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virG, a Plasmid-Coded Virulence Gene of *Shigella flexneri*: Identification of the *virG* Protein and Determination of the Complete Coding Sequence

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On the 230-kilobase-pair (kb) virulence plasmid of *Shigella flexneri* 2a strain YSH6000, at least seven separate genetic determinants have been identified. One of them, an approximately 4-kb region, *virG*, that is required for the Sereny reaction, was extensively studied to examine the role of the *virG* region. The phenotype of a VirG$^-$ mutant (M94) of YSH6000 in the cytoplasm of cultured MK cells was characterized by a kinetic study of the invading shigellae. The observed phenotype of M94 in the cytoplasm indicated that the *virG* locus is not required for multiplication of the invading shigellae, but is essential for their spread to adjacent cells. The DNA region necessary for the VirG function was localized to a 3.6-kb DNA sequence on the 230-kb plasmid. A 130-kilodalton polypeptide was confirmed to be the *virG* product. External labeling of bacteria with ^{125}I indicated that the 130-kilodalton *virG* protein is exposed on the bacterial surface. The nucleotide sequence of 4,472 bp, which contains the functional *virG* gene and its own regulatory sequence, was determined, and a large open reading frame encoding 1,102 amino acid residues was identified.

Shigellae cause bacillary dysentery in humans and monkeys. The early essential step leading to dysenteric symptoms is invasion of colonic epithelial cells (8), followed by intracellular bacterial multiplication and spread of invading bacteria to adjacent cells. Strains that lack any one of these properties are avirulent as measured by the Sereny test (25). At least three separate chromosomal loci have been identified genetically to be virulence determinants of *Shigella flexneri* (4–6, 19). In addition, a large plasmid (approximately 230 kilobase pairs [kb]) is required for invasion of colonic epithelial cells (19, 20).

By random Tn*5* insertions we have identified at least seven separate virulence-associated genetic regions on the 230-kb plasmid (pMYSH6000) of *S. flexneri* 2a strain YSH6000 (22, 24). Five contiguous regions making up a 31-kb DNA segment were associated with inducing invasion (22). A 786-bp *virF* gene, originally cloned in *Escherichia coli* K-12 by selecting for the ability to bind Congo red (15, 16), was recently shown to be a positive regulator for expression of at least four virulence-associated plasmid-encoded immunogenic polypeptides of 130, 57, 43, and 39 kilodaltons (kDa) (17). Finally, the *virG* region, of approximately 4 kb, has been shown to be essential for a positive Sereny test (10, 24).

Hale et al. (7) identified at least seven plasmid-encoded, virulence-associated peptides produced by *S. flexneri* 2a and 5 and enteroinvasive *E. coli* O143. By immunoblot analysis of extracts of whole cells, four peptides of 78, 57, 43, and 39 kDa were recognized by convalescent-phase monkey antisera. These workers proposed that these proteins function as components of the invasion phenotype and are expressed on the bacterial surface when grown at 37°C. Oaks et al. (13) identified an additional plasmid-encoded large surface peptide of approximately 140 kDa which was also specifically recognized by some convalescent-phase human and monkey sera.

To identify the genetic regions encoding those peptides, Maurelli et al. (11) shotgun cloned partial *Sau*3A digests of the large plasmid pWR100 into a high-copy cosmid vector, pJB8, which was subsequently introduced into a plasmid-free strain of *S. flexneri* 2a. A resulting clone, containing a 37-kb minimum sequence necessary for invasion, produced the four immunoproteins of 78, 57, 43, and 39 kDa (11). DNA fragments coding for the three immunogenic peptides of 57, 43, and 39 kDa were cloned into a λ expression vector by Buysee et al. (2), and the genetic determinants for the virulence-associated immunogenic peptides were localized on a 9-kb region by use of overlap of the cloned fragments (1, 2). However, nothing further is known about the genetic region encoding the 140-kDa antigen or about the association of the large antigen with the virulence of shigellae.

In this communication, we have extended our previous studies (10, 17, 24) to localize the *virG* gene and its 130-kDa protein product. We have determined a 4,472-bp DNA sequence which contained a 3,306-bp open reading frame and also the regulatory sequence for the gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. flexneri* 2a strain YSH6000 (23) and the 230-kb plasmid-free derivative, YSH6200 (15), were routinely used as positive and negative controls, respectively, for virulence assays. M94, a Tn*5*-induced VirG$^-$ mutant of YSH6000 (10), was used as a representative VirG$^-$ mutant of YSH6000. pMY6003 is a trimethoprim- and tetracycline-resistant derivative of pBR322 (10). pMYSH6601 (abbreviated as 103 in Fig. 2) is pMY6003 into which was cloned the 6.1-kb *Sal*I-*Eco*RI fragment that encodes the *virG* gene (10).

