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Determination of the Promoter Strength of the Gene Encoding *Escherichia coli* Heat-Stable Enterotoxin II

DAN F. SPANDAU AND CHAO-HUNG LEE*

Department of Pathology, Indiana University School of Medicine, Indianapolis, Indiana 46223

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We studied the promoter strength of the gene encoding the *Escherichia coli* heat-stable enterotoxin II (STII). The promoter region and a portion of the 5′ coding sequence of the STII gene were fused to the *lacZ* gene so that the production of β-galactosidase was under the control of the STII gene promoter. The strength of the STII gene promoter was compared with that of the *ompF* and *lac* operons, which were similarly fused to the *lacZ* gene. The β-galactosidase produced by the hybrid genes was assayed in vitro by using cell extracts. The mRNA transcribed by each promoter was assayed by Northern blot analysis and by in vitro transcription. The results suggest that the STII gene is regulated by a relatively weak promoter.

Enterotoxigenic *Escherichia coli* causes diarrhea in humans as well as in animals (16). The enterotoxins produced by enterotoxigenic *E. coli* are categorized according to their heat stability into heat-labile toxins and heat-stable toxins (ST). Heat-labile toxins are similar to the cholera toxin in molecular structure, antigenicity, and function (2, 4). Two types of ST have been identified, STI (STa) and STII (STb). The structural gene of STI encodes a peptide of 72 amino acids (13, 20). The mature toxin, which activates guanylate cyclase (5, 6), is composed of only 18 amino acids derived from the last 18 amino acids of the C-terminal end of the protoxin (1, 21). The STII gene has also been isolated and characterized (9, 14). It has the potential to produce a 71-amino-acid peptide. However, the molecular structure and the mode of action of the toxin are unknown.

Although the gene encoding the STII toxin has been identified, the protein has proven to be difficult to isolate and characterize. Our failure to detect toxin production from the cloned STII gene in mini- and maxicells may be due to the low level of expression of the toxin gene. Therefore, we began to look at the regulation of STII gene expression.

The regulatory region and NH_2 terminus of the structural gene of STII derived from pCHL6 (9) were fused to the *lacZ* gene of pORF1 (23), creating the plasmid $pSTII_{lac}$ (Fig. 1). This allowed the expression of the STII gene to be easily measured by utilizing a β-galactosidase assay. The correct translational reading frame at the STII-*lacZ* fusion was confirmed by the nucleotide sequencing method of Maxam and Gilbert (10). Deletion of the 10-base-pair *Bam*HI fragment of the pORF1 vector and a few base-pair deletions at the *Sma*I site of pORF5 (17) yielded *lacZ* fusions in which the expression of β-galactosidase was under the control of the *omp*F promoter ($pOMPF_{lac}$) or the native *lac* promoter ($pORF5_{lac}$). These constructions allowed us to compare the expression of the STII gene with that of two well-characterized promoters by determining β-galactosidase activities. The test plasmids were introduced into *E. coli* MH1000 (*lacZ ompR*) (23), and the β-galactosidase activity encoded by each of the test plasmids was assayed by using bacterial cell extracts as described by Miller (11). No detectable β-galactosidase activity was demonstrated in *E. coli* MH1000 without a plasmid or with pORF1. The specific activity of the STII-*lacZ* enzyme was only 10% of that of the *ompF-lacZ* protein and 0.04% of that of the *lac-lacZ* protein.

Since our STII-*lacZ* fusion gene contains STII translational signals, β-galactosidase activities may not truly reflect transcriptional efficiencies. Therefore, Northern blot analyses were performed to quantitate *lacZ*-specific mRNA. RNA was obtained from log-phase cell cultures by hot-phenol extraction in the presence of the RNase inhibitor guanidinium isothiocyanate (3). A portion of each extract was treated with DNase-free RNase A to ensure that the bands identified were not due to contaminating plasmid DNA. Samples were electrophoresed on a denaturing agarose gel and then transferred to a nitrocellulose membrane. β-Galactosidase-specific mRNA was identified by hybridization with a ^{32}P-labeled *lacZ* DNA probe. The mRNA transcribed from the *lacZ* gene was estimated to be approximately 3.0 kilobases (kb) in length. The autoradiograph showed *lacZ*-positive, RNase-sensitive bands greater than 2.7 kb derived from cells containing $pOMPF_{lac}$ and $pORF5_{lac}$ plasmids, whereas no band was detected from *E. coli* MH1000 without a plasmid or from MH1000 harboring pMLB1034 (19), a plasmid containing a promoterless *lacZ* gene (Fig. 2). RNA isolated from cells containing $pSTII_{lac}$ showed a smear on the radiograph. However, a faint band at a position identical to that of the *lac-lacZ* mRNA was seen after the overexposure of a separate autoradiograph (data not shown). Since no bands were observed in untransformed *E. coli* MH1000 cells, *lacZ*-specific RNA must result solely from the plasmids introduced into the cells. The $pORF5_{lac}$ transcripts always appeared as a pair of bands, demonstrating equally expressed heterologous initiation or termination sites. This observation is similar to that made by Stefano and Gralla (22). However, the reasons for our results are not certain. Dark bands above the mRNA sequences were extraneous plasmid DNA sequences, as evidenced by their resistance to RNase A digestion. The intensities of the RNA bands were determined by densitometer tracing. The STII transcript was assigned a value of one, and the relative values for the remaining bands were calculated accordingly. Each value is the average of at least three tracings. The mRNA of the STII-*lacZ* fusion gene was about 1,200 times less abundant than that transcribed by the *opmF* promoter

