Characterization of the *rcsA* and *rcsB* Genes from *Salmonella typhi*: *rcsB* through *tviA* Is Involved in Regulation of Vi Antigen Synthesis

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Synthesis of Vi antigen, a capsular polysaccharide expressed by *Salmonella typhi*, is controlled by the *viaA* and *viaB* chromosomal loci. It was previously shown that Vi antigen expression was regulated by a system similar to the *rcs* regulatory system involved in colanic acid synthesis in *Escherichia coli*. We have cloned the *rcsA*, *rcsB*, and *rcsC* genes from *S. typhi*. The predicted amino sequences of the RcsA and RcsB proteins showed a high degree of similarity to their *E. coli* homologs. The nucleotide sequence of the *rcsC* gene was partially determined and was shown to be homologous to that of its *E. coli* counterpart. Complementation experiments indicated that *rcsB* and *rcsC* were encompassed within the *viaA* locus. The RcsA protein was not involved in Vi antigen synthesis. In contrast, the RcsB protein acted as a positive regulator of Vi polysaccharide expression. By mRNA and gene fusion analyses, we studied the role of RcsB and TviA, a *viaB*-encoded regulatory protein characterized previously, in regulating Vi antigen synthesis. The transcriptional start point of *tviA* mRNA was not influenced by RcsB or TviA. In the absence of RcsB or TviA protein, transcription of *tviA* gave rise to only a monocistronic *tviA*-specific mRNA. The presence of RcsB and TviA not only increased the amount of monocistronic *tviA*-specific mRNA but also resulted in cotranscription of *tviA* and *tviB*, which is located immediately downstream of *tviA* on the *viaB* locus. In addition, TviA protein did not appear to be subject to degradation by the Lon protease. These results strongly suggest that TviA might act in concert with RcsB at the *tviA* promoter to activate transcription of the genes involved in Vi polymer synthesis in *S. typhi* in a Lon-independent manner.

Bacterial capsular polysaccharides are classified into two groups by chemical and physical criteria. In general, group I polysaccharides contain uronic acid as the acidic component, have high molecular mass, and are coexpressed with specific O polysaccharides. In contrast, group II polysaccharides contain a large variety of acidic components and have a relatively low molecular mass (21). There is considerable information on the regulation of biosynthesis of group I polysaccharides (8, 35). However, the regulatory strategies used for colanic acid biosynthesis, an extracellular slime produced by *Escherichia coli* K-12 under appropriate growth conditions, usually serve as the reference model for regulation of capsule synthesis. Colanic acid synthesis in *E. coli* K-12 is regulated by the *rcs* system, which includes three positive regulators (RcsA, RcsB, and RcsF) and two negative regulators (RcsC and Lon). RcsB and RcsC act as the effector and the sensor, respectively, of a two-component regulatory system by stimulating capsule synthesis from colanic acid synthesis *cps* genes (36). The environmental signals which activate RcsC have yet to be determined. RcsA is an auxiliary factor which may interact with RcsB to form a heterodimer required for increased transcription of *cps* genes. The availability of RcsA protein is normally limited because it is a substrate for the ATP-dependent Lon protease (39). In addition, Gervais and Drapeau (15) proposed that RcsF could activate the effector RcsB through kinase activity,

although phosphorylation of RcsB has not been directly demonstrated.

The Vi antigen is a capsular polysaccharide coexpressed with the somatic O:9 and 12 factors by *Salmonella typhi*, the agent of human typhoid fever (13). This polysaccharide is a linear homopolymer of α-1,4 2-deoxy-2-*N*-acetylgalactosamine uronic acid variably *O*-acetylated at the C-3 position (10, 18). Its molecular mass is estimated to be 10^6 Da (38). Based on these characteristics, the Vi antigen may be considered a group I capsular polysaccharide. All strains of *S. typhi* and *Salmonella paratyphi* C as well as a few strains of *Salmonella dublin* and *Citrobacter freundii* are capable of expressing Vi antigen (2, 14, 29).

The genetic determinants involved in Vi antigen expression occupy two widely separated chromosomal loci, designated *viaA* and *viaB*. The *viaA* locus, located at 43 min on the chromosome of *S. typhi* (24), is present not only in Vi-expressing *Salmonella* and *Citrobacter* strains but also in *E. coli* (23, 25). Interestingly, it was shown that expression of the RcsB protein of *E. coli* restored a Vi-positive phenotype to spontaneous *viaA* mutants of *S. typhi*, suggesting that Vi antigen synthesis in *S. typhi* might be regulated by the same regulatory proteins involved in colanic acid synthesis in *E. coli* (19). The *viaB* locus, which is specific to Vi-expressing strains, is located at 92 min on the chromosome of *S. typhi* (23, 25, 34). This locus contains at least 10 open reading frames (ORFs) transcribed in the same orientation, which were termed *tviA*, *tviB*, *tviC*, *tviD*, *tviE*, *vexA*, *vexB*, *vexC*, *vexD*, and *vexE* (17, 42, 43). Intracellular synthesis of the Vi polysaccharide is catalyzed by the TviB, TviC, TviD, and TviE polypeptides, and the cell surface localization of the Vi antigen is directed by the VexA, VexB, VexC,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
S. typhi		
Ty2	Wild-type strain expressing Vi antigen	
Ty2(*rcsA*)	Derived from Ty2, *rcsA*::*aph*	This study
Ty2(*rcsB*)	Derived from Ty2, *rcsB*::*aph*	This study
Ty2B	Derived from Ty2, *tviB*::*lacZ-cat*	42
Ty2B(*rcsB*)	Derived from Ty2B, *rcsB*::*aph*	This study
Ty2K	Derived from Ty2, *tviA*::*aph*Δ*T*	42
T643WSR	Vi-negative *viaA* mutant	24
T643B	Derived from T643WSR, *tviB*::*lacZ-cat*	42
E. coli		
MC1061	*araD139* Δ(*ara-leu*)-7697 *rpsL galU galK* Δ(*lacIPOZY*)*X74*	4
MC1061(*rcsB*)	Derived from MC1061 + P1(SG21035), *rcsB*::*aph*	This study
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*)	33
SG20250	Derived from MC4100, Δ*lacU169*	37
SG21034	Derived from SG20250, *lon rcsA*::*aph*	S. Gottesman
SG21035	Derived from SG20250, *lon rcsB*::*aph*	S. Gottesman
Plasmids		
pUC18	Cloning vector, carbenicillin resistant	41
pGB2	Cloning vector, spectinomycin resistant	6
pUC4K	Source of *aph* cassette	Pharmacia
pQF50	Promoter-probe vector, carbenicillin resistant	12
pVT1	Recombinant plasmid carrying the *viaB* locus	28
pVT1K	Derived from pVT1, *tviA*::*aph*Δ*T*	42
pRcsA	pGB2 carrying *rcsA*$_{Ty}$	This study
pRcsB	pGB2 carrying *rcsB*$_{Ty}$ and 3′ end of *rcsC*$_{Ty}$ from Ty2	This study
pRcsB*	pGB2 carrying *rcsB**$_{Ty}$ from T643WSR	This study
pRcsBZ	Transcriptional fusion *rcsB*$_{Ty}$′-*lacZ* in pQF50	This study
pVT21	Plasmid pGB2 carrying *tviA* and 5′ end of *tviB*	42
pVT42	Derivative of pVT21, *Bgl*II site of *tviA* filled in	42
pNAG	Transcriptional fusion *tviA*′-*lacZ* in pQF50	This study
pNAGF	Derivative of pNAG, *Bgl*II site of *tviA* filled in	This study
pNAC	Transcriptional fusion *tviA-lacZ* in pQF50	This study
pNACF	Derivative of pNAC, *Bgl*II site of *tviA* filled in	This study
pNO	Transcriptional fusion *tviAB*′-*lacZ* in pQF50	This study
pNOF	Derivative of pNO, *Bgl*II site of *tviA* filled in	This study

