translational but not of transcriptional fusions, and fusions in the leader region show no regulation (8, 11–13). Despite the complexity of the regulatory process, no *trans*-acting regulatory factors have been identified in numerous genetic screens (14). Of the many unlinked mutations selected in our laboratory for increased *btuB* expression in the presence of CN-Cbl, all were mapped either to genes involved in Cbl uptake (*btuB*, *tonB*, and *btuCD*) or to the *btuR*/*cobA* locus, which is involved in conversion of CN-Cbl to Ado-Cbl (2a).

We showed previously that the promoter region and first 350 nt of the transcribed portion of *btuB* contain the sequences needed for proper regulation (8). We describe here the effect of an extensive series of deletions in this region on Ado-Cbl-dependent repression and on *btuB-lacZ* expression. The results confirm and extend previous conclusions about the locations of the regions required for transcriptional and translational regulation. More importantly, the importance of the B12 box for gene expression is demonstrated.

A series of deletions entering the transcribed *btuB* regulatory region from the 5′ or 3′ end was fused to *lacZ* to form transcriptional and translational fusions. To facilitate this analysis, we used oligonucleotide-directed mutagenesis to introduce *Bam*HI recognition sequences at 15 positions from residues +1 to +450, all coordinates relative to the transcription start site. The choice of these positions was based on their locations relative to particular sequence elements in the leader, the ability to place each *Bam*HI sequence in the same reading frame within the *btuB* coding sequence, and the need for a minimal number of base changes. The *Bam*HI substitutions did not change the length of the leader. Mutations were constructed in the plasmid pALTER carrying the *Eco*RI fragment extending from −60 to +1435 in the *btuB* sequence (4). Following confirmation of the presence of the desired sequence changes, the series of 3′ deletions was made by ligating the appropriate *Eco*RI-*Bam*HI fragment to the corresponding fragments of the transcriptional *lac* fusion vector pRS415 or the translational *lac* fusion vector pRS414 (15). The series of 5′ truncations of the leader transcript left the *btuB* promoter intact. The promoter and transcribed sequence to the *Bam*HI

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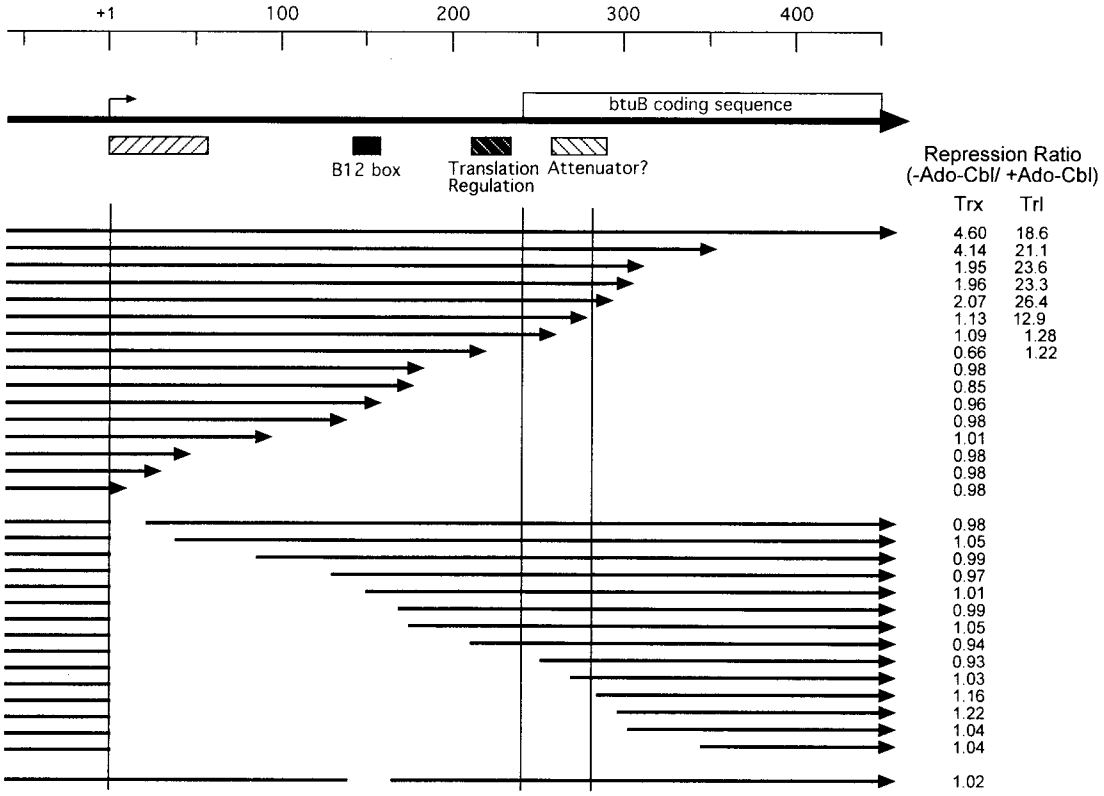

