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Identification of the Product of an *Agrobacterium tumefaciens* Chromosomal Virulence Gene

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The *chvB* operon of *Agrobacterium tumefaciens* is required for bacterial attachment to plant cells and for efficient crown gall tumor formation. As defined by the virulence phenotypes of mutants with transposon insertions mapping in the region, the operon was previously mapped to a 5-kilobase (kb) stretch of chromosomal DNA. We report here that the operon is actually about 8.5 kb long and that it contains a 7-kb gene coding for a large membrane protein involved in the synthesis of cyclic β-1,2-glucan. Mutants with transposon insertions within the 5-kb phenotypically defined operon do not synthesize this functional protein, do not synthesize β-1,2-glucan, and do not form tumors. However, mutants with insertions that map up to 3.5 kb downstream of the phenotypically defined operon synthesize truncated proteins that are active in β-1,2-glucan synthesis. These mutants form tumors. The truncated proteins correspond closely in size with the map positions of the insertions, suggesting that the insertions truncate the proteins by translational termination. A plasmid that contains only the phenotypically defined *chvB* operon also codes for a truncated protein. A fusion product between the protein and β-galactosidase carried on a Tn*3*-HoHo1 insertion was observed in one mutant. Partial trypsin digestion of wild-type inner membranes generated truncated proteins that were active in β-1,2-glucan synthesis, demonstrating that a large portion of the protein is not required for β-1,2-glucan synthesis. The correlation between β-1,2-glucan synthesis by the truncated proteins and tumorigenesis strongly implicates the polysaccharide product of this protein in tumor formation.

Additional keywords: attachment, chromosomal virulence genes, *ChvB*, crown gall, β-1,2-glucan synthesis, polysaccharides.

Crown gall tumor formation on dicotyledonous plants by *Agrobacterium tumefaciens* requires tumor-inducing (Ti) plasmid genes (Van Larebeke *et al.* 1974; Watson *et al.* 1975; Garfinkel and Nester 1980; Nester *et al.* 1984), as well as chromosomal genes. The latter are required for attachment of the bacteria to plant cells (Douglas *et al.* 1982, 1985; Cangelosi *et al.* 1987; Matthysse 1987; Thomashow *et al.* 1987). Three chromosomal virulence loci, *chvA*, *chvB*, and *exoC*, are directly or indirectly involved in the synthesis of a periplasmic and extracellular cyclic β-1,2-glucan (Puvanesarajah *et al.* 1985; Zorreguieta and Ugalde 1986; Cangelosi *et al.* 1987; Thomashow *et al.* 1987; Cangelosi and Nester 1988). One of them, *chvB*, has been shown to be required for β-1,2-glucan synthesis both *in vivo* (Puvanesarajah *et al.* 1985) and *in vitro* (Zorreguieta and Ugalde 1986). Analysis of virulence phenotypes of transposon insertion mutants indicated that the *chvB* operon was at least 5 kilobases (kb) long (Douglas *et al.* 1985).

β-1,2-glucan is synthesized by the successive transfer of glucose from UDP-glucose and requires a membrane-bound, protein-containing intermediate with a molecular size of approximately 235 kilodaltons (kD) (Zorreguieta *et al.* 1985; Zorreguieta and Ugalde 1986). This protein was absent in mutants with transposon insertions in the phenotypically defined *chvB* operon (Zorreguieta and Ugalde 1986). A similar protein was found to act as an intermediate in β-1,2-glucan synthesis by *Rhizobium meliloti* (Geremia *et al.* 1987; Zorreguieta and Ugalde 1986). We report here that the structural gene for this protein is part of the *chvB* operon in *Agrobacterium* and that β-1,2-glucan synthesis is the key function of this protein in tumorigenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. tumefaciens* strains (Table 1) and cosmid clones were described previously (Douglas *et al.* 1985). Bacteria were grown on tryptone-yeast extract (TY) media (Zorreguieta and Ugalde 1986). Kanamycin or carbenicillin was added to a final concentration of 150 μg/ml when appropriate. Merodiploid strains for complementation analysis were constructed by triparental mating as described (Ditta *et al.* 1980). Transconjugants were selected on AB agar medium (Douglas *et al.* 1985) with the appropriate antibiotic and purified by restreaking five times on selective media.

In vitro assays. The assay for *in vitro* β-1,2-glucan synthesis, and labeling of the protein β-1,2-glucan intermediate, were described previously (Zorreguieta *et al.* 1985; Zorreguieta and Ugalde 1986). Inner membranes from 24-hr cultures (Zorreguieta *et al.* 1985) and UDP-(^{14}C) glucose (Wright and Robbins 1965) were used. Polyacrylamide gel electrophoresis (PAGE), protein staining, and autoradiography were carried out as described (Zorreguieta and Ugalde 1986).

β-galactosidase assay. Whole *A. tumefaciens* cells grown overnight on TY medium were assayed for β-galactosidase activity as previously described (Douglas *et al.* 1985). Membrane-bound β-galactosidase activity was determined in 100-μl reactions containing purified inner membranes

(150 μg of protein), 10 mM *p*-nitrophenyl-β-galactoside, 0.1% Na-deoxycolate, and 100 mM Tris-HCl, pH 8.0. Units of β-galactosidase activity were calculated as described (Miller 1972).