VexD, and VexE proteins (17, 42, 43). Previous results suggested that the TviA polypeptide might be a regulatory protein which positively controlled transcription of the *tviA* and *tviB* genes from a promoter located upstream of *tviA* (42).

From these data, it appears that the TviA protein, the *viaA* products, and the *rcs* system may be involved in the same regulatory pathway modulating Vi antigen expression in *S. typhi*. This study was initiated to determine the mechanisms involved in this complex regulatory network. We report here the cloning, sequencing, and functional analysis of the *rcsA* and *rcsB* genes from *S. typhi*. In addition, we present evidence that the *rcsB* and *rcsC* genes are encompassed within the *viaA* locus. The RcsA protein is not involved in Vi antigen expression. In contrast, the RcsB protein acts as a positive regulator of Vi antigen synthesis in *S. typhi*. Furthermore, our results suggest that the TviA protein might interact with the RcsB polypeptide to promote transcription of the genes involved in Vi polymer synthesis in a Lon-independent manner.

The *rcs* genes from *S. typhi* and *E. coli* will henceforth be distinguished by the subscripts Ty and Ec, respectively.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. typhi* Ty2 was from the collection of the World Health Organization collaborating center for reference and research on *Salmonella* (Institut Pasteur, Paris). Strains were routinely grown at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA; Diagnostics Pasteur). When required, antibiotics were added at the following concentrations: carbenicillin, 100 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added at 20 μg ml^{-1}.

DNA manipulations. The methods used for constructing and manipulating recombinant DNA were essentially those of Sambrook et al. (33). Restriction endonucleases were purchased from Amersham. To clone the *rcsB*$_{Ty}$ gene, chromosomal DNA from *S. typhi* Ty2K was partially digested with the restriction endonuclease *Sau*3A, and DNA fragments were separated by gel electrophoresis. Fragments with an average size of 5 kb were recovered by electroelution and ligated to the *Bam*HI site of plasmid pGB2. Small-scale preparation of plasmid DNA was carried out as described by Birnboim and Doly (3) for *E. coli* strains and as described by Kado and Liu (26) for *Salmonella* strains. Plasmid transformation was performed in *E. coli* by the method of Humphreys et al. (20) and in *Salmonella* by electroporation with a Bio-Rad apparatus according to the manufacturer's instructions. Plasmid pUC4K (Pharmacia), which carried the aminoglycoside 3′-phosphotransferase gene, was used as a source of the *aph* cassette. P1D7H transductions were carried out as described by Miller (31). The *rcsA*::*aph* and *rcsB*::*aph* mutants of *S. typhi* were constructed by allelic exchange as described previously (42). The double recombination event was confirmed by Southern blot analysis.

Sequencing of double-stranded DNA was performed with the Sequenase sequencing kit (U.S. Biochemical Corp.) on both strands with universal, reverse, or specific synthetic primers. Nucleotide sequence data were analyzed with the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis software package (Data General UNIX computer at the Service d'Informatique Scientifique, Institut Pasteur).