Media and bacterial growth conditions. Cultures grown overnight at 37°C in LN broth (21) were diluted 50-fold with brain heart infusion medium (Difco Laboratories, Detroit,

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Mich.) and shaken at 37°C for 2 h. These freshly prepared bacterial cultures were used for virulence assays.

Sereny test and focus plaque assay. The methods for the Sereny test (25) and the focus plaque assay (14, 22) were described previously.

Measurement of bacterial multiplication in the cytoplasm and the percentage of infected cells. The cultured rhesus monkey kidney epithelial (MK) cells (26) were infected with bacteria at a multiplicity of infection of ca. 100, centrifuged at 350 × *g* for 10 min, and then incubated by standing at 37°C for 40 min in an atmosphere containing 5% CO_2. The cells were then washed twice with Hanks balanced salt solution, the medium was changed to Eagle minimal essential medium containing gentamicin (100 μg/ml), and the cells were then incubated for additional periods of 1, 2, 3, 5, 8, or 18 h. Monolayers were stained with Giemsa solution, and invading shigellae in the cytoplasm were counted by light microscopy. To count the number of bacteria in the cytoplasm of MK cells, we chose a total of 10 MK cells from each of four monolayers at random. To calculate the percentage of infected cells, we chose five microscope fields (×400) at random and determined the percentage by scoring cells invaded by at least five bacteria.

External labeling of bacteria by iodination. Peptides exposed on the surface of shigellae were labeled by the lactoperoxidase-catalyzed iodination method. A 2.5-ml preparation of freshly grown bacteria was washed and suspended in 1 ml of 0.05 M phosphate buffer (pH 7.2) (a total of approximately 10^9 cells). To the 1-ml bacterial suspension were added sequentially 800 mU of glucose oxidase (Boehringer Mannheim, Penzberg, Federal Republic of Germany), 80 mU of lactoperoxidase (Calbiochem-Behring, La Jolla, Calif.), 20 μCi of $Na^{125}I$ (Amersham Corp., Amersham, United Kingdom), and 2 mg of glucose. Iodination was performed at room temperature for 30 min and terminated by the addition of 20 μl of 2% NaN_3. After being washed twice with 1 ml of phosphate buffer containing 10^{-5} M NaI, the labeled bacteria were suspended in 50 μl of a lysing solution (9) and lysed by boiling, and a 15-μl portion was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Analysis of plasmid-encoded proteins. The minicell method was used to analyze plasmid-encoded proteins as described previously (16).

DNA sequence determination. The dideoxynucleotide procedure of Sanger et al. was used for DNA sequence determination (18). To eliminate ambiguities in the sequence, we used 7-deaza-dGTP (Takara Shuzo Co., Kyoto, Japan) in place of dGTP during the sequencing reaction, as described by Mizusawa et al. (12). The nucleotide sequence determination was carried out on both DNA strands of the 4,472 bp in the VirG region.

Primer extension analysis. For primer extension analysis, a synthetic oligonucleotide, 5'-CGTAGGTAATTCTCCG-3', was mixed with RNA prepared from YSH6700 (M94 carrying pMYSH6601) (50 ng of primer to 8.0 μg of RNA), and 10 U of avian myeloblastosis virus reverse transcriptase was added to a 10-μl reaction mixture containing 50 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$, 20 mM KCl, 5 mM dithiothreitol, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, and 0.33 μM [^{32}P]dCTP (3,000 Ci mmol^{-1}) (Amersham). The reaction was carried out at 42°C for 30 min and was followed by addition of unlabeled dCTP to 30 μM and further incubation for 20 min. One-eighth of the reaction mixture was analyzed on an 8% polyacrylamide–7 M urea sequencing gel along with the product of a Sanger sequencing reaction generated by using the same primer and the 1.6-kb *Hin*cII-(leftmost)-*Hpa*I(leftmost) fragment (see Fig. 2) single-strand template.