Trypsin digestion. *A. tumefaciens* inner membranes were partially digested with trypsin under two different conditions. Under condition 1, trypsin (Sigma, 0.02 mg) was added to purified inner membranes (0.2 mg of protein) in 50 mM Tris-HCl, pH 8.5, and 10 mM $MgCl_2$. After incubation for 5 min at 10° C, the reaction was stopped by the addition of 0.04 mg of soybean trypsin inhibitor (Sigma). UDP-(^{14}C)glucose (60,000 cpm, 320 Ci/mol) was then added, and after 10 min at 10° C the reaction was stopped by the addition of 10% TCA. The precipitate was subjected to PAGE and autoradiography as described (Zorreguieta and Ugalde 1986). Under condition 2, inner membranes were incubated with UDP-(^{14}C)glucose before being treated with trypsin. Controls were carried out as in condition 1 but with the addition of soybean trypsin inhibitor before adding trypsin.

RESULTS

Analysis of the β-1,2-glucan-protein intermediate encoded by plasmid pRAR205. The cosmid clone pRAR205 contains a 9-kb *Bam*HI fragment that was thought to cover the entire *chvB* operon, as previously defined by an avirulent phenotype (Fig. 1) (Douglas *et al.* 1985). The plasmid was introduced into avirulent *chvB* mutant A1011 (Douglas *et al.* 1985), which does not synthesize β-1,2-glucan *in vivo* or *in vitro* (Puvanesarajah *et al.* 1985; Zorreguieta and Ugalde 1986) and does not have the 235-kD protein or any other protein that functions as an intermediate in β-1,2-glucan synthesis (Zorreguieta and Ugalde 1986). Inner membranes prepared from the resulting merodipoid strain (SC5) synthesized β-1,2-glucan *in vitro* (Fig. 2).

The 235-kD protein-glucan intermediate was previously detected in inner membranes from wild-type bacteria by PAGE and Coomassie blue staining, and by autoradiography when the membranes were incubated with radiolabeled UDP-glucose (Zorreguieta and Ugalde 1986; Fig. 3, lanes 11 and 12). However, after electrophoresis of inner membranes from strain SC5, the 235-kD protein-glucan intermediate was not detectable by Coomassie staining or autoradiography (Fig. 3, lane 9). Instead, a 150-kD protein became labeled after incubation of the membranes with UDP-(^{14}C)glucose (Fig. 3B, lane 9). Like the wild-type 235-kD intermediate, the label in the 150-kD product could be chased by incubation with nonradioactive UDP-glucose (Fig. 3B, lane 10). These results suggest that pRAR205 codes for formation of a protein that is smaller than the wild type, but still active in β-1,2-glucan synthesis.

Analysis of the β-1,2-glucan-protein intermediate in strains with transposon insertions located downstream of the phenotypically defined *chvB* operon. One explanation for the truncated protein coded by pRAR205 is that the wild-type structural gene for the protein begins within the

Table 1. Strains of *Agrobacterium tumefaciens*

Strain	Genotype[a]	Phenotype[b]
A348	parent strain	wild type
A1011	Tn*5* in *chvB* region	avirulent, Km^R
SC5	A1011 with pRAR205	virulent, Km^R, Cb^R
ME3	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R
ME4	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R
ME32	Tn*3*-HoHo1 in *chvB* region	avirulent[c], Cb^R
ME35	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R
ME101	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R
ME103	Tn*3*-HoHo1 in *chvB* region	avirulent, Cb^R
ME111	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R
ME112	Tn*3*-HoHo1 in *chvB* region	virulent, Cb^R

[a] Construction of all strains and plasmids is described in Douglas *et al.* (1985). All strains have a C58 chromosomal background. Precise map positions of transposon insertions are indicated in the text and in Figure 1.
[b] Abbreviations: Km, Kanamycin; Cb, carbenicillin.
[c] Strain ME32 is avirulent, but this phenotype is not complemented by cosmid clone pCD523 (Douglas *et al.* 1985), which carries the entire *chvA* and *chvB* region. Avirulent mutants A1011 and ME103 are complemented by this plasmid. Therefore, the avirulent phenotype of strain ME32 appears to be due to a second mutation and not to the Tn*3*-HoHo1 insertion it carries in the *chvB* region.

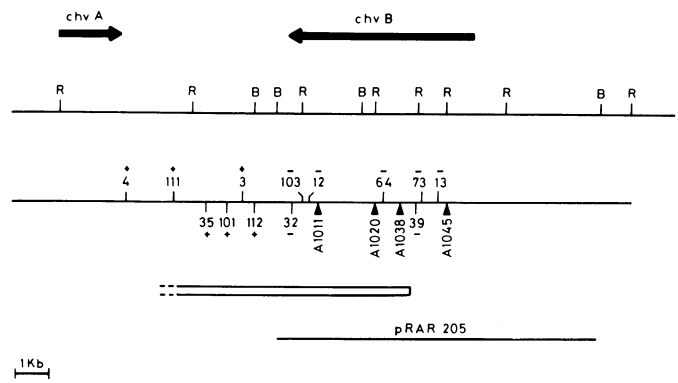

Fig. 1. Restriction endonuclease map of the *Agrobacterium tumefaciens chvA* and *chvB* region, showing Tn*5* transposon insertions (arrow heads) that inactivate virulence, β-1,2-glucan synthesis, and β-1,2-glucan-protein intermediate formation. Vertical lines show the location of Tn*3*-HoHo1 transposon insertions. Virulence of Tn*3* HoHo1 insertions are indicated by plus (virulent) or minus (avirulent) signs. The *Bam*HI fragment cloned in pRAR205 is shown. Open bar shows the probable map position of the structural gene for the β-1,2-glucan-protein intermediate. B, *Bam*HI; R, *Eco*RI. Horizontal arrows indicate the positions and sizes of *chvA* and *chvB* as defined by virulence phenotypes and their directions of transcription. Restriction map and positions of transposon insertions taken from Douglas *et al.* (1985).