To construct *tviA*′-*lacZ*, *tviA-lacZ*, and *tviAB*′-*lacZ* fusions in plasmids pNAG, pNAC, and pNO, respectively (Table 1), the DNA fragment to be cloned into pQF50 was amplified by PCR with a commercial kit (Amersham) and a couple of degenerate primers (Fig. 1). The amplified fragment was digested with appropriate enzymes, ligated into pQF50, and transformed into *E. coli* strains. To construct the *rcsB*$_{Ty}$′-*lacZ* fusion in plasmid pRcsBZ (Table 1), plasmid pRcsB was digested with *Sma*I and *Eco*RV enzymes. The *Eco*RV site was located within the *rcsB*$_{Ty}$ coding sequence. This fragment was cloned in the *Sma*I restriction site of pQF50. The correct orientation of the insert with regard to the *lacZ* reporter gene was verified by restriction analysis.
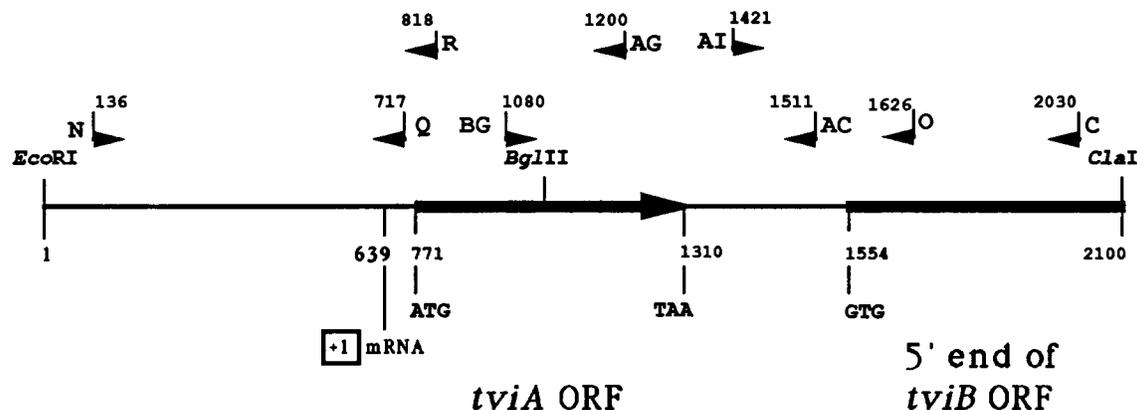
FIG. 1. Schematic representation of the *tviA-tviB* region on plasmid pVT21. Coordinates refer to nucleotide sequence X67785 in the EMBL data library. Above the 2.1-kb *Eco*RI-*Cla*I DNA fragment, flags represent primers used in this study. These primers (30-mers) are identified by letters and by the coordinates of their 5′ endpoints. Newly created *Sph*I and *Hin*dIII sites were introduced in primer N and in primers AG, AC, and O, respectively. The *Bgl*II restriction site located within the *tviA* coding sequence was filled in for constructing plasmids pVT42, pNAGF, pNACF, and pNOF (see Table 1). Below the DNA stretch, the transcriptional start point of *tviA* mRNA, the translational start and end points of *tviA*, and the translational start point of *tviB* are indicated.

RNA manipulations. Total RNA was prepared by the procedure of Glatron and Rapoport (16). After ethanol precipitation, the RNA-containing pellet was dissolved in DEPC-treated water, quantitated by the spectrophotometric method, precipitated again, and resuspended in sterile bidistilled water to a final concentration of 4 µg µl⁻¹. Aliquots were stored at −80°C. Primer extension experiments followed a previously described protocol (33). Oligonucleotide Q or R (Fig. 1) was radiolabelled with T4 polynucleotide kinase (Pharmacia) and [γ-³²P]ATP (Amersham). Primer extension reactions were carried out with 50 µg of RNA, 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim GmbH), the buffer supplied by the manufacturer, and deoxynucleoside triphosphates at 42°C. After ethanol precipitation and air drying, the pellet was resuspended in 10 µl of loading dye. A portion of the mixture was loaded onto a 6% sequencing gel next to a sequencing ladder of plasmid pVT21 with primer Q or R.

For Northern (RNA blot) analysis, total RNA (20 µg) was separated on a 1.2% agarose–2 M formaldehyde gel and transferred to a Hybond-N⁺ membrane according to the recommendations of the manufacturer (Amersham). Molecular weight markers (RNA ladder; Gibco BRL) were visualized by ethidium bromide staining in a piece of the gel cut off before transfer. Probe was generated by PCR with two of the primers in Fig. 1 and labelled with [α-³²P]dCTP (Amersham) by using the Megaprime oligolabelling system (Amersham). Prehybridization and hybridization, carried out at 40°C in 50% formamide–5× SSPE–2× Denhardt's reagent–0.1% sodium dodecyl sulfate (SDS) solution, and washings were done as described by Sambrook et al. (33).

Production of anti-Vi MAbs. BALB/c mice were immunized by intraperitoneal injection with 5 µg of purified Vi polysaccharide contained in 0.2 ml of sterile phosphate-buffered saline (PBS, pH 7.2). The purified Vi polysaccharide, prepared from a Vi-expressing strain of *C. freundii*, was a kind gift of S. C. Szu and J. B. Robbins, United States Department of Health and Human Services, National Institutes of Child Health and Human Development, Bethesda, Md. Antibody to the Vi antigen was assayed 8 days following immunization by an enzyme-linked immunosorbent assay (ELISA) with purified Vi polysaccharide as described by Tsang and Chau (40). Hybridomas were prepared, and purified ascites was obtained as described by Duffey et al. (11). Briefly, spleen cells obtained from two mice showing the highest ELISA reaction were pooled and fused with P3X63-ag8.653 plasmacytoma cells. Hybridoma cultures reactive with Vi polysaccharide by ELISA were cloned twice by limiting dilution in soft agarose, and ascites was prepared by intraperitoneal injection of approximately 2.5 × 10⁶ cells into Pristane (Sigma)-treated BALB/c mice. The ascites was partially purified by precipitation in 50% saturated ammonium sulfate. The precipitate was brought to 1/2 its original volume in PBS and then dialyzed against three changes of PBS overnight, filter sterilized (0.2 µm filter; Whatman), and mixed with an equal volume of sterile, reagent-grade glycerol (Sigma). Isotyping was performed with an Isostrip kit (Boehringer Mannheim). Two cell lines, T1 and T3 (immunoglobulin G3 [IgG3], κ), which were separate clones of the same hybridoma cell line reacted with purified Vi polysaccharide by ELISA and also slide-agglutinated Vi-positive but not Vi-negative strains of *S. typhi*, *S. paratyphi* C, and *C. freundii*. The anti-Vi monoclonal antibody (MAb) T3 used in this study.

Vi antigen assays. The presence of Vi antigen associated with the cell surface was determined by slide agglutination with Vi-specific antiserum (Diagnostics Pasteur) and further confirmed by lysis with the Vi-specific phage IV (9). Accumulation of intracellular Vi antigen or release of the polysaccharide into the culture supernatant was demonstrated by immunoprecipitation as described pre-

viously (28) with the anti-Vi MAb T3. By using a highly purified preparation of Vi antigen (Typhim Vi; Institut Merieux) as a reference standard, it was possible to detect as little as 0.1 µg of soluble Vi antigen ml⁻¹ with MAb T3.

