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Diphtheria toxin promoter function in *Corynebacterium diphtheriae* and *Escherichia coli*

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ABSTRACT

The expression of the diphtheria tox228 gene encoding the nontoxic, serologically related CRM228 mutant diphtheria toxin has been analyzed in Corynebacterium diphtheriae and Escherichia coli. The diphtheria toxin promoter has been used to direct the expression of β-galactosidase in E.coli, and the efficiency of promotion has been compared to that obtained with the lac promoter. Expression in C.diphtheriae is known to be dependent on the absence of iron, and we present for the first time direct evidence that this regulation occurs at the level of transcription. The 5' end of toxin mRNA maps at the same position in C.diphtheriae and E.coli, suggesting identical sequences to be recognized by C.diphtheriae and E.coli RNA polymerase. The diphtheria toxin promoter carries at position -34 a TTGATT sequence closely related to the E.coli -35 consensus sequence and in the -14 to -8 region a set of overlapping sequences with complete or partial homology to the E.coli -10 consensus sequence.

INTRODUCTION

Diphtheria toxin is produced by Corynebacterium diphtheriae lysogenic for a phage carrying the toxin gene. A non-toxic precursor is secreted which is subsequently cleaved to yield two fragments, the enzymatically active A fragment catalyzing an ADP ribosylation of elongation factor EF2, and the B fragment which binds to a receptor present in the membranes of almost all eukaryotic cells and plays a role for delivering the A fragment into the cytosol (1).

A number of mutant diphtheria toxins have previously been obtained by mutagenesis of bacteriophage β carrying the toxin gene (2). These encode immunologically cross-reactive diphtheria toxins (CRMs) which have no or reduced activity of fragment A, B or both.

The first diphtheria toxin gene to be cloned and sequenced was that of such a mutant, tox228 (3). Subsequently the nucleotide sequences of the wild type gene and of additional CRM encoding mutants became also known (4-6).

It has been known for many years that expression of diphtheria toxin is

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regulated by iron and is maximal only under conditions of iron starvation (7). Bacterial (8) and bacteriophage β (9, 10) mutants have been isolated for which toxin production is insensitive to iron. One of the mutations in bacteriophage β has recently been mapped upstream of the tox structural gene (11). Several models have been proposed to explain the regulation of diphtheria toxin synthesis by iron (12, 13) but direct biochemical confirmation for the actual mechanism of regulation is lacking.

Diphtheria toxin expression has been observed in an in-vitro system from E. coli programmed by bacteriophage β DNA (14). More recently, expression of the cloned tox228 gene has been obtained in E. coli (3). Fragment A has also been expressed in E. coli and secretion into the periplasm has been observed (15). In addition, a DNA fragment from upstream of the coding region of the toxin gene has been shown to promote expression of galactokinase in E. coli (16).

We are interested in developing an iron-controlled expression and secretion system for Corynebacteria and have now undertaken an analysis of the diphtheria toxin promoter function both in C. diphtheriae and in E. coli.

MATERIALS AND METHODS

Detection of CRM228 in E. coli

E. coli strains were grown in L-broth to A_{600} of 0.8, collected by low speed centrifugation, washed twice with 50 mM Tris·HCl, pH 8.0, 30 mM NaCl, and were then resuspended in one tenth of the original volume in the same buffer containing lysozyme (1 mg/ml) and 30 µg/ml p-toluene sulfonyl fluoride. Bacteria were incubated for 30 min at 0°C and lysed by three cycles of freezing and thawing. Cell debris were removed by centrifugation at 10^5g for one hour. Supernatants were used for the detection of CRM228.

For immunoprecipitation experiments cells were grown in L-broth to A_{600} of 1. 15 to 30 min before the end of the culture 150 µCi/ml of ^{35}S-methionine (110 Ci/mmol) were added. Bacterial extracts were then prepared as described above. Immunoprecipitation of labelled proteins was carried out as described by Charnay et al. (17), using rabbit IgG directed against diphtheria toxin and protein A-Sepharose beads (Pharmacia). Samples of total bacterial extracts or of the immunoprecipitates were then subjected to SDS/polyacrylamide electrophoresis (18).