FIG. 1. Regulation of *btuB-lacZ* fusions in deletions of the *btuB* regulatory region. The upper portion is a schematic representation of the *btuB* regulatory region, showing the locations of the transcription start site, designated +1, and the *btuB* coding sequence. Nucleotide coordinates are relative to the transcription start site. The three hatched boxes indicate the positions of regions of hyphenated dyad symmetry, including one region that contains the translation initiation sequence and another region that forms the putative Rho-independent attenuator. The solid rectangle indicates the position of the B12 box conserved in Cbl-regulated transcripts. Below the map are represented the sequences present in the various deletion derivatives used in this study, with *lacZ* fused at the *Bam*HI site indicated by the arrowhead. To the right of each variant is tabulated the repression ratio for each deletion, i.e., the ratio of β-galactosidase activity in the absence and presence of Ado-Cbl. These values are given for transcriptional fusions in the column marked "Trx" and for translational fusions in the column marked "Trl." The three vertical guidelines indicate, from left to right, the start of transcription, the start of translation, and the region in which 3′ deletions resulted in a loss of transcriptional repression.

substitution at +2 was ligated to the *Bam*HI sites in distal portions of the leader region fused to *lacZ* at +450 in the transcriptional or translational fusion vectors. The expression of β-galactosidase (5) was determined with plasmid-borne copies of the *lac* fusion constructs in the *E. coli* strain RK5046 [*araD139* Δ(*argF-lac*)*U169 rpsL50 relA1 deoC1 flb5301 gyrA219 non metE70 recA56*] (9). The regulation of *btuB* expression is not affected by its gene copy number (1, 8). Cells were grown in Luria-Bertani medium (10) containing ampicillin (100 μg/ml) to maintain plasmid carriage and supplemented when indicated with 5 μM Ado-Cbl. All assays were performed at least three times, and the standard deviations of enzyme activities were <10% of the means.

Effects of deletions of the *btuB* regulatory region on regulation. The repression by Ado-Cbl of β-galactosidase expression from the constructs containing transcriptional *lac* fusions to the 5′- and 3′-deletion derivatives demonstrated the importance of sequences at both ends of this region for regulation (Fig. 1). The 3′-deletion derivatives with fusion junctions at +450 or +345 exhibited a roughly fourfold repression by Ado-Cbl. The 3′-deletion derivatives with endpoints at +303, +297, and +285 displayed a reduced degree of repression (repression ratio of around 2), and further 3′ deletions to +270 or beyond resulted in a complete loss of transcriptional regulation. The latter truncations extended into the proposed Rho-independent transcription attenuator in the coding sequence for the BtuB signal sequence.

None of the deletions that removed sequences from the 5′ end of the transcript resulted in any repression by Ado-Cbl, even though the promoter was intact (Fig. 1). Thus, proper transcriptional regulation requires the integrity of both the 5′ end of the transcript and the putative attenuator at residues +258 to +288, although full transcriptional repression requires sequences extending past +303.

Seven of the 3′-deletion derivatives, along with one deletion that ends before the start of the coding sequence, were used to construct translational fusions to *lacZ*, making use of the fact that the *Bam*HI sites lay in the same reading frame. The levels of β-galactosidase activity expressed from the translational fusions were only 2.2 to 8.7% (mean ± standard deviation, 5.9% ± 2.2%) of those expressed from the transcriptional fusions with the same endpoints (Fig. 2A). This difference could indicate that translation initiation is a regulated or inefficient process, but this conclusion does not account for possible differences in the stability of the BtuB-LacZ hybrid proteins or the effect of varying portions of the BtuB signal sequence on protein localization and activity. The repression ratios for the translational fusions at +285 or further downstream ranged from 19 to 26 (Fig. 1), showing again that translational fusions exhibited a greater degree of regulation than did transcrip-