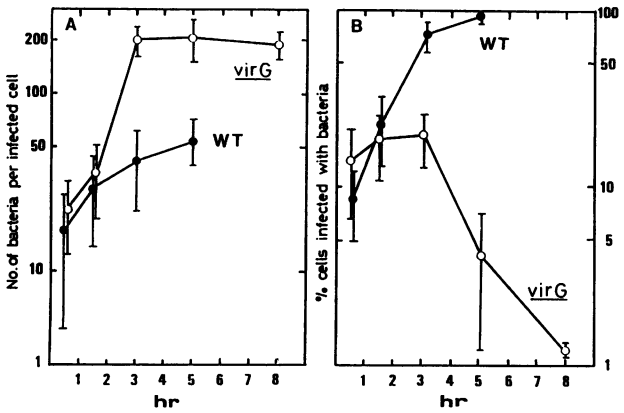


FIG. 1. Kinetics of invading YSH6000 and M94 in the cytoplasm of MK cells. (A) Kinetics of the multiplication of invading bacteria in the cytoplasm of MK cells. The number of bacteria in the cytoplasm at each time interval is shown (mean and standard deviation). (B) Kinetics of the percentage of infected cells. Cells invaded by more than five bacteria were considered to be infected. Symbols: ●, YSH6000 (wild type); ○, M94 (VirG$^-$).

RESULTS

Phenotype of the VirG$^-$ mutant (M94) in the cytoplasm of epithelial cells. The numbers of bacteria in the cytoplasm of MK cells infected with either YSH6000 or M94 and the population of cells infected were measured at 1, 2, 3, 5, and 8 h by microscopic observation. Up to 1 h after invasion, the shapes of invading bacteria and the numbers of M94 and YSH6000 bacteria were apparently similar. At 3 h after infection, YSH6000 had spread freely within the cytoplasm and sometimes into adjacent cells. Also, the number of M94 bacteria per cell was higher than the number of YSH6000 bacteria in the cytoplasm. The apparent increase in the number of M94 bacteria in the cytoplasm was also greater than that of YSH6000. However, M94 was confined to the cytoplasm and was converted to a spherical shape (10), with the number of bacteria at around 200 per cell (Fig. 1A). In contrast, while the proportion of cells infected by YSH6000 increased up to 100% by 5 h, the proportion of cells infected with M94 did not increase after 3 h and decreased thereafter, presumably owing to peeling off of the infected cells (Fig. 1B). These observations support the premise that the function of VirG is related to the ability of the bacteria to survive and/or spread within the cytoplasm.

Determination of the minimum coding region for the VirG phenotype. To determine the minimum DNA sequence necessary for the VirG phenotype, we performed an insertion and deletion analysis of this sequence (Fig. 2). Each mutated DNA ligated to pMY6003 was introduced into M94, which was then tested for the VirG phenotype by the Sereny test and the focus plaque assay (14, 22). The functional VirG region lies between the leftmost *Hin*cII site and the *Xma*I site (Fig. 2).

To examine whether the VirG region consists of a single cistron, we introduced each of the five avirulent linker- or fill-in mutants (M2, 1526, 620, 1474, and M6) into each of six Tn*5* insertion VirG$^-$ mutants (10). None of the complementation tests among mutants resulted in a positive Sereny