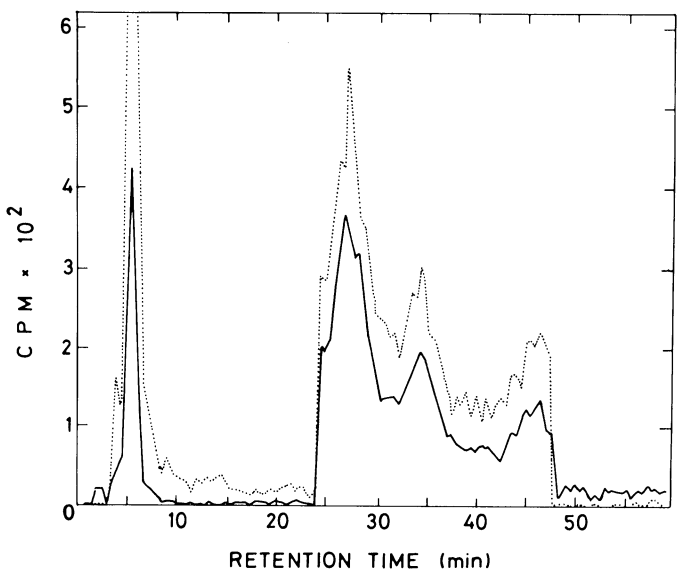

Fig. 2. HPLC pattern of the neutral soluble fraction formed by *A. tumefaciens* wild type and mutant A1011 complemented with plasmid pRAR205. Neutral fractions (about 12,000 cpm) formed by inner membranes of *A. tumefaciens* A723 (wild type) incubated with UDP-(^{3}H)glucose (dotted line) and by *A. tumefaciens* SC5 (mutant A1011 complemented with plasmid pRAR205) incubated with UDP(^{14}C)glucose (about 8,500 cpm, solid line) were subjected to HPLC as described (Zorreguieta and Ugalde 1986).

phenotypically defined *chvB* operon and extends beyond the *Bam*HI fragment carried on the plasmid. To test this possibility, we examined inner membranes from strains that have transposon insertions in the region immediately downstream of the phenotypically defined operon (Fig. 1). Inner membranes from each mutant were incubated with UDP-(^{14}C)glucose and subjected to PAGE and autoradiography. Strain ME103, which has an insertion within the phenotypically defined operon (Fig. 1), had no labeled protein (Fig. 3B, lanes 3 and 4, and Fig. 4B, lane 10). This agrees with previous results that the protein does not appear in avirulent insertion mutants (Zorreguieta and Ugalde 1986). Strain ME112, which has an insertion 0.7 kb downstream of the phenotypically defined operon, produced two labeled proteins of 150 kD and 177 kD (Fig. 3B, lane 1, and Fig. 4B, lane 7). Both behaved as intermediates in β-1,2-glucan synthesis, as indicated by chasing with nonlabeled UDP-glucose (Fig. 3B, lane 2) (Zorreguieta and Ugalde 1986). Labeled proteins of increasing size were detected in insertion mutants ME3, ME35, and ME111 (Fig. 4B, lanes 6, 4, and 3). The apparent molecular weight of the largest labeled protein synthesized by each of these mutants corresponded to the map position of the insertion. For example, the 177-kD protein produced by ME112 is 27 kD larger than the 150-kD protein encoded by pRAR205, and this corresponds well with the map position of insertion 112 (0.7 kb downstream of the *Bam*HI site at the end of pRAR205).

Strain ME111 synthesized a protein that is almost as large as the wild type (Fig. 4B, lane 3), and strain ME4 synthesized a protein that is indistinguishable from the wild type (Fig. 4B, lane 2). If the truncated proteins in the other strains arise from premature termination of transcription or translation of the structural gene, the results for these two strains suggest that the structural gene ends in the vicinity of insertion 111 (Fig. 1). Six labeled proteins were observed in strain ME101, one of which had a molecular weight higher than that of the wild-type protein (Fig. 3B, lane 7). These results are discussed in the next section.