* Corresponding author.

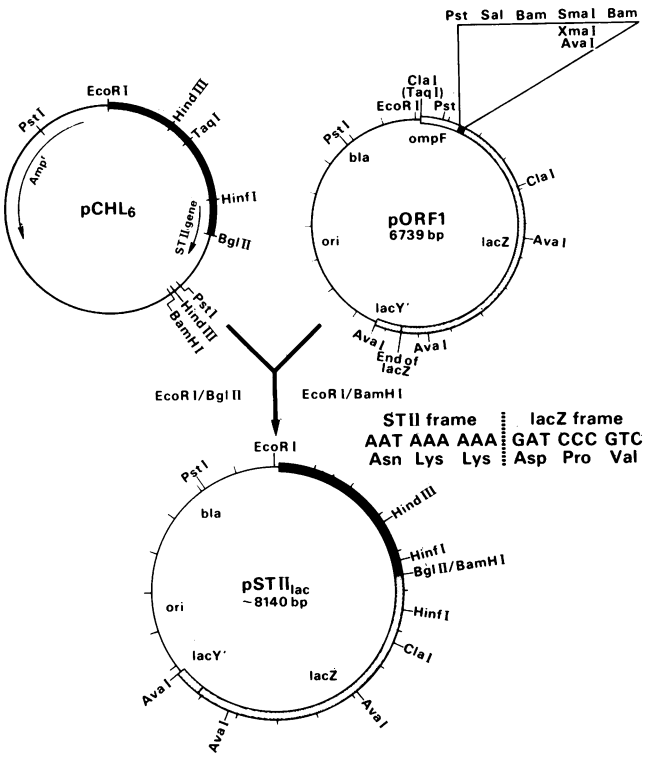

FIG. 1. Construction of plasmid $pSTII_{lac}$. The 1.9-kb *Eco*RI-*Bgl*II fragment from pCHL6 containing the STII promoter was cloned into the *Eco*RI-*Bam*HI sites of pORF1, which contains the structural gene for β-galactosidase. bp, Base pairs.

and 5,500 times less abundant than mRNA initiated from the *lac* promoter.

Since transcription may be affected by cellular *trans*-acting factors, Northern blot results may not accurately represent native promoter strengths. Therefore, in vitro transcriptions were performed. The templates for transcription included either the *ompF*, *lac*, or STII promoter region fused only to the amino-terminal region of the *lacZ* structural gene. The templates were created as outlined in Fig. 3, and the in vitro transcription reactions were performed by the method of Kajitani and Ishihama (8) and Mizuuchi (12). Equimolar quantities of the DNA templates were added separately to in vitro transcription reaction mixtures containing [α-^{32}P]UTP, and the reactions were stopped at 5-min intervals.

Results from the densitometer tracing of the RNA gel autoradiograph are shown in Fig. 4. As was done for the in vivo transcription, the STII transcript was arbitrarily set at 1 transcript per min. After 5 min, transcripts from both *ompF* and *lac* promoters were apparent, but transcription products from the STII promoter could not be seen until 20 min after the reaction was initiated. The data show that *ompF* and *lac* transcripts both increased at a steady rate for the entire assay period. At 30 min, over 450 times more RNA had been transcribed from the *lac* template compared with the STII template. The *ompF* promoter was almost 7 times more active than the STII promoter. These ratios are a relative measure of the native ability of each of the promoters to bind RNA polymerase and to transcribe the adjoining genes. Since the assay was performed in the absence of cellular factors, it demonstrated the innate strength of the promoters more accurately than any of the other methods.