β-galactosidase assay

β-galactosidase activity was measured according to Miller (19) in the E. coli lacZ$^-$ strain MC1000 (20) harbouring one of the plasmids pSKS105,

pSKS107, or pTG113. Aliquots were withdrawn from cultures of exponentially growing bacteria and were lysed by toluene, and β-galactosidase was assayed by hydrolysis of o-nitrophenyl-β-D-galactoside.

mRNA

E. coli harbouring pTD134 was grown to A_{600} of 0.8. Total RNA was prepared using the procedure described by Barry et al. (21). Total RNA from C. diphtheriae C7($\beta^{tox-228}$) (2) was obtained from a culture grown at 34°C to A_{600} of 5 in YT medium (not induced). For induction, cells were harvested and resuspended in the same medium which had been treated for 12 h at 4°C with Chelex-100 (BIO-RAD) to remove metal ions (22). C. diphtheriae was lysed by French press. Subsequent isolation of RNA was as described for E. coli.

Dot blots

Aliquots of preparations of total RNA from E. coli harbouring pTD134 and from C. diphtheriae C7 ($\beta^{tox-228}$) were fixed on nitrocellulose using a Minifold apparatus (Schleicher & Schuell). DNA fragments from pTD134 were labelled (3 x 10^7 cpm/µg) with {^{32}p} by nick-translation (23) and were hybridized to the RNAs fixed on nitrocellulose.

S1 nuclease mapping

A 119 base pair (bp) HaeIII/HgiAI fragment from pTD134, labelled at the HaeIII site (18000 cpm) was co-precipitated with 50 µg of the RNA to be analysed and then resuspended in 50 µl hybridization buffer containing 0.4 M NaCl, 40 mM Pipes (pH 6.4), 1 mM EDTA and 80% formamide. After heating for 10 min to 75°C the samples were incubated for 14 h at 49.5°C for hybridization.

S1 nuclease digestion was performed for 1 h at 20°C by addition of 5000 units of enzyme (Boehringer Mannheim) in 300 µl of buffer containing 0.28 M NaCl, 50 mM NaOAc (pH 4.5), 4.5 mM $ZnSO_4$ and 20 µg/ml sonicated single stranded salmon DNA. After phenol extraction and isopropanol precipitation the samples were resuspended in 8 µl of sequencing dye (24).

Primer extension

AMV reverse transcriptase was purchased from Life Sciences (St. Petersburgh, Flo., USA). A 5' {^{32}p} labelled 173 bp HindIII/HaeIII fragment from pTD134 was cut by HhaI, and a 17 bp HhaI/HaeIII fragment was isolated. Aliquots (8000 cpm) were co-precipitated with 50 µg of the RNA to be analyzed and redissolved in 50 µl H_2O. After 5 min heating to 90°C the samples were incubated for 2 h at 20°C for hybridization. After precipitation the samples were redissolved in H_2O. Reverse transcription was carried

out in 25 µl of a buffer containing the four dNTPs (1mM each), and 34 units of reverse transcriptase. After phenol extraction and ethanol precipitation the samples were resuspended in 8 µl of sequencing dye.

DNA sequencing

The 119 bp HgiAI/HaeIII fragment was sequenced according to Maxam and Gilbert (24).

RESULTS

The diphtheria toxin gene

The diphtheria toxin gene, schematically represented in Figure 1a, encodes a 25-amino acid signal peptide not present in the mature toxin, a 193-amino acid sequence representing the A fragment and a 342-amino acid sequence corresponding to the B fragment.

The amino acid sequence of the CRM228 mutant diphtheria toxin derived from the nucleotide sequence of the tox228 gene had previously been compared to the wild type amino acid sequences of the A fragment and a preliminary version of the B fragment (3). The direct comparison of the nucleotide sequences of the mutant and of the wild type gene is now possible.