Strain ME32 is avirulent, which suggests that insertion 32

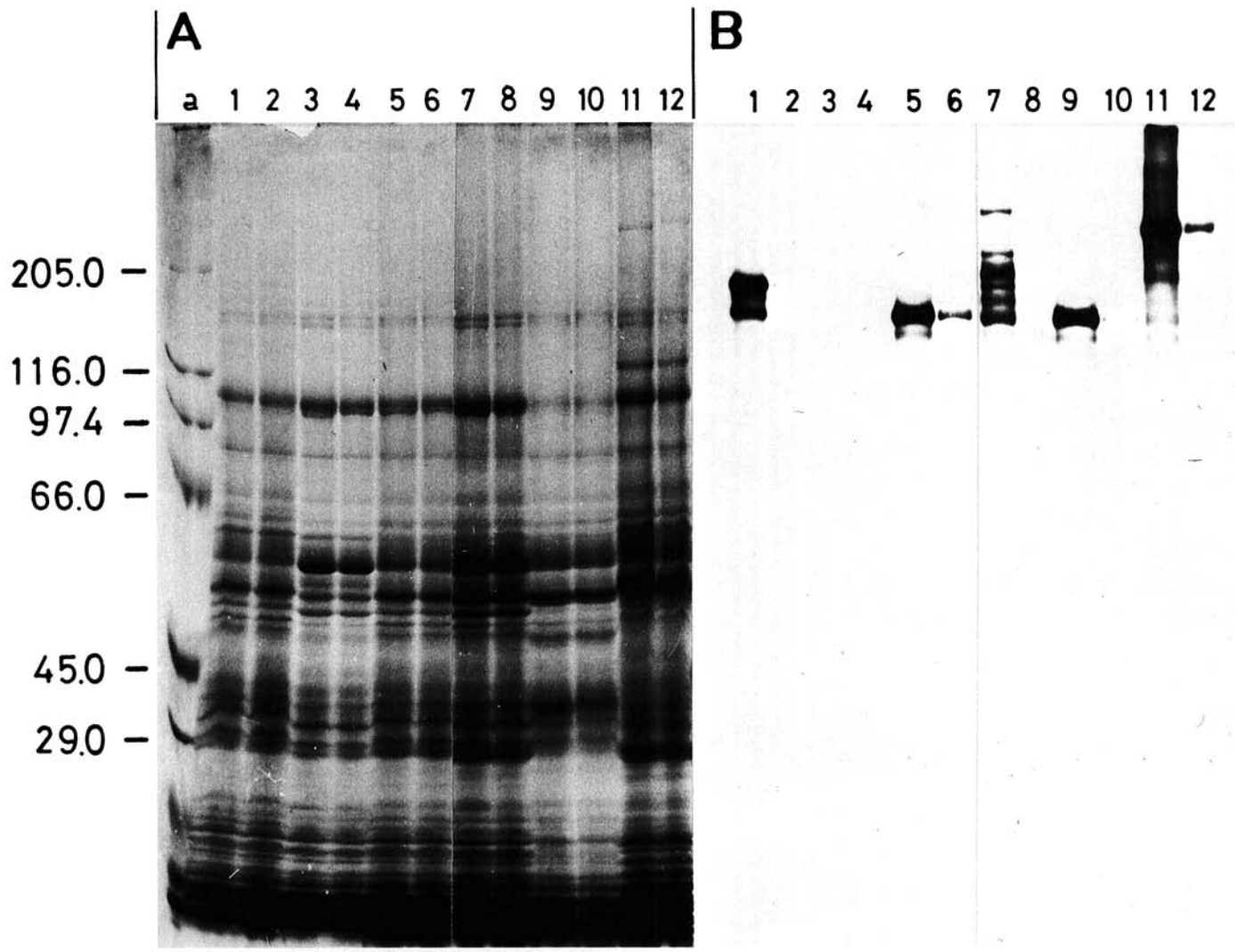

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) of inner membranes of *A. tumefaciens* incubated with UDP(^{14}C)glucose. Inner membranes (0.2 mg of protein) were incubated with UDP(^{14}C)glucose for 15 min. The reaction was stopped with trichloroacetic acid and the precipitate subjected to gel electrophoresis as described (Zorreguieta and Ugalde 1986). Proteins were stained with Coomassie blue (**A**) and radioactivity detected by autoradiography (**B**). Lanes 2, 4, 6, 8, 10, and 12 correspond to chase experiments in which, after 15 min of incubation with UDP(^{14}C)glucose, 2 mM of nonradioactive UDP-glucose was added and incubation was continued for 60 min. The reaction was stopped with trichloroacetic acid and the precipitate was subjected to gel electrophoresis. Lanes 1 and 2, insertion ME112; lanes 3 and 4, insertion ME103; lanes 5 and 6, insertion ME32; lanes 7 and 8, insertion ME101; lanes 9 and 10, *A. tumefaciens* SC5 (mutant A1011 complemented with plasmid pRAR205); lanes 11 and 12, *A. tumefaciens* wild type. Lane a: molecular weight standards. Numbers indicate molecular weight of standards in kilodaltons.

lies within the phenotypically defined operon (Fig. 1) (Douglas *et al.* 1985). This strain produced a truncated protein of about the same size as that encoded by pRAR205, which was expected since insertion 32 maps very near the *Bam*HI site (Fig. 1). Unexpectedly, this protein became labeled after incubation with UDP-(^{14}C)glucose, and the label was chased by incubation with nonradioactive UDP-glucose (Fig. 4B, lane 9, and Fig. 3B, lanes 5 and 6). This result suggests that ME32 produces a protein that functions in β-1,2-glucan synthesis, which would distinguish it from other phenotypically defined *chvB* mutants such as ME103 and A1011 (Zorreguieta and Ugalde 1986). On closer examination, however, we found that this strain was competent for attachment to plant cells, in addition to *in vitro* β-1,2-glucan synthesis (data not shown). Genetic complementation data showed that the avirulent phenotype of this mutant is due to a second mutation outside of the *chvB* region (Table 1).