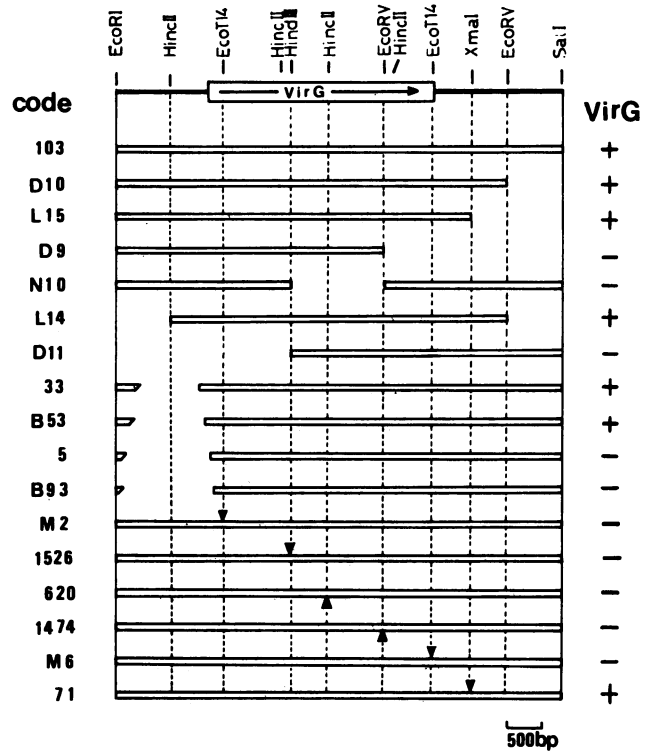


FIG. 2. Physical and genetic map of the VirG region. Shown is a restriction map of the 6.1-kb *Eco*RI-*Sal*I fragment of pMYSH6601 (abbreviated 103). The open box at the top line represents the DNA region encoding the VirG function, as determined by insertion and deletion mutations and by the nucleotide sequence (see Fig. 5). The open box in each line represents the DNA region remaining in each mutant derivative of 103. The endpoints of the four BAL 31-generating deletions, 33, B53, 5, and B93, were determined by DNA sequencing (Fig. 5). Symbols: ▼, ▲, fill-in and linker insertion mutations, respectively.

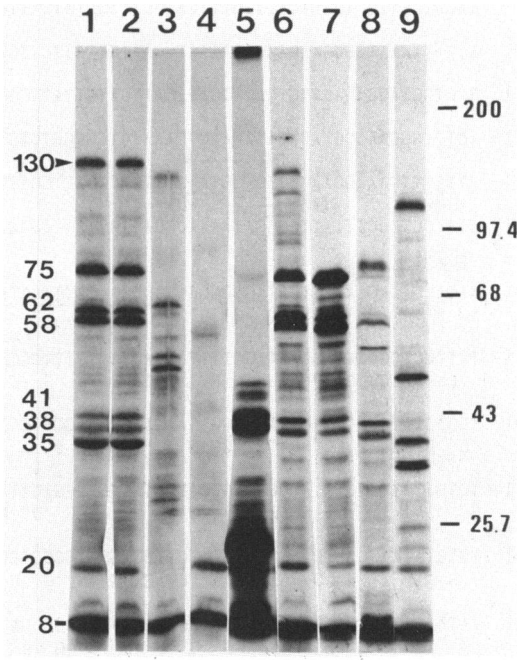


FIG. 3. Minicell analysis of peptides expressed by the VirG region. Polypeptides of either deletion, linker insertion, or fill-in mutants were labeled with [^{35}S]methionine and electrophoresed in an SDS–12.5% polyacrylamide gel. Samples were applied to the gel as follows: lane 1, 103; lane 2, L15; lane 3, D9; lane 4, N10; lane 5, D11; lane 6, M2; lane 7, 1526; lane 8, 620; lane 9, 1474 (Fig. 2). The numbers shown on the left were the apparent molecular masses of the major products of 103 (in kilodaltons). The arrowhead indicates the 130-kDa peptide and the solid bar indicates the 8-kDa trimethoprim-resistant dihydrofolate reductase expressed by the vector pMY6003 (16).

reaction (data not shown), suggesting that the VirG region contains a single cistron.

Identification of the proteins encoded by the *virG* gene. To identify the *virG* product(s), minicells carrying pMYSH6601 (10, 17) or its deletion, linker insertion, or fill-in derivatives were prepared (Fig. 2) and labeled with [^{35}S]methionine, and the polypeptides were fractionated on SDS-polyacrylamide gels. At least nine polypeptides, which migrated at apparent molecular masses of 130, 75, 62, 58, 41, 38, 35, 20, and 8 kDa were expressed from the two VirG$^+$ chimeric plasmids, 103 (pMYSH6601) and L15 (Fig. 3). However, the smallest peptide (8 kDa) in each lane shown was the product of the trimethoprim-resistant dihydrofolate reductase expressed by the vector used (pMY6003 [16]). None of the other peptides was seen in the pMY6003 vector when a foreign DNA was inserted into the tetracycline resistance gene (16). In contrast, a VirG$^-$ derivative (D11) with a deletion between *Eco*RI and *Hin*dIII did not express the four largest peptides. In another VirG$^-$ strain, N10, a 1.4-kb *Hin*dIII-*Eco*RV(left) deletion derivative, most of the peptides also disappeared. D9, a 2.6-kb *Eco*RV(left)-*Sal*I deletion derivative, showed a protein profile of the four largest peptides, similar to that observed with the two VirG$^+$ plasmids, 103 and L15, although the four proteins uniformly decreased in size by approximately 10 kDa (Fig. 3, lane 3). A fill-in mutant (M2) with a 4-bp insertion at the leftmost *Eco*T14I produced seven of the peptides (75, 62, 58, 41, 38, 20, and 8 kDa) but not the