After correction of nucleotide 665 (A instead of G) in our previously established tox228 sequence (3), four mutations leading to amino acid

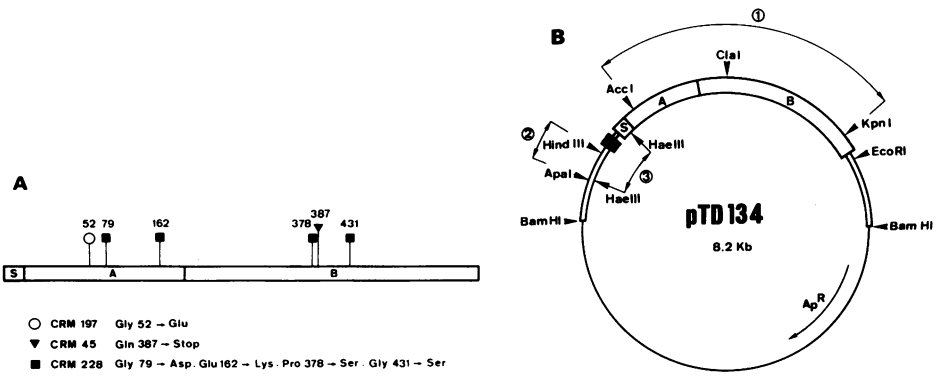

Figure 1. The diphtheria toxin gene. (a) Location of mutations in CRM 45 (▼), CRM197 (○), and CRM228 (■). S, A and B designate the signal peptide, fragment A, and fragment B, respectively. (b) Map of pTD134 carrying a 3.9 kb BamHI fragment from $\beta_c^{tox-228}$DNA inserted into the BamHI site of pBR322. The presumptive tox promoter is boxed in black. Some restriction sites and three fragments used are indicated. 1 : tox probe, 2 : upstream probe, 3 : promoter containing fragment.

exchanges in CRM228 remain, two each in A and in B (Figure 1a). These are Gly → Asp (wt→ CRM228) and Glu → Lys in positions 79 and 162, respectively, in the A fragment, and Pro → Ser and Gly → Ser in the B fragment (amino acids 378 and 431, respectively). The genes of two additional non-toxic diphtheria toxin mutants, CRM197 and CRM45, have since been sequenced (6), and the corresponding amino acid exchanges are included in Figure 1a. All of the amino acid exchanges indicated are due to single point mutations (transitions). The nucleotide sequences preceding the coding region are identical in tox228 and in the wild type gene.

Cloning of a 3.9 kb BamHI fragment carrying the toxin gene from Corynephage $\beta_c^{tox-228}$ DNA in the BamHI site of pBR322 has yielded plasmid pTD134 (Figure 1b). Restriction sites and some of the corresponding restriction fragments used in the subsequent work are indicated.

Expression of diphtheria toxin in E. coli

For a quantitative evaluation of diphtheria toxin expression in E. coli we have developed an enzyme-linked immunosorbent assay (ELISA) using horse antibodies directed against wild type toxin and coupled to peroxidase.

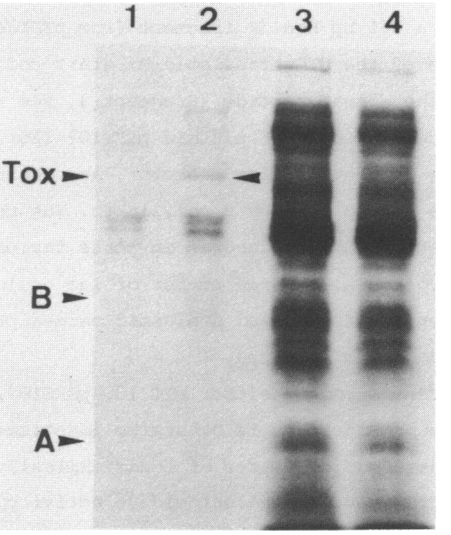

Figure 2. Expression of CRM228 in E. coli. An SDS/polyacrylamide gel of ^{35}S methionine labelled proteins is shown. Immunoprecipitates from E. coli carrying pBR322 (1) or pTD134 (2) were obtained with antitoxin antibodies. Total proteins from the same strains (3, 4). The positions of intact toxin and of fragments B and A run in the same gel are indicated.

pTD134 and the independently isolated plasmids pTD44 and pTD76 (3) carrying tox228 in opposite orientations were used to transform E. coli, and lysates of bacteria from logarithmically growing cultures were assayed. With wild type toxin as reference, the level of CRM228 expression was estimated to be 50-100 ng/ml of culture, irrespective of the orientation of the toxin gene in pBR322.