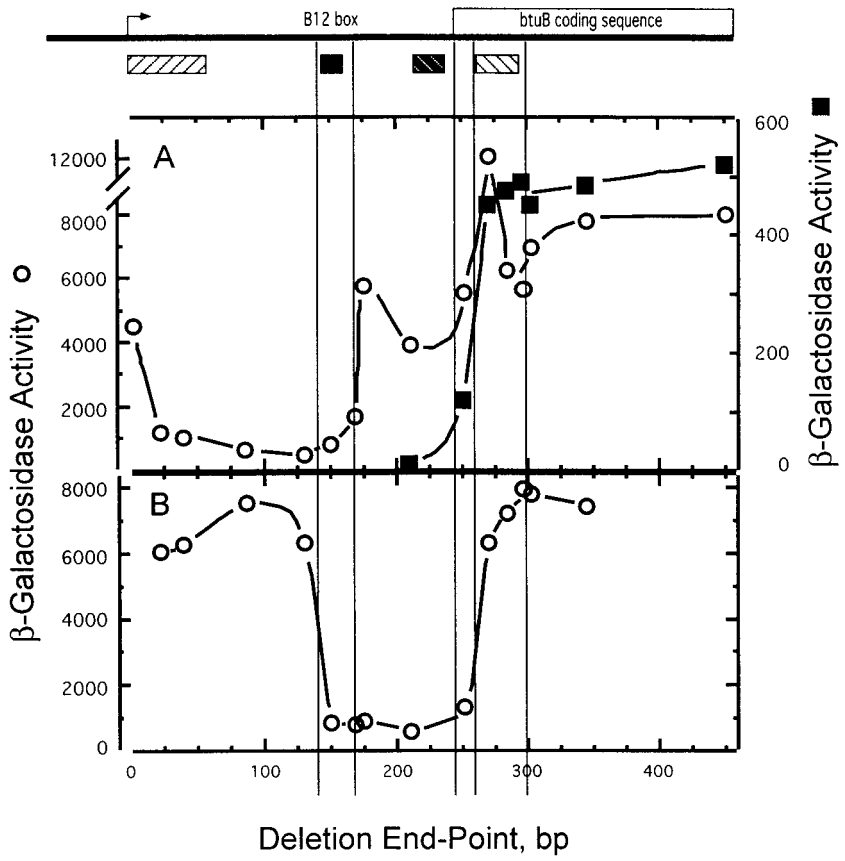

FIG. 2. Level of β-galactosidase expressed in derivatives with deletions of the *btuB* regulatory region. At the top is the map of the *btuB* regulatory region aligned with the level of β-galactosidase activity expressed in the absence of Cbl by the 3′-deletion derivatives (A) and the 5′-deletion derivatives (B). Values are for transcriptional fusions (open circles, left vertical axis) and for translational fusions (filled squares, right vertical axis). The five vertical guidelines indicate, from left to right, the region where 5′ deletions cause reduced expression, the region where 3′ deletions cause reduced expression, the start of translation, the region in which 5′ deletions recover high expression and where translational fusions lose activity, and the region where 3′ deletions lose full transcriptional regulation.

tional fusions. Deletion past residue +285 to +270 resulted in reduced repression (13-fold), and deletion to +252 in codon 5 resulted in an almost complete loss of repression (1.3-fold) and a 4-fold decrease in the level of expression. Thus, the minimal sequence needed for full repression of translational fusions extends to +285.

Effects of deletions of the regulatory region on *btuB* expression. In addition to their effect on regulation, the 5′ and 3′ deletions had a dramatic effect on the basal levels of *btuB-lacZ* expression (Fig. 2). For the series of 3′ deletions, transcriptional expression (Fig. 2A) showed a moderate and gradual decrease as the fusion junction was moved from +450 to +175, with the exception of the elevated activity for the fusion at +270. There was a sharp decrease in β-galactosidase levels when the deletion junction moved past +175 to +169 or beyond. Expression remained low upon further truncations to +22, ranging from 6% of the activity of the full-length fusion at +130 to 21% at +169. Expression returned to its high but unregulated level in the construct with the truncation to +2. Thus, removal of sequences near the B12 box, located between residues +140 and +160, resulted in a substantial reduction in *btuB-lac* expression. Expression of the *btuB* promoter in the absence of any transcribed sequence is not regulated by AdoCbl, and thus, the presence of transcribed sequences near the 5′ end results in reduced expression when the B12 box is missing.

The expression levels in the 5′-deletion derivatives showed similar behavior (Fig. 2B). All of the 5′ deletions that removed *btuB* sequences between +22 and +130 conferred constitutive, high-level expression. Further deletion of sequences from +150 to +252 resulted in marked reduction in β-galactosidase expression, down to 7 to 16% of that of the full-length fusion. Strikingly, further deletion past +252 to +270 and beyond resulted in restoration of high-level constitutive expression. These results show that the B12 box is necessary for high-level expression as long as the attenuator sequence is present.