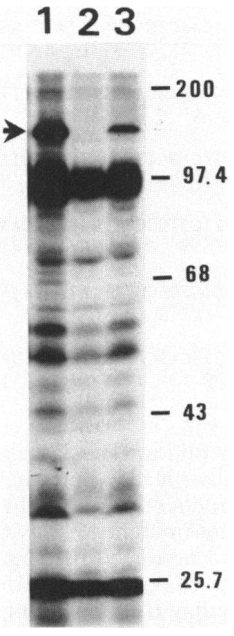
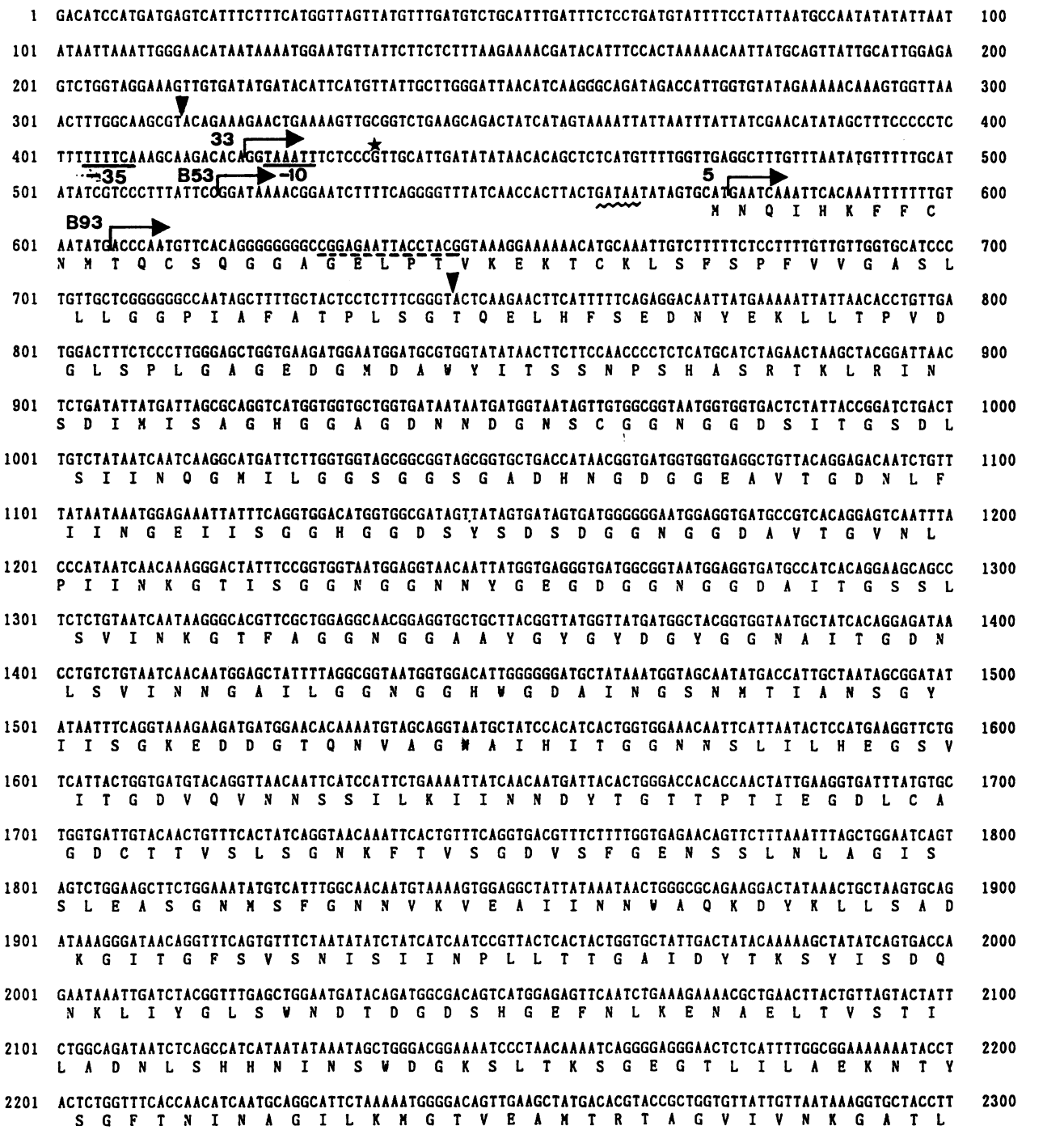


FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins of three strains, YSH6700 (M94 carrying pMYSH6601) (VirG$^+$) (lane 1), M94 (VirG$^-$) (lane 2), and YSH6000 (wild type) (lane 3), subjected to radioiodination as described in the text. The arrow indicates the VirG protein.