Measurements of β-galactosidase activity. The assays for β-galactosidase activity were performed on overnight cultures as described by Miller (31). Average values (± 1 standard deviation) of activity units were calculated from at least three independent assays in each case.

RESULTS AND DISCUSSION

Cloning and characterization of the *rcsB*$_{\text{Ty}}$ gene. The *tviB* gene, located immediately downstream of *tviA* in the *viaB* locus, encoded the NAD-dependent enzyme required to synthesize the nucleotide sugar of the Vi polymer (17, 42). We have previously shown that a *tviB*::*lacZ* transcriptional fusion was not expressed in *S. typhi* T643B, a derivative of the Vi-negative *viaA* mutant T643WSR (Table 1). Providing *rcsB*$_{\text{Ec}}$ or *tviA* in *trans* on a multicopy plasmid restored expression of this fusion (42). We took advantage of these data to clone the *S. typhi* gene functionally homologous to *rcsB*$_{\text{Ec}}$. This gene will henceforth be referred to as *rcsB*$_{\text{Ty}}$. After ligation of size-fractionated *Sau*3A fragments of *S. typhi* Ty2K total DNA in the *Bam*HI site of pGB2, recombinant plasmids were transformed into *S. typhi* T643B. Since the *tviA* coding sequence was disrupted by the *aphΔT* cassette in strain Ty2K (Table 1), only recombinant plasmids carrying *rcsB*$_{\text{Ty}}$ were expected to restore expression of the *tviB*::*lacZ* fusion of strain T643B and, in turn, to give rise to β-galactosidase-expressing blue colonies on TSA plates supplemented with X-Gal. Five β-galactosidase-expressing clones were found among approximately 10³ Sp^r colonies. Analysis by restriction endonucleases showed that the recombinant plasmids present in these clones had a common DNA sequence of about 2 kb. Plasmid pRcsB, which contained a 2.3-kb insert, restored a Vi-positive phenotype to *S. typhi* T643WSR (Table 2) and was selected for further study.

The nucleotide sequence of a 1,230-bp fragment on pRcsB was determined. Analysis of the sequence revealed one complete ORF of 648 bp (from bp 166 to bp 816). This ORF defined a protein of 216 amino acids (aa), with a predicted molecular mass of 24 kDa, which showed 98% identity with the RcsB protein of *E. coli*. The C-terminal domain of this product contained a possible helix-turn-helix DNA-binding motif (po-

TABLE 2. Detection of Vi antigen in isogenic strains
of *S. typhi* Ty2 and T643WSR

Strain	Plasmid	Presence or amount (μg ml^{-1}) of Vi antigen[a]		
		Surf	Intra	Super
Ty2		+	6 ± 2	37 ± 9
Ty2(*rcsB*)		−	<0.1	<0.1
Ty2	pRcsB	+	9 ± 4	46 ± 6
Ty2(*rcsB*)	pRcsB	+	7 ± 1	33 ± 8
	pVT21	−	<0.1	<0.1
	pRcsB*	−	<0.1	<0.1
	pRcsB* + pNO	+	4 ± 2	28 ± 6
T643WSR		−	<0.1	<0.1
T643(*rcsB*)		−	<0.1	<0.1
T643WSR	pVT21	+	8 ± 3	19 ± 4
T643WSR	pRcsB	+	12 ± 4	24 ± 5
T643WSR	pRcsB*	−	<0.1	<0.1
Ty2(*rcsA*)		+	4 ± 2	41 ± 3
Ty2	pRcsA	+	9 ± 1	42 ± 5
Ty2(*rcsA*)	pRcsA	+	9 ± 3	35 ± 8

[a] The presence of Vi antigen at the bacterial cell surface (Surf) was determined by slide agglutination. +, positive reaction; −, negative reaction. The amount of Vi antigen in the intracellular compartment (Intra) and in the culture supernatant (Super) was measured by an immunoprecipitation assay with MAb T3. The results represent averages of three independent experiments $\pm$ 1 standard deviation.

sitions 170 to 185) which was identical to the corresponding motif identified on *E. coli* RcsB (37). In addition, as reported for *rcsB*$_{Ec}$ (36), a sequence with homology to the consensus RpoN-activated promoter was identified at bp 32 to 44 upstream of the ATG codon. Moreover, introduction of pRcsB into *E. coli* SG21035 conferred a mucoid phenotype on this *rcsB* mutant. From these results, we assumed that this sequence was the *rcsB*$_{Ty}$ gene of *S. typhi* Ty2. The 3′ end of a second ORF, from bp 919 to 1230, was identified in the *rcsB*$_{Ty}$ downstream region on the complementary strand. The 103-residue polypeptide predicted for this DNA stretch showed 86% identity with the C-terminal region of *E. coli* RcsC protein, strongly suggesting that the underlying gene was the *rcsC*$_{Ty}$ gene of *S. typhi* Ty2. The intergenic region between *rcsB*$_{Ty}$ and *rcsC*$_{Ty}$ (102 bp) was shorter than the corresponding region (196 bp) in *E. coli* and did not contain any direct repeat or repetitive extragenic palindromic sequence as in *E. coli* (36). From these results, and since pRcsB complemented the *viaA* mutation of strain T643WSR, it was likely that the *rcsB* and *rcsC* genes were encompassed within the *viaA* locus of *S. typhi*.

Role of the *rcsB*$_{Ty}$ gene in Vi antigen expression. The *aph* cassette of plasmid pUC4K (Table 1) was cloned into the unique *Eco*RV site located within the *rcsB*$_{Ty}$ coding sequence on plasmid pRcsB. The *Eco*RI-*Pst*I fragment carrying the *rcsB*::*aph* gene was further subcloned in pUC18. This recombinant plasmid was transformed into *S. typhi* Ty2, and the *rcsB*::*aph* mutation was recombined into the host chromosome by allelic exchange. The resulting *S. typhi* Ty2(*rcsB*) mutant was defective in Vi antigen synthesis. It was no longer agglutinated with Vi antiserum, and Vi polymer could not be detected by the immunoprecipitation assay in the culture supernatant or in the intracellular compartment of this mutant (Table 2). Introduction of plasmid pRcsB fully restored a Vi-positive phenotype to Ty2(*rcsB*). This construct was slide agglutinated with Vi antiserum and lysed by phage IV. The amount of Vi polysaccharide in its culture supernatant or in its intracellular compartment was similar to that measured for the parental strain Ty2 in the presence or absence of pRcsB (Table 2).