Fusion of the β-1,2-glucan-protein intermediate to β-galactosidase. As mentioned above, insertion mutant ME101 produced several labeled proteins, one with an apparent molecular weight higher than that of the wild type. The label was chased by incubation with nonradioactive UDP-glucose (Fig. 3B, lanes 7 and 8). The transposon used to generate the mutants used in this study, Tn*3*-HoHo1, can cause translational fusions between a target gene product and β-galactosidase (Stachel *et al.* 1985). The labeled protein of higher molecular weight produced by ME101 could be such a fusion product, since the Tn*3*-HoHo1 insertion in this strain is oriented in the direction of transcription of *chvB* (Douglas *et al.* 1985). This possibility is supported by the observation of β-galactosidase activity in inner membranes from strain ME101 (Table 2). Such activity is not observed in wild-type *Agrobacterium* (Table 2) and is normally found in the soluble fraction of bacteria

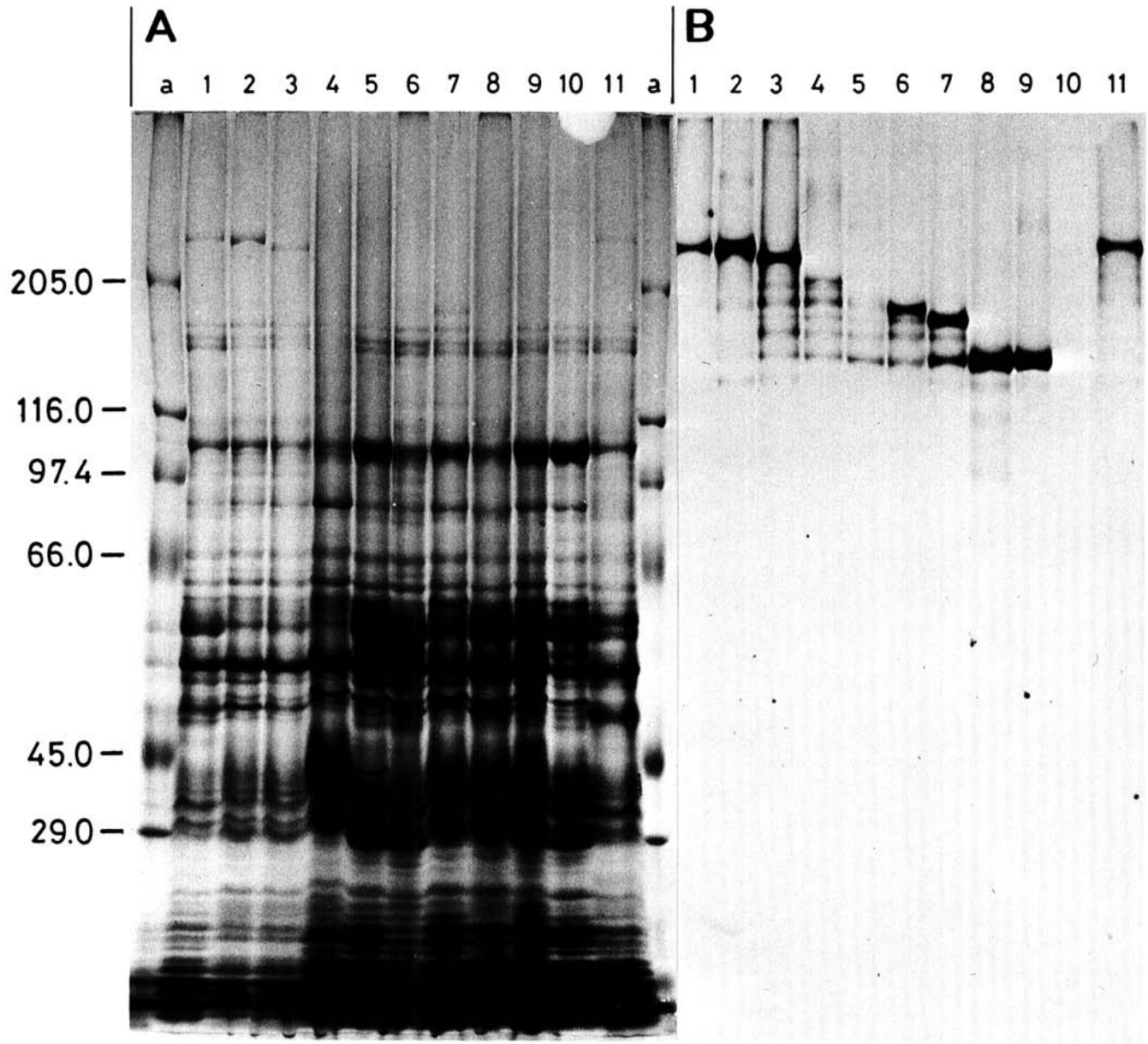

Fig. 4. PAGE of inner membranes of *A. tumefaciens* incubated with UDP(^{14}C)glucose. PAGE was carried out as in Fig. 3. Proteins were stained with Coomassie blue (**A**) and radioactivity detected by autoradiography (**B**). Lane 1, *A. tumefaciens* wild type; lane 2, insertion ME4; lane 3, insertion ME111; lane 4, insertion ME35; lane 5, insertion ME101; lane 6, insertion ME3; lane 7, insertion ME112; lane 8, *A. tumefaciens* SC5 (mutant A1011 complemented with plasmid pRAR205); lane 9, insertion ME32; lane 10, insertion ME103; lane 11 *A. tumefaciens* wild type. Lane a: molecular weight standards. Numbers indicate the molecular weight of standards in kilodaltons.