Deletion of the B12 box. The effect of deletion of the B12 box on expression and regulation was examined in transcriptional and translational fusions. Oligonucleotide-directed mutagenesis was used to remove *btuB* sequences between +140 and +163. In both types of fusions, removal of the B12 box resulted in a complete loss of regulation and very low levels of β-galactosidase expression, down to 165 U (2.4% of the full-length fusion) for the transcriptional fusion and 2 U (0.7% of the full-length fusion) for the translational fusion.

Discussion. The results presented here demonstrate the importance for both the expression and the Cbl-dependent regulation of the *E. coli btuB* gene of three segments of the transcript, sequences at the 5′ end, the B12 box element conserved in Cbl-repressed genes, and the putative transcriptional attenuator at residues +258 to +288. Deletion of any one of

these elements eliminated transcriptional regulation and had marked effects on gene expression.

Although the *S. typhimurium btuB* gene is thought to be regulated solely at the translational level (11), the expression of *btuB* in *E. coli* is regulated at both the transcriptional and translational levels. The four- to fivefold repression of transcriptional *btuB-lacZ* fusions by Ado-Cbl was dependent on the presence of the putative attenuator element. Transcriptional regulation was lost when 3′ deletions removed this element. All 5′-deletion derivatives lacking the B12 box had very low levels of expression unless this attenuator element was also deleted. Both results are consistent with its proposed action as an attenuator. The relatively low degree of transcriptional repression (four- to fivefold) suggests that this terminator is at best 80% efficient, which is consistent with the relatively low stability predicted for its G+C-rich stem structure and from the short run of four uridine residues at its 3′ end.

Derivatives with translational fusions to *btuB* exhibit repression ratios in the range of 20 to 30 (8, 11). This degree of regulation could result from the independent combination of a fivefold repression of transcription and a comparable degree of control of translation initiation. However, derivatives with fusions at +270 in the middle of the attenuator lacked transcriptional regulation (repression ratio, 1.13) but retained a substantial degree of translational regulation (repression ratio, 12.9), suggesting that the major process subject to regulation is at initiation of translation, rather than mRNA synthesis. The somewhat longer fusions with deletions to +285, +297, and +303 displayed full translational repression but only partial transcriptional repression. Thus, the minimal sequence required for translational regulation is shorter than the minimal sequence required for transcriptional regulation; the latter includes the attenuator, whereas the former does not.

The key new finding of this work is that the level of β-galactosidase expression by derivatives with transcriptional fusions was strongly affected by the extent of *btuB* sequences present in the transcript. Both 5′- and 3′-deletion derivatives showed high β-galactosidase levels until the deletions neared the B12 box, between residues +130 and +150 on the 5′ side and between residues +169 and +175 on the 3′ side. These deletions resulted in an 8- to 10-fold reduction in β-galactosidase levels. Upon further deletion in both directions, the levels of β-galactosidase remained low until the deletion extended beyond +22 from the 3′ direction or beyond +252 from the 5′ direction. These results indicate that the presence of two regions of the transcript result in decreased *btuB* expression, namely, an element at or near the 5′ end of the transcript, and the putative attenuator at +258 to +288. The mechanism for the inhibitory effect of the attenuator element is likely to be the premature termination of transcription. The effect of the element at the 5′ end of the transcript is not obvious, and even its size is unknown. One possibility is that this element controls transcript stability, as seen with the 5′ termini of other transcripts (3). The low expression caused by the presence of these regions is counteracted by the presence of the B12 box. Deletion of the B12 box from a leader sequence that contains both regions resulted in even greater reduction of β-galactosidase levels than occurred with the deletions from either end, suggesting that the inhibitory elements act independently.

Experiments to determine whether these effects operate on the synthesis or stability of *btuB* RNA are in progress. However, these results strongly suggest that the B12 box interacts with the inhibitory elements at the 5′ end and the attenuator. This interaction could operate through formation of a long-range RNA secondary structure or through some indirect manner. It remains to be determined whether the B12 box is involved in Cbl regulation or mRNA level, since the repressed level of β-galactosidase (1,600 to 1,800 U) is much higher than that seen upon deletion of the B12 box (as low as 170 U). Since removal of the B12 box has such a strong effect on the transcription level, it is not possible to show whether it also affects translational expression. Future studies are directed toward investigation of the effect of Cbl on *btuB* mRNA levels and stability.

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