It was previously reported that *S. typhi* Ty2B which carried a *tviB*::*lacZ* transcriptional fusion (Table 1) expressed about 200 Miller units of β-galactosidase activity (42). In an attempt to unravel the role of RcsB in Vi antigen expression, the *aph* cassette was recombined in *rcsB* on the *S. typhi* Ty2B chromosome, yielding *S. typhi* Ty2B(*rcsB*) (Table 1). Disruption of the *rcsB* coding sequence dramatically decreased expression of the *tviB*::*lacZ* fusion (4 ± 2 Miller units). Providing *rcsB* in *trans* on plasmid pRcsB in *S. typhi* Ty2B(*rcsB*) fully restored expression of the *lacZ* fusion (229 ± 31 Miller units).

Taken together, these results demonstrated that RcsB protein was essential for Vi antigen synthesis and acted as a positive regulator of Vi antigen expression in *S. typhi*. Interestingly, multicopy RcsB did not increase Vi polysaccharide synthesis in *S. typhi* (Table 2), in contrast to the stimulating effect of multicopy RcsB on colanic acid synthesis in *E. coli*. Thus, there may be some differences in the regulatory mechanisms controlling Vi antigen synthesis in *S. typhi* and colanic acid synthesis in *E. coli*. This observation provided additional evidence for subtle variations in the pattern of regulation by the *rcs* system, as shown with other group IA capsules of *E. coli* (22).

Cloning and characterization of the *rcsA*$_{Ty}$ gene. Since RcsA is a positive regulator of colanic acid synthesis in *E. coli* and is thought to act with RcsB to promote transcription of the *cps* genes (37), we looked for an *rcsA* gene in *S. typhi*. The oligonucleotides used to amplify the *rcsA*$_{Ty}$ gene from strain Ty2 by PCR were derived from the *rcsA*$_{Ec}$ sequence (37). These oligonucleotides, 5′-CAC TCA CAT ATC GCA ACA TTT ACT TTA CTT-3′ and 5′-GCA TCA GGA CGG TAT CTT TGT GGA GAA AGC-3′, mapped at bp 21 to 50 and bp 912 to 883 of the *rcsA*$_{Ec}$ sequence, respectively. The amplified fragment was ligated into the *Sma*I restriction site of the vector pUC18 and used to transform *E. coli* TG1. The majority of transformants exhibited a mucoid phenotype. Complete sequencing of the insert was performed for recombinant plasmids from two clones. These two plasmids conferred a mucoid phenotype on the *rcsA* mutant strain *E. coli* SG21034. Examination of the sequence revealed a single ORF of 621 bp encoding a highly basic (pI = 9.7) protein of 207 aa, with a predicted molecular mass of 23 kDa. This product showed 87% identity with the RcsA protein of *E. coli* and contained a helix-turn-helix DNA-binding motif (positions 157 to 172) located in the carboxy terminus, as reported for the RcsA proteins so far characterized (27, 37). From these results, we assumed that the cloned fragment contained the *rcsA*$_{Ty}$ gene. For further experiments, this fragment was subcloned into vector pGB2, yielding pRcsA (Table 1).

Role of the *rcsA*$_{Ty}$ gene in Vi antigen expression. The RcsA protein acts as a positive regulator of colanic acid synthesis in some *E. coli* strains (22, 37) and of capsule synthesis in *Erwinia* spp. (5, 7, 39) and *Klebsiella pneumoniae* (1), but as a negative regulator of K54 capsule synthesis in *E. coli* (32). To study the role of RcsA in Vi antigen expression, the *aph* cassette from plasmid pUC4K (Table 1) was inserted into the *Cla*I restriction site of *rcsA*$_{Ty}$ cloned in pUC18 and recombined by allelic exchange into the chromosome of *S. typhi* Ty2. The resulting *S. typhi* Ty2(*rcsA*) mutant retained a Vi-positive phenotype. It was slide agglutinated by Vi antiserum and lysed by phage IV. Since RcsA acts with RcsB to increase colanic acid or decrease K54 capsule synthesis in *E. coli*, the amount of Vi antigen present in the intracellular compartment or released into the culture supernatant of strains Ty2, Ty2(*rcsA*), and their pRcsA-carrying derivatives was measured by the immunoprecipitation assay. As reported in Table 2, the four strains produced similar amounts of Vi antigen. Then, we examined the
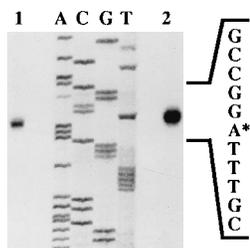
FIG. 2. Mapping of the 5′ end of *tviA* mRNA. The ^{32}P-labelled primer Q (Fig. 1) was annealed to total RNA isolated from *S. typhi* Ty2 (lane 1) and *E. coli* MC1061 carrying plasmid pVT1 (lane 2). The primer was extended with reverse transcriptase, and the products were subjected to electrophoresis on a sequencing gel. The DNA sequencing ladder (lanes A, C, G, and T) was prepared by using the same primer to sequence the pVT21 template (Table 1). The start site is indicated by an asterisk on the sequence shown at the right of the panel.

possibility that Vi antigen synthesis in *E. coli* might require functional RcsA protein. For this purpose, plasmid pVT1 was used to transform *E. coli* SG21034 (Table 1). Ten transformants were assessed in slide agglutination and phage IV assays. All were agglutinated by Vi antiserum and lysed by phage IV. These results demonstrated that $rcsA_{Ty}$ in *S. typhi* and $rcsA_{Ec}$ in *E. coli* were not involved in Vi antigen expression. This finding was unexpected since, until now, the RcsA protein through the RcsB protein, was always involved in activating (7, 27, 30) or in decreasing (32) the transcription of polysaccharide genes. As TviA was shown to be a regulatory protein modulating Vi antigen expression in *S. typhi* (42), we wondered whether the auxiliary product interacting with RcsB might be the TviA protein instead of the RcsA product.