To analyse whether complete toxin molecules were present in E. coli, proteins were labelled by ^{35}S-methionine, immunoprecipitated by rabbit antitoxin antibodies and submitted to polyacrylamide gel electrophoresis (Figure 2). A single protein band was detected which was absent from lysates of E. coli harbouring pBR322. This band corresponds to a protein of molecular weight 60 000 and comigrates with uncleaved diphtheria toxin. No cleavage products corresponding to fragments A or B were observed.

Diphtheria toxin promoter-dependent expression of β-galactosidase

Since the synthesis of diphtheria toxin in E. coli was independent of the orientation of the toxin gene in pBR322, the promotion of expression was likely to occur from the toxin promoter. Additional evidence for diphtheria toxin promoter functioning in E. coli was obtained by expression of β-galactosidase under the control of the toxin promoter.

For this purpose, a 365 bp HaeIII fragment from pTD134 carrying 310 bp of sequences upstream of the GTG translational start codon and 55 bp from the region encoding the signal peptide (Fragment 3, see figure 1) was inserted into the unique SmaI site of plasmid pSKS107 (25). pSKS107 carries a truncated lacZ gene without the lac promoter, and the region encoding the first six amino acids is replaced by a polylinker. The recombination with the toxin gene fragment should produce an in-phase fusion of the signal peptide with β-galactosidase. A large number of lac$^+$ colonies were obtained; one of them was chosen at random, and a plasmid termed pTG113 and encoding a hybrid β-galactosidase was isolated.

E. coli MC1000 was transformed by either pTG113, pSKS107, or by pSKS105 which carries the lac promoter but is otherwise identical to pSKS107 (25). β-galactosidase was assayed in lysates of logarithmically growing bacteria. In the absence of a promoter no β-galactosidase activity was observed. With the toxin promoter a level of expression was achieved (480 units/ml) which corresponded to 14% of the level attained with the lac promoter itself under these conditions (3480 units/ml).

Regulation of diphtheria toxin transcription

We have compared the efficiency of the diphtheria toxin promoter in C. diphtheriae and in E. coli and have investigated the role played by iron

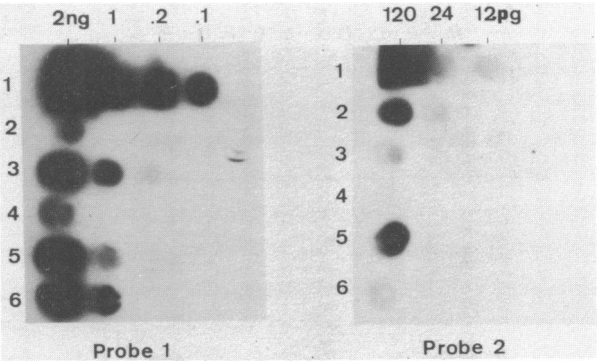

Figure 3. Quantitation of mRNA by dot blot hybridization. Probe 1 : 1437 bp AccI/KpnI fragment from pTD134 (tox probe, see figure 1). Probe 2 : 129 bp ApaI/HindIII fragment from pTD134 (upstream probe, see Figure 1). The following samples were applied, 1 : pTD134 containing 2 - 0.1 ng (120 - 12 pg) of DNA corresponding to each probe. 2-6 : 25 and 2.5 µg of total RNA from the following bacterial strains, C.diphtheriae C7 ($\beta^{tox-228}$) grown in the presence (2) and in the absence (5) of iron, E. coli pTD134 (3, 6) E. coli/pBR322 (4). The RNA blotted in (6) has been treated by DNAase I.

in the regulation of diphtheria toxin expression. Transcripts of the toxin gene were quantitated by dot blot hybridization using RNA from E. coli carrying pTD134 and from C. diphtheriae C7 ($\beta^{tox-228}$) carrying a single copy of a $\beta^{tox-228}$ prophage (Figure 3).