that carry the gene for it. Its occurrence in the inner membranes of strain ME101 suggests that it is fused to the membrane-bound protein intermediate. Strains ME32 and ME3 also have Tn*3*-HoHo1 insertions in the same orientation as *chvB*, but they do not produce detectable fusion proteins (Fig. 4B, lanes 9 and 7) and they have no detectable membrane-bound β-galactosidase activity (Table 2). These are presumably nontranslational fusions (Stachel *et al.* 1985).

Partial trypsin digestion of the β-1,2-glucan-protein intermediate. Our results indicate that truncated protein-glucan intermediates with apparent molecular weights as low as 150 kD function as well as the 235-kD wild-type protein in β-1,2-glucan synthesis. This suggests that the carboxy-terminal one-third of the protein is dispensable for β-1,2-glucan synthesis. We tested this interpretation by subjecting wild-type inner membranes to partial trypsin digestion before and after incubation with UDP-(^{14}C)glucose, respectively, as described.

Wild-type inner membranes partially digested under condition 1 showed a strong reduction in the amount of 235-kD protein labeled after incubation with UDP-(^{14}C)glucose. Instead, radioactivity was found in three main bands of approximately 200, 130, and 95 kD (Fig. 5A, lane 3, and Fig. 5B, lane 2). The labeled trypsin digestion products were active as intermediates in β-1,2-glucan synthesis, since the label was chased by incubation with nonradioactive β-1,2-glucan in the same manner as the undigested control (Fig. 5A, lanes 2 and 4). Trypsin treatment did not alter the amount or type of β-1,2-glucan produced by the inner membranes *in vitro* (data not shown).

Table 2. β-Galactosidase activity of whole cells and inner membranes

Strain	β-Galactosidase activity (units/mg of protein)	
	Whole cells	Inner membranes
A 723	12.4	<0.1
ME32	23.8	<0.1
ME3	18.7	<0.1
ME101	111.4	61.2