Transcription of *tviA* and *tviB* genes. The results reported so far indicated that RcsB was a positive regulator of Vi antigen synthesis in *S. typhi* and was required for *tviB* transcription. In addition, it was shown that the TviA protein positively controlled *tviB* transcription from a promoter located upstream of *tviA* (42). To unravel the roles of RcsB and TviA, transcription of the *tviA* and *tviB* genes was studied in strains expressing both of these proteins, only one, or neither.

We first determined the 5′ end of *tviA* mRNA by a primer extension assay with oligonucleotide Q or R (Fig. 1) and total RNA prepared from the RcsB- and TviA-expressing strains *S. typhi* Ty2 and *E. coli* MC1061 harboring pVT1. As shown in Fig. 2, when primer Q was used, RNA from the two strains gave rise to a primer extension product of the same size with an A nucleotide at position bp 639 identified as the transcriptional start point. The same start point was visualized with primer R (data not shown). Then, primer extension assays were repeated with oligonucleotide Q or R and total RNA from strains *S. typhi* Ty2K and *E. coli* MC1061 carrying pVT1K, in which the *tviA* coding sequence was disrupted by the *aphΔT* cassette, and from strain *E. coli* MC1061(*rcsB*) carrying pVT1 or pVT1K (Table 1). Detection of the start point of *tviA* transcription required a longer exposure, but in no case was a transcriptional start site which deviated from that shown in Fig. 2 observed, even after overexposure of the autoradiograms (data not shown).

To determine the length of the *tviA* transcript, Northern blots of total RNA from *S. typhi* Ty2 and *E. coli* MC1061 harboring pVT1 were hybridized to a *tviA* intragenic probe generated by PCR amplification with primers BG and AG (Fig. 1). Two transcripts of approximately 650 and 800 nucleotides (nt) were observed within the resolution of the Northern blot experiments (data not shown). This result did not preclude the possibility that one or both of the transcripts arose by

processing of a longer transcript not detected in our Northern blot assay. Indeed, a longer transcript was expected, since at least *tviA* and *tviB* were cotranscribed in the presence of the TviA and RcsB products (42). Therefore, blots of total RNA prepared from *S. typhi* Ty2 and *E. coli* MC1061 harboring plasmid pVT21, which carried *tviA* and the 5′ end of *tviB* (Table 1), were hybridized to the BG-AG probe. As shown in Fig. 3A, three transcripts of approximately 1,300, 800, and 650 nt were observed. As these three transcripts were evident in all experiments with different RNA preparations, it was unlikely that their detection was due to artifacts associated with Northern blots.

The size of the 1,300-nt transcript correlated with the calculated length of 1,465 nt of a run-off transcript originating at the transcriptional start point (bp 639) of *tviA* mRNA and terminating at the *Cla*I restriction site of plasmid pVT21. To test this possibility, Northern blots of total RNA from *S. typhi* Ty2 and *E. coli* MC1061 harboring pVT21 were hybridized to a second probe generated by PCR with primers AI and C (Fig. 1). This probe contained part of the *tviA-tviB* intergenic region and the 5′ end of *tviB*. As expected, only one transcript of 1,300 nt that hybridized to this probe was detected (Fig. 3B). This result demonstrated that the 1,300-nt transcript corresponded to a polycistronic *tviAB′* mRNA.

The size of the 800-nt transcript correlated with the length of a transcript originating at the transcriptional start point of *tviA* mRNA and terminating in the *tviA-tviB* intergenic region. Indeed, a search for possible secondary structure in the *tviA-tviB* intergenic region revealed a dyad symmetry (from bp 1469 to bp 1489) which might form a putative hairpin structure, with a free energy of formation of $\Delta G = -15$ kcal mol^{-1}. Transcription starting at the *tviA* promoter and terminating at this inverted repeat would give rise to a run-off transcript of about 830 nt. This size corresponded well to that of the 800-nt transcript visible in Fig. 3A. The lower intensity of the 650-nt band probably reflected the fact that it was a degradation product.

Because the TviA and RcsB proteins were shown to be required for *tviB* gene transcription, we further determined whether the 1,300-nt transcript was present in RNA extracted from *E. coli* MC1061 harboring plasmid pVT42. Plasmid pVT42, a derivative of plasmid pVT21 in which the *tviA* coding sequence was disrupted by filling in the *Bgl*II site (Table 1), did not express TviA protein (42). The labelled DNA probe was the BG-AG fragment. The autoradiograph (Fig. 3C) showed that only the 800-nt transcript, not the 1,300-nt transcript, was detected in the absence of the TviA protein in our experimental conditions. The presence of the 650-nt band in the absence of the 1,300-nt transcript indicated that it might correspond to a degradation product of the 800-nt transcript. Furthermore,
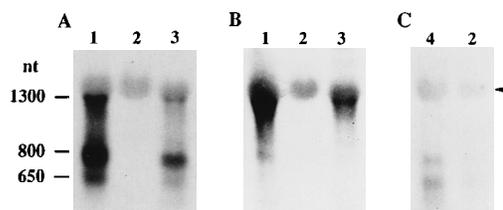

FIG. 3. Northern blot analysis of *tviA* and *tviAB* transcripts. Total RNA isolated from *E. coli* MC1061 carrying pVT21 (lane 1), *E. coli* MC1061 carrying pGB2 (lane 2) as a control, *S. typhi* Ty2 carrying pVT21 (lane 3), and *E. coli* MC1061 carrying pVT42 (lane 4) was hybridized to a ^{32}P-labelled probe generated by PCR amplification with primers BG and AG (A and C) or primers AI and C (B). The lengths of transcripts are indicated in the left margin. The size of each mRNA was determined by using a commercial RNA ladder. The position of 16S rRNA is indicated by the arrowhead.