130- and 35-kDa peptides. The largest peptide expressed from M2 was slightly smaller than the largest band of the 130-kDa peptide produced by both 103 and L15 (lane 6). Another fill-in mutant (mutant 1526) with a 4-bp insertion at the *Hin*dIII site also produced the same seven peptides as those produced by M2 (lane 7). However, in a linker insertion mutant (mutant 620), only four peptides (41, 38, 20, and 8 kDa) were expressed (lane 8), and in another linker insertion mutant (mutant 1474), the two smallest peptides only were produced (lane 9). These results suggest that the proteins of 130, 75, 62, 58, 41, and 38 kDa are probably translated from left to right and may indicate that the six peptides are encoded by the same open reading frame. Thus, it would appear that the 130-kDa protein is large enough to encompass the region of *virG* defined by the mutations shown in Fig. 2.

The 130-kDa virG protein is a surface-exposed antigen. Since a 130-kDa polypeptide has been shown to be an immunogenic polypeptide encoded by the VirG region (17), it was of interest to examine whether the *virG* product exists as a surface-exposed or cell-free form. To determine the site of this protein, we performed a simple enzyme labeling experiment on intact *Shigella* strains (see Materials and Methods). After labeling, the intact cells were washed,

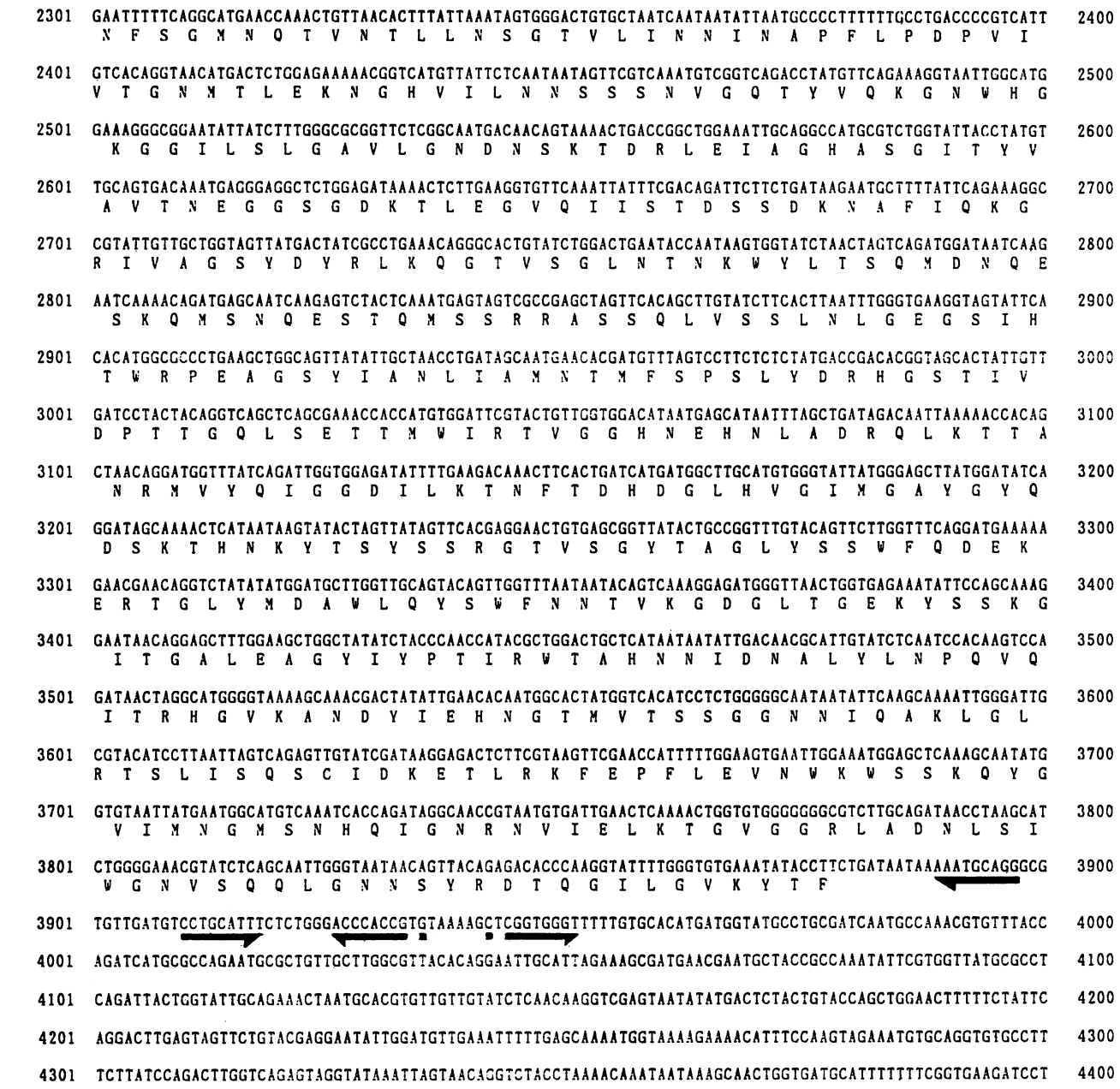


FIG. 5. Nucleotide sequence of the VirG region. The sequence of the sense strand is shown, as well as the amino acid code for the open reading frame corresponding to the VirG protein. The sequence is numbered from the *Hin*cII(left) to the *Xma*I sites (Fig. 2). Arrows over the sequence point to the region of DNA that remains in the four BAL 31-generated deletion mutants (Fig. 2). The −10 and −35 regions of the possible promoter are indicated. Symbols: ∿∿, Shine-Dalgarno sequence; ★, transcriptional startpoint; →, possible terminator structure; ----, region homologous to the 16-mer primer used in the primer extension experiment (Fig. 6); ▼, relevant *Rsa*I sites (GTAC).

suspended in lysis buffer, and boiled, and the labeled products were analyzed on SDS-polyacrylamide gels. The 130-kDa protein was labeled by this procedure, whereas the corresponding band was not observed at all in the VirG$^-$ mutant, M94 (Fig. 4, lane 2), indicating that the *virG* gene produces the 130-kDa surface-exposed peptide.