As probe an AccI/KpnI fragment comprising most of the region encoding fragments A and B (probe 1, see figure 1) was used. pTD134 spotted on the same filter served as reference (Figure 3, No.1). No hybridization above background could be detected with RNA from C. diphtheriae grown in the presence of iron (No. 2, 4). When the culture medium was deprived of iron, the signal obtained (No.5) corresponded approximately to 0.5 ng of pTD134 (No.1). A similar level of hybridization was observed with RNA from E. coli carrying pTD134 (No.3). Pre-treatment of the E. coli RNA with DNAase I did not decrease the hybridization signal which proves that the hybridization was not due to contaminating pTD134 (No.6).

The same RNA preparations were hybridized to a second probe, comprising sequences from further upstream of the toxin gene (probe 2, see figure 1). In this case, only Corynebacterium RNA hybridized detectably, though very weakly (Figure 3), and the amount of RNA hybridized was independent of the presence of iron (Nos.2 and 5). This may suggest the presence of another promoter in this region, but has not been further investigated.

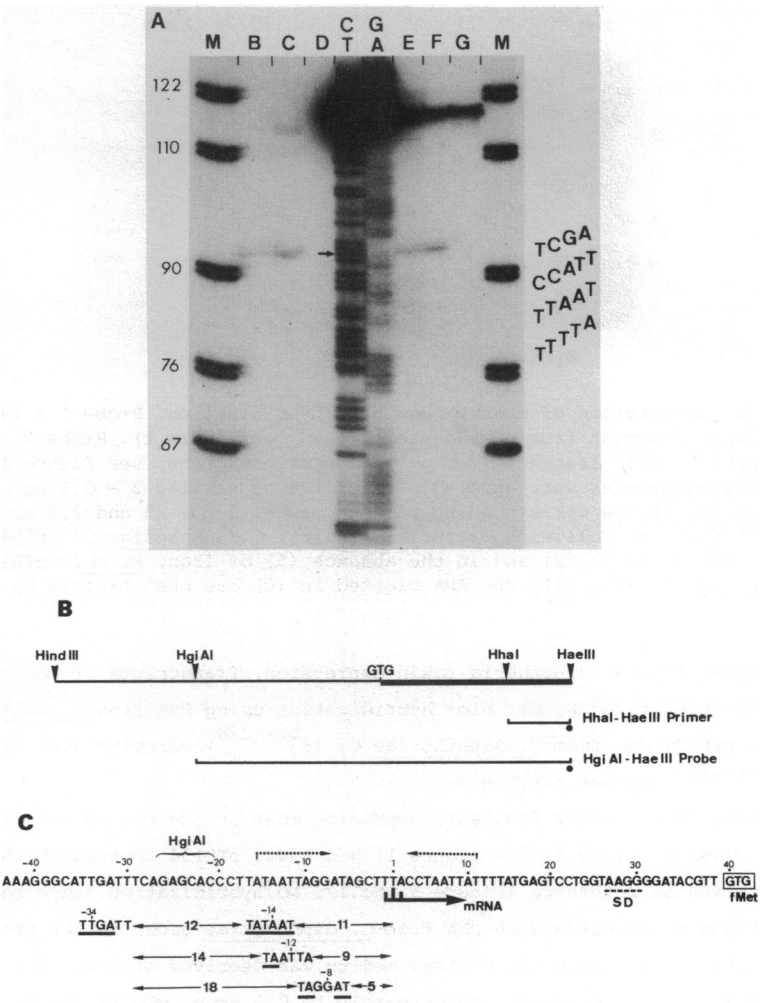

Figure 4. Mapping the 5' end of toxin mRNA. (a)8% polyacrylamide/urea gel. The following samples were applied, M : pBR322/HpaII, labelled at the 3' end with α^{32}p-dCTP. Sizes of fragments are given in bp. B-D : Primer extension, E-G : S1 mapping with total RNA from E. coli/pTD134 (B, E) or C. diphtheriae grown in the absence (C, F) or the presence (D, G) of iron. C,T and G, A : fragments obtained by cleavage at pyrimidines and purines, respectively (Maxam and Gilbert, 1980) of the HgiAI/HaeIII fragment 5' labelled at the HaeIII site shown in (b). (b) localization of the primer and the S1 probe. (c) Localization of the startpoint of transcription (position 1). Possible promoter sequences are indicated and their distances are given (bp). Nucleotides identical to the E. coli consensus sequences are underlined. The dotted arrows indicate an interruped 9 bp palindrome. SD indicates a possible Shine-Dalgarno sequence.

Mapping the initiation site of diphtheria toxin transcription

The previous results have demonstrated that sequences upstream of the diphtheria toxin gene function as promoter both in E. coli and in C. diphtheriae but it is unclear whether these sequences are the same. To approach this question, we have mapped the 5' end of the toxin mRNA from C. diphtheriae and E. coli. Two complementary techniques were used, S1 digestion of RNA/DNA hybrids and reverse transcription of RNA using a specific DNA primer (primer extension).

Identical results were obtained with RNA from E. coli and from C. diphtheriae deprived of iron. Primer extension yielded a single labelled DNA fragment, and a major fragment of the same size was detected after S1 digestion (Figure 4a). The 5' end of these fragments was identified by coelectrophoresis with fragments obtained by Maxam-Gilbert sequencing of a DNA fragment labelled at the same HaeIII site as the primer and the DNA fragment used for S1 mapping (Figure 4b). After correction by 1.5 nucleotides to account for the higher mobility of DNA fragments obtained by chemical cleavage (26), the T residue indicated (position 1 in Figure 4c) corresponds most likely to the 5' end of the toxin mRNA although the neighbouring A or T residues cannot be excluded with certainty by these experiments.