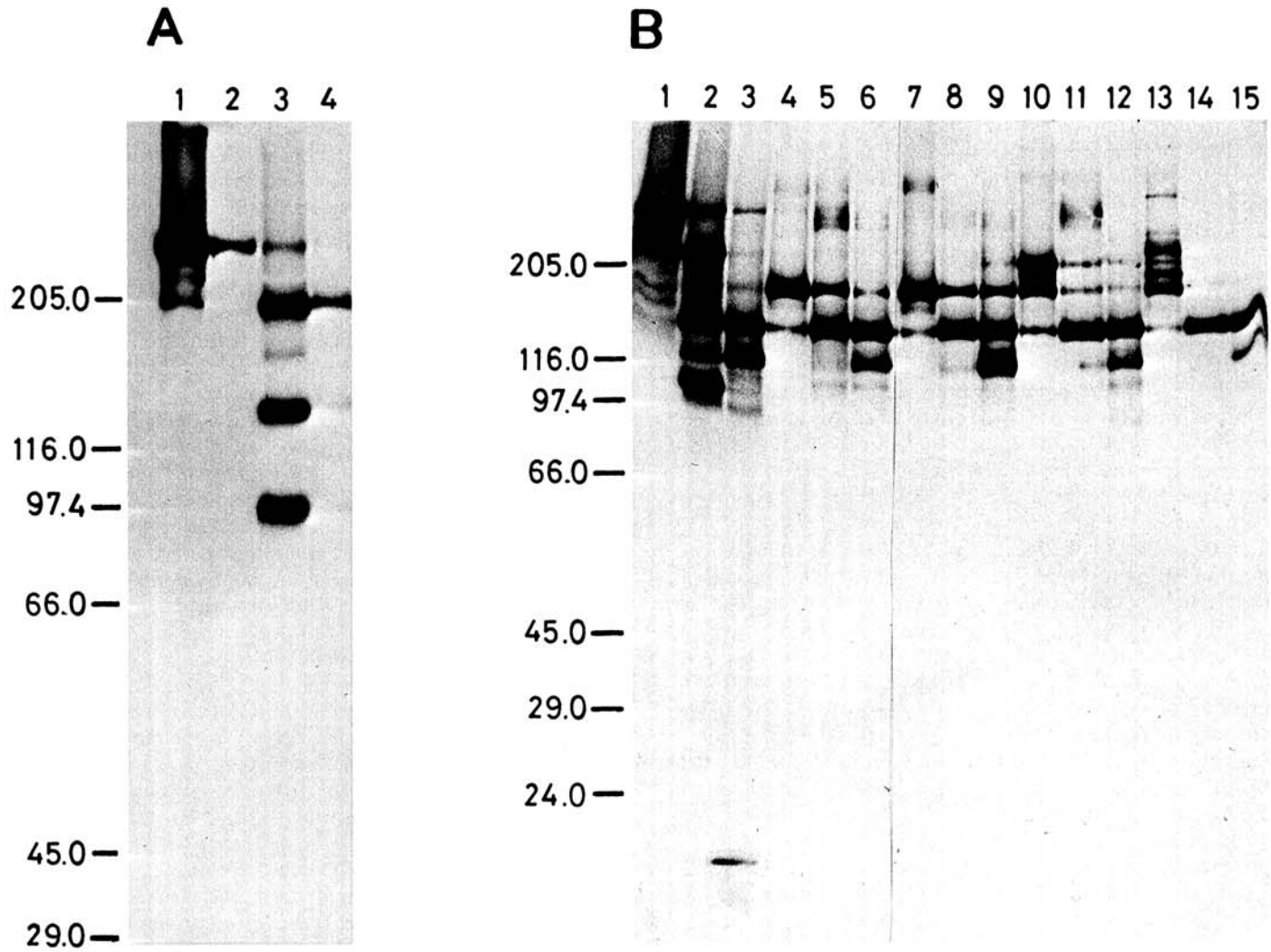

Fig. 5. PAGE of trypsin-treated inner membranes of *A. tumefaciens*. PAGE and autoradiography were carried out as in Fig. 3. Digestion with trypsin and incubation with UDP(^{14}C)glucose were as described in Materials and Methods. Panel A, lane 1 and panel B, lanes 1, 4, 7, 10, and 13 correspond to controls (soybean trypsin inhibitor added before trypsin). Panel A, lane 3 and panel B, lanes 2, 5, 8, 11, and 14 correspond to digestion with trypsin following condition 1. Panel B, lanes 3, 6, 9, 12, and 15 correspond to digestion with trypsin following condition 2. Panel A, lanes 2 and 4 correspond to a chase experiment in which, after 10 min of incubation of inner membranes digested following condition 1, 2 mM of nonlabeled UDP-glucose was added and incubated for another 30 min. Panel A corresponds to wild-type inner membranes. Panel B, lanes 1–3 correspond to wild-type inner membranes; lanes 4–6, strain SC5; lanes 7–9, strain ME32; lanes 10–12, strain ME112; lanes 13–15, strain ME101.

A 130-kD polypeptide also became labeled when inner membranes from strains SC5, ME32, ME112, and ME101 were partially digested with trypsin and incubated with UDP-(^{14}C)glucose, following condition 1 (Fig. 5B, lanes 5, 8, 11, and 14). The smallest (95 kD) labeled protein observed in the wild-type membranes was not observed in the other strains. This may be due to differing tertiary structures, which expose different trypsin cleavage sites, or it may indicate that a region downstream of insertion 101 is able to bind radiolabeled UDP-glucose.

When inner membranes were digested with trypsin following condition 2, a slightly different pattern was observed. Wild-type inner membranes had labeled peptides of 130, 110, and less than 20 kD (Fig. 5B, lane 3). Strains SC5, ME32, ME112, and ME101 had 130 and 110 kD proteins, but the 20-kD band was not visible (Fig. 5B, lanes 6, 9, 12, and 15).

DISCUSSION

The biosynthesis of β-1,2-glucan by *Agrobacterium tumefaciens* proceeds through a membrane-bound protein-glucan intermediate with an apparent molecular size of at least 235 kD (Zorreguieta *et al.* 1985; Zorreguieta and Ugalde 1986). Avirulent *chvB* mutants do not synthesize β-1,2-glucan *in vivo* (Puvanesarajah *et al.* 1985) or *in vitro* (Zorreguieta and Ugalde 1986), presumably because they do not synthesize the protein-glucan intermediate. The size of the *chvB* operon was estimated at about 5 kb, based on the analysis of virulence phenotypes of a large number of *Agrobacterium* insertion mutants (Douglas *et al.* 1985). Since this did not represent sufficient coding capacity for a 235-kD protein, it was suggested that the *chvB* operon might be a regulatory locus.

The results presented in this paper strongly suggest the alternative possibility, that the *chvB* locus contains the structural gene for the 235-kD protein. The structural gene begins within the *chvB* operon and extends approximately 3.5 kb beyond the phenotypically defined 3′ end (Fig. 1). This conclusion is drawn from the apparent molecular weights of protein-glucan intermediates observed in the inner membranes from *chvB* mutant A1011 complemented with pRAR205 (strain SC5) and from strains with transposon insertions located downstream of the phenotypically defined locus. We suggest that transcription or translation of the structural gene for the protein is terminated by the insertions, or in the case of strain SC5, by the end of the cloned *chvB* fragment in pRAR205.

The apparent molecular weights of the truncated proteins produced in each of these strains correlated very well with the map positions of the mutations that caused them. The plasmid pRAR205 coded for a protein with an apparent molecular weight of 150. Strains ME112, ME3, ME101, ME35, and ME111 synthesize proteins with apparent molecular weights of 177, 186, 208, 218, and nearly 235, respectively. The increased molecular weight of these proteins, relative to the 150-kD protein coded by pRAR205, are roughly proportional to the map positions of the corresponding insertions, relative to the *Bam*-HI site at the end of the cloned *chvB* fragment in pRAR205. Insertions 112, 3, 101, 35, and 111 map 0.7, 1.1, 1.4, 1.9, and 2.9 kb downstream of the *Bam*HI site, respectively (Fig. 1). Strain ME101 has a protein apparently larger than the wild-type protein. This probably represents a translational fusion between the membrane protein and β-galactosidase, caused by insertion of the transposon Tn*3*-HoHo1, since β-galactosidase activity was observed in inner membranes from this strain. In addition to these proteins, most of the mutants have peptides of lower molecular weight that were labeled after incubation with UDP-(^{14}C)glucose (Figs. 3 and 4). These bands may have resulted from posttranscriptional processing or degradation of the mutant gene products.