TABLE 3. Expression of *tviA'-lacZ*, *tviA-lacZ*, and *tviAB'-lacZ* transcriptional fusions in *E. coli* MC1061 and MC1061(*rcsB*)

Plasmid(s)	Relevant characteristics	β-Galactosidase activity (Miller units)	
		MC1061	MC1061(*rcsB*)
pQF50		<1	<1
pNAG	*tviA'-lacZ*	451 ± 6	327 ± 10
pNAG + pVT21	*tviA'-lacZ* + *tviA*	4,205 ± 168	324 ± 8
pNAG + pRcsB	*tviA'-lacZ* + *rcsB*$_{Ty}$	650 ± 69	437 ± 34
pNAGF	*Bgl*II site of *tviA* filled in on *tviA'-lacZ*	248 ± 27	214 ± 44
pNAGF + pVT21	*Bgl*II site of *tviA* filled in on *tviA'-lacZ* + *tviA*	4,273 ± 152	209 ± 10
pNAC	*tviA-lacZ*	521 ± 76	6 ± 1
pNAC + pVT21	*tviA-lacZ* + *tviA*	388 ± 53	4 ± 1
pNAC + pRcsB	*tviA-lacZ* + *rcsB*$_{Ty}$	355 ± 30	364 ± 23
pNACF	*Bgl*II site of *tviA* filled in on *tviA-lacZ*	3 ± 2	5 ± 1
pNACF + pVT21	*Bgl*II site of *tviA* filled in on *tviA-lacZ* + *tviA*	517 ± 47	5 ± 1
pNO	*tviAB'-lacZ*	665 ± 16	4 ± 2
pNO + pVT21	*tviAB'-lacZ* + *tviA*	781 ± 87	4 ± 1
pNO + pRcsB	*tviAB'-lacZ* + *rcsB*$_{Ty}$	389 ± 37	218 ± 5
pNOF	*Bgl*II site of *tviA* filled in on *tviAB'-lacZ*	6 ± 1	4 ± 1
pNOF + pVT21	*Bgl*II site of *tviA* filled in on *tviAB'-lacZ* + *tviA*	429 ± 39	11 ± 1

comparison of the intensity of signals obtained for the 800-nt transcript in Fig. 3A and C suggested a decrease in *tviA* mRNA in the absence of the TviA protein. Similarly, when blots were performed with RNA extracted from *E. coli* MC1061(*rcsB*) carrying plasmid pVT21 and hybridized with the BG-AG probe, only the 800-nt transcript was detected (data not shown).

Together, these results demonstrated that the same transcriptional start point of *tviA* mRNA was detected in the presence and absence of the TviA and RcsB proteins. In addition, they showed that transcription initiated at bp 639 gave rise to a monocistronic *tviA* mRNA independently of the presence of the TviA and RcsB$_{Ty}$ proteins. However, the amount of *tviA* mRNA was increased in TviA- and RcsB-expressing strains. Finally, they strongly suggested that cotranscription of the *tviAB* genes from the *tviA* promoter occurred only in the presence of functional TviA and RcsB proteins.

Expression of cloned *tviA'-lacZ*, *tviA-lacZ*, and *tviAB'-lacZ* fusions in *E. coli* MC1061 and its *rcsB* mutant. To confirm the interpretation of the Northern blot analysis, we first examined expression of the *tviA'-lacZ* transcriptional fusion from plasmid pNAG (Table 1) in *E. coli* MC1061 and its *rcsB* mutant. The results are reported in Table 3. The *tviA'-lacZ* fusion was expressed at a similar level in both strains. Overproduction of TviA did not have any effect on transcription at the *tviA* promoter in the absence of RcsB. Similarly, expression of *rcsB* either from the *E. coli* MC1061 chromosome or from the multicopy plasmid pRcsB did not have any significant effect on *tviA* transcription in the absence of TviA. In contrast, providing *tviA* in *trans* on plasmid pVT21 increased expression of the *tviA'-lacZ* fusion in *E. coli* MC1061 10-fold. These results showed that *tviA* was transcribed at a significant basal level in the absence of TviA or RcsB and that *tviA* transcription was enhanced when both of these proteins were present.

We repeated the assays after filling in the *Bgl*II restriction site located in the *tviA* coding sequence on plasmid pNAG (Fig. 1) because the results reported below were based on such constructions. This genetic manipulation had less than a two-fold effect on expression of the *tviA'-lacZ* fusion (Table 3).

To investigate the role of the TviA and RcsB proteins on *tviAB* transcription, we then constructed plasmid pNAC(*tviA-lacZ*), in which the promoterless *lacZ* gene was located immediately downstream of the putative hairpin structure located in the *tviA-tviB* intergenic region, and plasmid pNO(*tviAB'-lacZ*),

in which the promoterless *lacZ* gene was located downstream of the ATG start codon of *tviB* (Table 1 and Fig. 1). As shown in Table 3, the *tviA-lacZ* and *tviAB'-lacZ* fusions clearly displayed *tviA*- and *rcsB*-dependent transcription. In *E. coli* MC1061 carrying pVT21, about 400 U of β-galactosidase were expressed from plasmid pNAC, and about 800 β-galactosidase units were expressed from plasmid pNO. In the absence of TviA or RcsB, the *tviA-lacZ* and *tviAB'-lacZ* fusions were not expressed. These results provided proof that transcription initiated at the *tviA* promoter proceeded across the putative hairpin structure into *tviB* only in the presence of the TviA and RcsB proteins, thereby confirming the Northern blot analysis. They also corroborated and extended our previous data obtained with a *lacZ-cat* cartridge recombined into the chromosome of *S. typhi* (42).

Effect of the TviA protein in an *E. coli* *lon* *rcsA* mutant. Colanic acid synthesis in *E. coli* is normally regulated at the transcriptional level by RcsA and RcsB. The RcsA positive regulator is a natural substrate for the *E. coli* Lon protease. The results reported above indicated that TviA, instead of RcsA, was involved in conjunction with RcsB in Vi antigen expression in *S. typhi*. It was of interest, therefore, to examine the effect of *tviA* in a *lon* *rcsA* mutant background.