No band could be detected when RNA from C. diphtheriae grown in the presence of iron was used (Figure 4a, lanes D and G), indicating that no initiation of transcription of the toxin gene occured under these conditions. In the primer extension experiment, but not by S1 mapping, with RNA from C. diphtheriae deprived of iron a second band was detected. Whether this corresponds to the primer binding to another RNA or to an artefact of reverse transcription has not been investigated.

DISCUSSION

An approximate estimation of the efficiency of the toxin promoter in E. coli was obtained by a comparison of the β-galactosidase activity in a strain harbouring either pSKS105 or pTG113, which differed in the promoter, the 5' untranslated region and the beginning of the coding region of lacZ but were otherwise identical. It is not unreasonable to assume that the copy number of the two plasmids is similar. In addition, the 5' untranslated regions of the corresponding two mRNAs are equally long, and the presumptive Shine-Dalgarno sequences have the same distances from the translational start codon. Replacing the ATG codon of lacZ by the GTG codon from the toxin gene, as well as changing the sequence context around the start codon may

influence the initiation of translation, however, this is not known. If one makes the assumption that the efficiency of translation is similar for the corresponding two mRNAs and takes into account that about one quarter of the lac operons in E. coli carrying pSKS105 will be shut off by lac repressor, then the ß-galactosidase activities obtained suggest that the toxin promoter has about 10% of the efficiency of the lac promoter in E. coli. These results confirm and extend an earlier report that a 230 bp fragment from upstream of the toxin coding region can promote galactokinase activity in E. coli (16).

We have also made a rough estimate of the efficiencies of the diphtheria toxin promoter in C. diphtheriae and in E. coli. Equal amounts of total RNA from E. coli transformed by pTD134 and from C. diphtheriae C7 ($\beta^{tox-228}$) isolated from approximately the same number of cells yielded similar hybrization signals with a tox gene probe. Since C. diphtheriae C7($\beta^{tox-228}$) is a monolysogen, i.e. contains a single copy of the toxin gene, whereas it is present on a multicopy plasmid in E. coli, the results have to be corrected for copy number. If we assume that pTD134 is present at 40 copies per cell, then the amount of toxin mRNA per gene copy is 40 times higher in C. diphtheriae than in E. coli, suggesting a 40 times higher efficiency of transcription of the toxin gene in C. diphtheriae than in E. coli.