Analysis of the data derived from β-galactosidase assays, Northern blots, and in vitro transcription (Table 1) suggested that the failure to isolate the STII toxin protein was due to the low level of expression of the STII gene. The expression of the STII-*lacZ* fusion gene was about 3 logarithms (2,500-fold in β-galactosidase activity and 5,500-fold in mRNA determined by Northern blots) lower than the *lac* promoter in vivo. However, in vitro transcription demonstrated only 2.5 logarithms (450-fold) difference in expression. Since in vitro transcription was performed in the absence of cellular factors that could influence the expression of the gene, it best reflects the native affinity of RNA polymerase to each promoter region. The greater difference in transcription seen in vivo could be due to *trans* activation of the *lac* promoter, repression of the STII promoter, or both. The expression of the *ompF* promoter was about 10-fold more than that of the STII promoter, based on the β-galactosidase activity and in vitro transcription. However, the Northern results showed a 1,200-fold difference in the amount of mRNA. This may be because the *ompF* mRNAs are more stable. In the autoradiogram of the Northern blot (Fig. 2), a smear is seen in lanes D and E but not in lane C, which contained RNA isolated from $pOMPF_{lac}$-containing cells, suggesting that *ompF* mRNAs may be more resistant to degradation. This may explain why the Northern blot results are not consistent with the other results.

Although other factors, such as translation efficiency and mRNA stability, may affect the production of STII, the overall results of this study suggest that the STII promoter is weaker than that of both the *ompF* and *lac* operons. Since the nucleotide sequence of the gene responsible for the production of the toxin is known (9, 14), it is possible to examine the structure of the STII gene for irregularities that might contribute to the apparent low production of the toxin. It was discovered that the promoter region of the STII gene did not conform with the consensus sequence of promoters

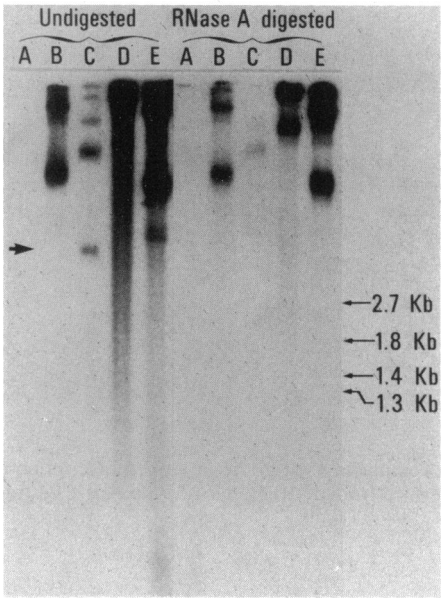

FIG. 2. Radiograph of Northern transfer of total cellular RNA extracted from *E. coli* MH1000 containing different plasmids and probed with *lacZ* DNA. Lanes: A, no plasmid; B, pMLB1034; C, $pOMPF_{lac}$; D, $pSTII_{lac}$; E, $pORF5_{lac}$. Samples on the right were digested with RNase A before electrophoresis. The arrow in the left margin is pointing to the position of the STII-*lacZ* transcript.