Determination of the nucleotide sequences and the promoter region for the *virG* gene. The sequence of the 4,472-bp region encompassing the *virG* gene was determined (Fig. 5). The largest open reading frame runs from the ATG at position 574 to position 3880, corresponding to a protein of 1,102 amino acids with a molecular mass of 117 kDa. Since a short open reading frame with ATG at position 604 was found, a number of deletions from the *Hin*cII(leftmost) site were generated by BAL 31 digestion and tested for VirG function as well as for the protein products by the minicell method (data not shown). On the basis of the sequencing endpoints of the four deletion derivatives, 33 (VirG$^+$), B53 (VirG$^+$), 5 (VirG$^-$), and B93 (VirG$^-$) (Fig. 2 and 5), which defined the minimum *virG* region, only the largest 3,306-bp open reading

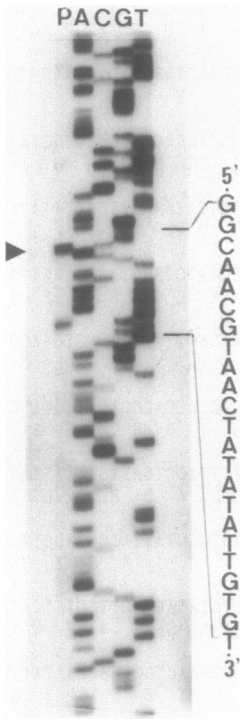


FIG. 6. Primer extension analyses. A synthetic 16-mer oligonucleotide complementary to the sequence from positions 630 through 645, which corresponds to the sequence from positions 57 through 72 from the N-terminal end of the *virG* gene (Fig. 5), was used. The major product is marked by an arrowhead. Lanes G, A, T, and C are sequencing ladders generated by using the same primer and the 1.6-kb *Hin*cII(leftmost)-*Hin*cII(the next) fragment. P is the product(s) extended from the primer along the complementary sequence across the 5′ end of the mRNA indicated beside the gel.

frame could be relevant to this phenotype. To further confirm the correct orientation of the *virG* gene and localize the promoter sequences, the 430-bp *Rsa*I fragment (Fig. 5, arrowheads) that contains the 168-bp N-terminal DNA sequence of the *virG* gene was inserted into a restriction site located just before the promoter-deficient chloramphenicol resistance gene (*cat*) of pKK232-8 (3) in both orientations. In the correct orientation, the *Rsa*I fragment produced a high level of chloramphenicol resistance (200 μg/ml), suggesting that a functional promoter sequence exists within the 261-bp region before the large open reading frame for the *virG* gene. To confirm this, we performed a primer extension RNA sequencing on total RNA prepared from M94 harboring pMYSH6601. For this purpose, a primer that hybridizes to mRNA sequences within the coding region for the *virG* gene, comprising the 16 bases from positions 630 through 645, was used. A major extension product ended at the G residue at position 435 that is 138 bp away from the open reading frame for the *virG* gene (Fig. 5 and 6).