By hybridization of a tox specific probe to RNA from C. diphtheriae grown in presence or absence of iron we have presented evidence that starvation of iron increases considerably the level of toxin mRNA. This is the first evidence that iron regulates diphtheria toxin expression at the level of transcription. Moreover, the absence of RNA carrying the 5' end of toxin mRNA in bacteria grown in the presence of iron indicates that iron acts on the initiation of transcription of the toxin gene. It had previously been shown that the fraction of C. diphtheriae RNA hybridizing to ß phage DNA was increased in the absence of iron but whether this concerned toxin mRNA had not been demonstrated (22).

Since E. coli is much less dependent on iron than C. diphtheriae and will continue to grow in a medium containing only traces of iron, in-vivo studies on the effect of iron on diphtheria toxin expression are difficult to perform with E. coli. However, Murphy et al. (14) have previously shown that even very high concentrations of iron have no inhibitory effect on the expression of the diphtheria toxin gene in vitro using an E. coli extract.

S1 mapping and primer extension have revealed the same 5' terminal nucléotide of diphtheria toxin mRNA in C. diphtheriae and E. coli. Although we

have not demonstrated the presence of a triphosphate group at this nucleotide and therefore cannot rigorously exclude the possibility of processing at the 5' end, we consider it unlikely that this should be the same in C. diphtheriae and E. coli.

Initiation of transcription at the same nucleotide in C. diphtheriae and E. coli suggests recognition of the same DNA sequences by C. diphtheriae and E. coli RNA polymerase. The consensus sequences of E. coli promoters are well known (27). In the TTGATT hexanucleotide sequence located at position -34 the four most characteristic nucleotides of the -35 consensus sequence of E. coli, TTGACA, are conserved. In the -8 to -14 region a set of overlapping sequences occur which show complete or partial homology to the E. coli -10 consensus sequence (Figure 4c). The "perfect" -10 sequence, TATAAT, is found in the toxin promoter around position -14. However, the distance of this sequence to the TTGATT sequence and to the start site of transcription is outside the range normally found in E. coli. This suggests that it may not be used in E. coli. The most likely candidate is the TAGGAT hexanucleotide centered around position -8 in which the three most characteristic nucleotides are conserved and which has a distance of 18 nucleotides from the TTGATT sequence and of 5 nucleotides from the start site of transcription. However, to which of these sequences E. coli and C. diphtheriae RNA polymerase will actually bind remains speculative for the time being.

The occurrence of sequences characteristic of E. coli promoters in the promoter of this Corynebacterium gene is reminiscent of a class of promoters in Bacillus subtilis which carry the same sequences and specifically interact with RNA polymerase containing a sigma subunit known as σ^{55} (28).

The same sequences have been found in other genes of gram-positive bacteria e.g. the β-lactamase gene of Staphyloccocus aureus (29). There is also remarkable homology to the cholera toxin promoter. However, none of the repeated sequences probably involved in the positive regulation of cholera toxin expression (30) are found in the diphtheria toxin promoter.

A unique feature of the diphtheria toxin promoter is its regulation by iron. Whether this occurs through negative regulation, iron being a co-repressor of a bacterial aporepressor as previously suggested (13), through positive regulation or through some other mechanism remains speculative at the moment. The mapping of the transcriptional start site to within an interruped 9 bp palindrome (Figure 4c) would be compatible with a model of negative regulation if this palindrome serves as repressor binding site. The analysis of iron-independent mutants of bacteriophage β (10,11) should

further clarify this question.

Corynebacteria are non-pathogenic and are used at large scale, mainly for the fermentation of amino acids. The genetic engineering of Corynebacteria may therefore have important applications, and the diphtheria toxin promoter should be particularly useful for such a system.

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