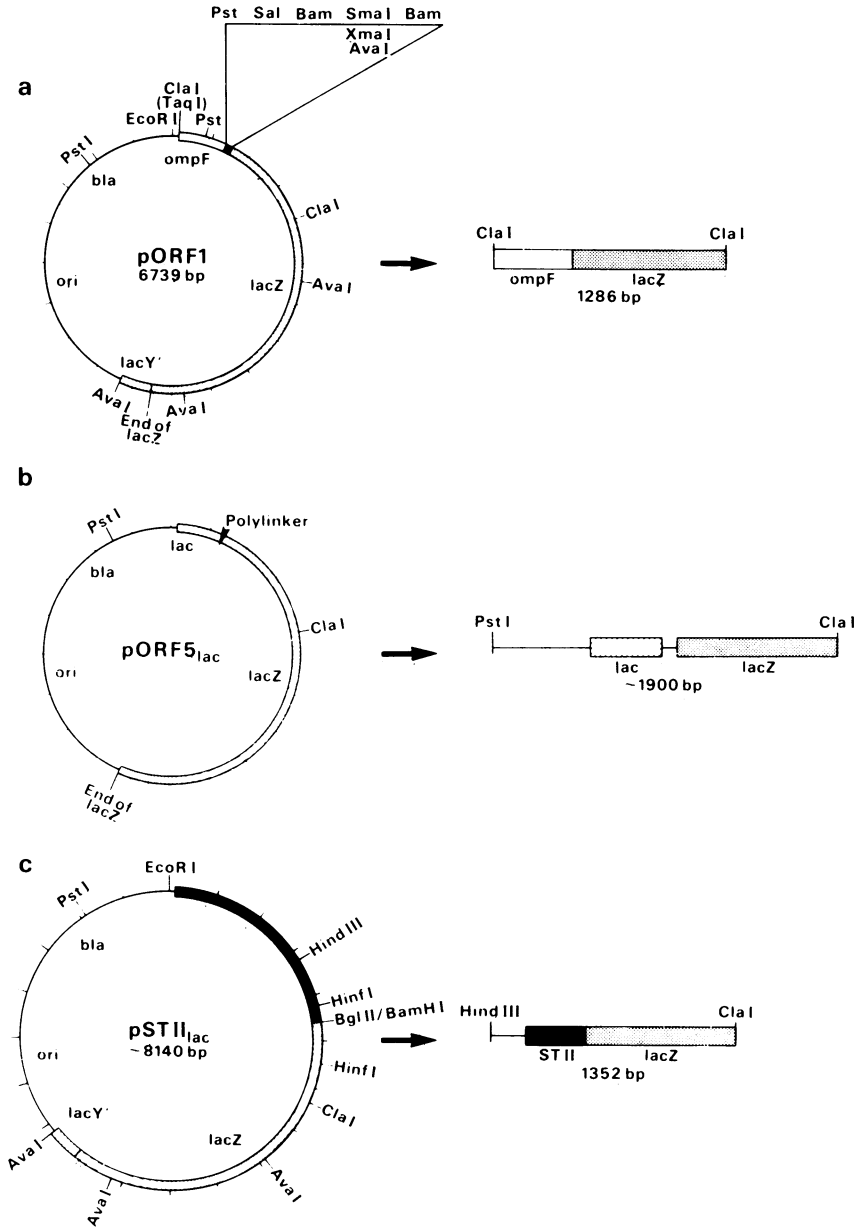

FIG. 3. Templates used in the in vitro transcription assay. (a) *ompF* template isolated as the 1.3-kb *Cla*I fragment from pORF1. (b) *lac* template derived from $pORF5_{lac}$ as a 1.9-kb *Pst*I-*Cla*I fragment. (c) STII template isolated as a 1.3-kb fragment from $pSTII_{lac}$. bp, Base pairs.

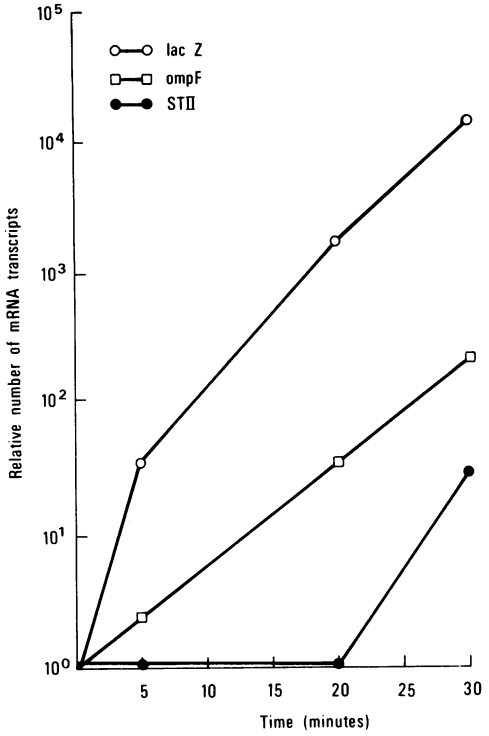

FIG. 4. In vitro transcription. Relative numbers of mRNA transcripts were determined by densitometer tracing of the autoradiograph from the RNA gel. The level of STII transcription was set at 1.0 transcript per min.

derived from hundreds of *E. coli* genes. The Pribnow box (−10 region) for the STII gene demonstrated good homology with the consensus sequence except for one important base, the final T in the sequence (9). Among the 170 promoter sequences that have been identified to date, only 4 do not contain a T in that position, called the invariant T of the Pribnow box (7). Although it may not be essential for the binding or recognition of RNA polymerase, it has been theorized to be important in the release of RNA polymerase from the promoter region (18). Of the four promoters that do not contain the final T, none contains a G residue in that position as does the STII promoter (9). When the −35 regions are compared, the STII promoter is highly homologous to the −35 consensus sequence. The −35 region is thought to play an important role in RNA polymerase recognition and binding (15). It is possible that the STII promoter is capable of binding RNA polymerase but is a poor initiator of transcription. Therefore, very little STII is produced. This hypothesis suggests that STII may be very potent since very little expression of the STII gene is required for activity. This notion is consistent with our initial problem of isolating the STII protein from cell culture supernatants that had demonstrated toxin activity in the pig ligated-ileal-loop assay. If indeed the STII toxin is as potent as it appears to be, then overexpression of the STII gene could be deleterious to the bacterial cell itself. Therefore, low expression of the gene might be favored for the well-being of the bacterial cell.

TABLE 1. Comparison of promoter strengths determined by different methods

Test	Promoter strength[a]	
	ompF	*lacZ*
β-Galactosidase activity	10	2,500
Northern blot	1,200	5,500
In vitro transcription	7	450

[a] Calculated relative to the STII gene promoter.

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