DISCUSSION

To characterize the role of the VirG region, we examined the kinetics of invasion by M94 and YSH6000 in the cytoplasm of MK cells (Fig. 1). M94 (VirG$^-$) was shown to multiply slightly faster than YSH6000 in MK cells. Interestingly, this apparent difference in increase of bacterial number in the cytoplasm has always been observed in Inv$^+$ Ser$^-$ mutants of YSH6000, such as KcpA$^-$ mutants (M. Yamada, C. Sasakawa, N. Okada, S. Makino, and M. Yoshikawa, Mol. Microbiol., in press), KcpB$^-$ mutants (manuscript in preparation), and the VirG$^-$ mutant compared with the Inv$^+$ Ser$^+$ wild type. Since the growth rates of YSH6000 and M94 were essentially the same under standard in vitro growth conditions at 37°C (24), the observed apparently different increases in bacterial numbers in the cytoplasm (Fig. 1A) may not be due to the different bacterial growth rates, but are probably due to the different behaviors of the two strains in the cytoplasm. The apparently smaller number of YSH6000 bacteria than M94 bacteria in the cytoplasm may be a reflection of the ready spread of YSH6000 to adjacent cells.

The 230-kb plasmid of *S. flexneri* has been shown to produce at least six plasmid-encoded polypeptides designated a (78 kDa), b (57 kDa), c (43 kDa), d (39 kDa), e (24 kDa), and f (20 kDa) (7). In addition, one large (approximately 140-kDa) plasmid-encoded polypeptide has been noted as an immunogenic peptide that was recognized in immunoblots by convalescent-phase sera of patients with shigellosis or of monkey challenged with shigellae (13). The 140-kDa immunopeptide, however, was recognized by only half of the convalescent-phase sera, while the other three virulence-associated immunogenic peptides (57, 43, and 39 kDa) were almost always recognized (13). Even though the biological implications of antibody-eliciting plasmid-encoded polypeptides are not yet clear, the different response to these immunogenic peptides in sera may be a reflection of the different cellular locations or the nature of the peptides themselves. The VirG protein is localized on the surface of bacteria in a conformation that can be labeled externally by ^{125}I. Perhaps a surface-exposed region acts as both the antigen and the factor promoting intercellular spread. Clearly, several other proteins labeled by this procedure (Fig. 4) may be exposed on the bacterial surface, but they are not relevant to this study.

A series of genetic and biochemical experiments indicated that the VirG region is a single transcriptional unit encoding a single gene for a polypeptide comprising 1,102 amino acids. This is based on the lack of complementation between a series of Tn*5*-induced *virG* mutants (10) and linker insertion or fill-in mutants, as well as the identification by SDS-polyacrylamide gel electrophoresis of a 130-kDa peptide that is large enough to saturate the coding capacity of this region. However, as can be seen in the minicell analysis of the VirG products, at least eight polypeptides were always observed in the chimeric plasmids coding for VirG function. Because a total of 32 initiation codons exist in the same open reading frame for the *virG* gene, some of them might be translated into peptides. Although the significance of these smaller peptides and their role in the function of the *virG* gene are still unclear, we believe that the largest peptide is the most plausible candidate for the VirG protein, since the large (130-kDa) polypeptide was recognized by patient sera (17) and can also be labeled externally with ^{125}I. In addition, the sera did not react with any smaller peptides when tested with the same cloned 6.1-kb VirG region (pMYSH6601) in a 230-kb plasmid-free derivative of YSH6000 (17).

Finally, it should be mentioned that the level of the VirG protein, as well as those of the other three proteins (57, 43, and 39 kDa), has been shown to be regulated positively at the transcriptional level by the VirF protein (17), another virulence-associated 30-kDa protein of pMYSH6000 (16). The significance of the regulatory control by the *virF* gene is not yet clear, but it is interesting to speculate that the levels of those virulence-associated immunogenic polypeptides on the

bacterial surface could be stringently controlled to allow shigellae to express those antigens only at appropriate times during initial invasion, intracellular multiplication, and intercellular spread. Hence, the detailed information on the regulatory region for the *virG* gene from this study should facilitate the investigation of the regulatory mechanisms of the *virF* protein upon the *virG* gene.

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