On the basis of these results, we estimate that the structural gene for the protein intermediate begins about 3.5–4.0 kb upstream of the *Bam*HI site (1.0–1.5 kb from the 5′ end of the phenotypically defined *chvB* operon) and extends about 7.0 kb to a point downstream of the phenotypically defined operon, near insertion 111. However, this estimate can only be approximate, since it is impossible to precisely determine the molecular weights of large glycoproteins by SDS-PAGE. A 7.0-kb gene should code for a protein that is larger than the previously estimated 235 kD. It is particularly difficult to estimate the molecular weights of peptides that migrated more slowly than myosin, the largest (205 kD) molecular weight standard used in these studies. Therefore, our estimates on size and map position of the structural gene were calculated from the apparent molecular weights of the smaller truncated peptides. Attempts to test our conclusions by a different method, measuring the sizes of *chvB* mRNA by northern blot analysis, were unsuccessful due to the short half-life of the *chvB* transcript (data not shown). Accordingly, nucleotide sequencing will be required to accurately determine the size of the structural gene and the total number of genes in the *chvB* operon.

It is surprising that truncated polypeptides, which apparently are less than two-thirds the size of the wild-type peptide, are fully functional in β-1,2-glucan synthesis and virulence. Therefore, we partially digested wild-type inner membranes with trypsin, to determine if truncated polypeptides could be obtained that were active as intermediates in β-1,2-glucan synthesis. Fragments of 200, 130, and 95 kD were labeled upon incubation with UDP-(^{14}C)glucose, and the label was chased by incubation with nonlabeled UDP-glucose. The amount of β-1,2-glucan formed was the same as when undigested membranes were used. These data show that regions of the wild-type protein are dispensable for β-1,2-glucose synthesis. The dispensable portions may carry out a different, unidentified function. The 130-kD fragment was also present in trypsin-digested membranes from all of the mutants that were examined. This portion of the protein is apparently coded within the phenotypically defined *chvB* locus. Labeled fragments of reduced size, including a 130-kD fragment, were also obtained when incubation with UDP-(^{14}C)glucose preceded trypsin digestion (condition 2). This result precludes the possibility that the altered mobilities of the proteins in the digested membranes are due to digestion of a second protein that is involved in glycosylation of the 235-kD protein.

Even small conjugated polysaccharides can have dramatic effects on the mobility of glycoproteins in SDS-PAGE. However, it is very difficult to attribute all of our observations to spurious effects on molecular weight determination. It is not likely that our results are due to mutations in a separate gene that is downstream of *chvB* and is involved in modification or glycosylation of the 235-kD protein, since such a gene would be wild type in strain SC5. We observed altered mobilities of protein-glucan intermediates in SC5, in several insertion mutants, and in wild-type and mutant inner membranes digested with trypsin before and after glycosylation. The best explanation for all of the observed alterations in electrophoretic mobility

is that they are due to truncation of the polypeptide.

Certain previous published data were difficult to reconcile with our biochemical mapping. Transposon insertions in two strains, ME2 and ME116, were previously reported to map within the phenotypically defined *chvB* operon, but had no effect on virulence (Douglas *et al.* 1985). Insertion 116 was previously mapped between Tn*5* insertions A1011 and A1020, which would place it within the proposed 7.0-kb structural gene (Fig. 1). We remapped insertions 2, 116, and several others by digesting DNA from the insertion mutants with six different restriction enzymes, Southern blotting, and hybridizing with cosmid clones of the region (pCD521 and pCD526) (Douglas *et al.* 1985). The avirulent insertions (12, 13, 32, 39, 64, 73, and 103 in Fig. 1) all mapped at their previously reported positions, but strains ME2 and ME116 had wild-type *chvB* restriction patterns (data not shown). The revised map of the region (Fig. 1) contains no insertions within the phenotypically defined *chvB* operon that do not affect virulence.

Three chromosomal loci of *A. tumefaciens*—*chvA*, *chvB*, and *exoC*—are involved in β-1,2-glucan synthesis or its export, in addition to being required for attachment to plant cells and tumor formation (Douglas *et al.* 1985; Puvanesarajah *et al.* 1985; Zorreguieta and Ugalde 1986; Cangelosi *et al.* 1987; Thomashow *et al.* 1987; Cangelosi and Nester 1988). Mutational analysis has also correlated β-1,2-glucan synthesis with nodulation of alfalfa by *Rhizobium meliloti* (Geremia *et al.* 1987). Although these data suggest that β-1,2-glucan plays a role in both types of bacterium-plant interaction, a direct requirement for the polysaccharide has not been demonstrated. The results presented here show that the portion of the *chvB* gene product that is required for tumorigenesis is the portion that is also required for β-1,2-glucan synthesis. This is strong evidence for a role for β-1,2-glucan in attachment and tumor formation by *A. tumefaciens*.

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