Plasmid pNO (*tviAB'-lacZ*) was transformed into the *rcsA* *lon* mutant *E. coli* strain SG21034 (Table 1). The resulting strain remained nonmucoid, indicating that the TviA product was not functionally homologous to the RcsA protein, the presence of which in multicopy conferred a mucoid phenotype on *E. coli* SG21034. In addition, the *tviAB'-lacZ* fusion was expressed at a similar level in *E. coli* SG21034 (414 ± 51 Miller units) and in the parental strain *E. coli* SG20250 (468 ± 67 Miller units). This latter result showed that TviA was insensitive to the *lon* proteolytic system.

Genetic data suggesting an interaction between TviA and RcsB. The results reported above led us to first consider the possibility that the basal level of *tviA* transcription might provide the necessary amount of TviA to activate *rcsB* transcription. For this purpose, we constructed plasmid pRcsBZ, which carried a transcriptional *rcsB'-lacZ* fusion in pQF50 (Table 1). *E. coli* MC1061 and *E. coli* MC1061(*rcsB*) containing pRcsBZ expressed 117 ± 12 and 46 ± 13 Miller units of β-galactosidase, respectively. These β-galactosidase levels were not affected in the presence of pVT21 (123 ± 8 and 43 ± 8 Miller

units, respectively). Thus, it was unlikely that the role of TviA was to initiate *rcsB* transcription.

An interaction between TviA and RcsB was further suggested by the following observations. The original *viaA* mutant *S. typhi* T643WSR did not express Vi antigen even though it carried a functional *viaB* locus (25). In particular, the *tviA* gene was shown to be functional in T643WSR (42). This strain might be considered an $rcsB_{Ty}$ mutant because it was complemented by $rcsB_{Ec}$ (19) or $rcsB_{Ty}$, as reported above, on a multicopy plasmid. Moreover, a high copy number of *tviA* restored a Vi-positive phenotype to *S. typhi* T643WSR (42). In contrast, the presence of *tviA* in multicopy on plasmid pVT21 (Table 1) did not restore a Vi-positive phenotype to *S. typhi* Ty2(*rcsB*) (Table 2). This discrepancy prompted us to identify the defect in the *rcsB* gene of *S. typhi* T643WSR. This gene was cloned in vector pGB2 after PCR amplification with oligonucleotides 5′-GAA AGA TGC TCC AGG TAT AG-3′ and 5′-GAC GCT GGC GGT GTA TGC CG-3′, which mapped at bp 83 to bp 102 and bp 967 to bp 948, respectively, on the $rcsB_{Ty}$ sequence. Complete sequencing of the insert was performed on four recombinant plasmids, designated pRcsB*. Compared with the $rcsB_{Ty}$ sequence from strain Ty2, the same point mutation was identified in the $rcsB_{Ty}$ coding sequence from strain T643WSR on the four pRcsB* plasmids. This mutation was a G to A transition at bp 695 which altered the Arg-177 residue to a His. This change is located in the DNA-binding motif of the RcsB product. Hereafter, this altered form of the $rcsB_{Ty}$ gene and RcsB protein is designated $rcsB*_{Ty}$ and RcsB*, respectively.

The presence of pRcsB* did not confer a Vi-positive phenotype on either strain Ty2(*rcsB*) or strain T643WSR (Table 2). To demonstrate that RcsB* protein was expressed but could positively control Vi antigen expression only when TviA was overexpressed from a multicopy plasmid, pNO, which encoded TviA (Table 1), was further transformed into *S. typhi* Ty2(*rcsB*) carrying pRcsB*. As shown in Table 2, the resulting strain exhibited a Vi-positive phenotype. These results indicated that the $rcsB*_{Ty}$ gene encoded an altered form of RcsB, the activity of which was conditional to overexpression of TviA protein. In addition, they strongly suggested that TviA might act in concert with RcsB to promote expression of the genes involved in Vi antigen synthesis in *S. typhi*. If TviA interacts with RcsB to promote cotranscription of *tviA* and *tviB*, and possibly of other *viaB*-associated downstream genes, it is tempting to speculate that TviA is required either for activation of RcsB bound at the *tviA* promoter or for DNA binding by RcsB. Characterization of the $rcsB*_{Ty}$ mutation in *S. typhi* T643WSR favors the latter possibility, although no direct binding of RcsB to DNA has been demonstrated yet.

In conclusion, three other proteins have been shown to stimulate capsular polysaccharide synthesis in concert with the RcsB protein. RcsA is a protein of 207 aa residues with a calculated pI of 9.9, which contains a DNA-binding domain in the C-terminal domain (37). RcsF is a protein of 133 aa residues with a calculated pI of 10.8, which requires RcsA to increase colanic acid synthesis in *E. coli* (15). MucZ is a *Coxiella burnetti* protein of 270 aa residues with a calculated pI of 10.5, which induces capsule expression in an *E. coli rcsA* null mutant (44). Similarly, TviA is a protein of 170 aa residues with a calculated pI of 10.2, which enhances Vi polysaccharide synthesis in *S. typhi* (17, 42, 43). TviA, RcsF, and MucZ do not contain a DNA-binding motif and are not subject to Lon-dependent degradation. We could not detect any significant homology between TviA, RcsA, RcsF, and MucZ. However, these four small proteins share the characteristic of being highly basic. These proteins might form part of a family of

bacterial proteins that interact directly or indirectly with the response regulator RcsB to enhance capsular polysaccharide synthesis.

Direct analysis of transcripts by DNA-RNA hybridization showed that transcription of *tviB* was initiated at the promoter of the preceding gene, *tviA*. From comparison of the signal intensity obtained for the 1,300- and 800-nt transcripts in Fig. 3A, it might be roughly estimated that about half of the transcripts initiated at the *tviA* promoter were terminated in the intercistronic region between *tviA* and *tviB*, where a terminating stem-loop structure might be formed. As confirmed by the *lacZ* fusion data (Table 3), transcription was strongly attenuated at that point. This suggested that the *tviB* gene was less actively transcribed than the *tviA* gene. If so, such an attenuation mechanism would allow the desired amount of readthrough of *tviB* and downstream genes and thus ensure balanced production of the polypeptides involved in Vi polysaccharide synthesis and transport. Further studies of transcriptional and posttranscriptional events associated with the *viaB* locus should allow a more detailed understanding of the complex regulatory mechanisms involved in Vi antigen expression in *S. typhi*.

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