MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 6, pp. 301-308, 1989

Differences in Induction of Ti Plasmid Virulence Genes *virG* and *virD*, and Continued Control of *virD* Expression by Four External Factors

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Received 27 March 1989. Accepted 26 June 1989.

We investigated external factors controlling the expression of *virG* and *virD* in the *vir* region of Ti plasmids. *virG* expression was measured by immunological demonstration of the protein in western blots. Cells grown in YEB medium at pH 7.2 contain little if any of the protein. Induction requires two factors, temperatures below 28° C and sucrose; the protein is detectable after less than 1 hr. Expression in the presence of these factors is stimulated by acetosyringone and acidic pH. The regulation of *virD* was investigated with a *virD* promoter-reporter (*galK*) gene construct in the presence of a Ti plasmid containing the complete *vir* region (pGV2260). Temperatures below 28° C, sucrose, acetosyringone, and acidic pH are strict requirements for induction, indicating that the expression of *virD* is controlled more stringently than is *virG*. Preincubation of the cells with combinations of three of these four factors did not significantly reduce the length of the *virD* lag phase, indicating that all four factors must be present simultaneously for the initiation of expression. Removal of any one of the four factors after the onset of induction stopped or reduced further expression. This indicates that the activation of the *vir* genes does not represent an irreversible commitment to induction.

Additional keyword: Agrobacterium tumefaciens.

Gene transfer from *Agrobacterium tumefaciens* (Smith and Townsend) Conn to plants requires a set of genes localized in the (tumor-inducing) Ti plasmid *vir* region. The organization and function of these genes have been studied intensively during the past few years (see Koukolíková-Nicola *et al.* 1987 for a recent review). Most of the transcription units are silent in free-living bacteria and must be induced for transfer of the T-region to plants. It is thought that the protein products of *virA* and *virG* form a two-component regulatory system for induction of the *vir* regulon (Stachel and Zambryski 1986; Winans *et al.* 1986; Melchers *et al.* 1986, 1987; Leroux *et al.* 1987).

The induction of the virulence functions requires several factors: plant signals and acidic pH (Stachel and Zambryski 1986; Bolton *et al.* 1986; Rogowsky *et al.* 1987; Veluthambi *et al.* 1987), and temperatures below 28° C and sucrose (or another easily metabolized sugar or glycerol) (Alt-Mörbe *et al.* 1988). The latter two factors were identified by an immunological western blot demonstration of the protein encoded in *virD2* (a *vir* region product essential for processing of the T-region). The experiments also showed that the protein was detectable only after a surprisingly long lag period of 2 hr after the onset of induction. Even longer lags of 6 hr have been reported by others (Winans *et al.* 1986, 1988). These authors suggested that the activities of *virA* and *virG* create a feedback loop of positive autoregulation of these two genes, and that the lag phase in the expression of the other *vir* genes could be explained by a slow initial rate caused by low initial concentrations of the proteins encoded in *virA* and *virG*. In this study, we focus on the following points that are of interest for the regulation of *vir* genes.

First, *virG* expression has been studied so far by transcriptional analysis. Most of the published data indicate that the transcription is constitutive and also inducible, but it was not clear whether this actually correlates with the presence of the protein. We raised antiserum against the *virG*-encoded protein and investigated (on the level of the protein product) the regulation of gene expression by various external factors.

Second, a long lag phase in *vir* gene induction could also be explained by the possibility that one or several of the factors required for induction are necessary to change the cells into a physiological state in which they are able to respond to plant signals (competence). This could be a time-consuming process only very indirectly related to specific regulation by plant signals. This was investigated by experiments testing whether the lag period can be shortened by preincubation of the cells with combinations of factors that by themselves do not allow induction (preconditioning). The analysis was performed with cells containing a Ti plasmid with a complete *vir* region in addition to a plasmid with a promoter-reporter construct in which the *virD* promoter region from the nopaline plasmid pGV3850 is fused to a *galK* structural gene.

Third, one of the interesting questions in models involving positive autoregulation of the expression of *virG* and *virA* (Winans *et al.* 1988) is whether induction, once initiated, becomes independent of external signals. This was analyzed by switch-off experiments in which one of the signals is removed several hours after the initiation of induction. These experiments were performed with cells containing the *virD* promoter-reporter plasmid and a Ti plasmid with the complete *vir* region.

MATERIALS AND METHODS

Agrobacterial strains and plasmids. The descriptions of

plasmid-free APF2 (Hynes *et al.* 1985), the broad host range vector plasmid pCP13 (Darzins and Chakrabarty 1984), C58C1(pGV3850) (Zambryski *et al.* 1983), and C58C1(pGV2260) (Deblaere *et al.* 1985) have been published. The plasmid pGV3850 is a derivative of the nopaline plasmid pTiC58; it contains the complete *vir* region, but a part of the T-region is replaced by pBR322. The plasmid pGV2260 is a derivative of the octopine plasmid pTiB6S3; it harbors the complete *vir* region, but the T-region and some adjoining sequences are replaced by pBR322. The bacteria were routinely kept on YEB agar (0.5% Bacto beef extract, 0.1% Bacto yeast extract, 0.5% Bacto peptone, 0.5% sucrose, 2 mM $MgSO_4$; pH 7.2) (Van Larebeke *et al.* 1977) at 28° C and with the appropriate antibiotics (rifampicin, 50 μg/ml; carbenicillin, 50 μg/ml; and tetracycline, 2 μg/ml).

***Escherichia coli* strains and plasmids.** HB101(recA) (Maniatis *et al.* 1982) was used for propagation of plasmids, DS410 (Reeve 1979) for minicell production, C600 (Maniatis *et al.* 1982) for expression of the *trpE-virG* fusion protein, and S17-1 (which contains the *tra* genes of the broad host range IncP-type plasmid RP4 in the chromosome) (Simon *et al.* 1983) for conjugational crosses. The expression vector pATH11 was obtained from O. Schmidt (Freiburg); the features essential for this work are shown in Figure 1. Cells were grown on Luria-Bertani agar medium (Maniatis *et al.* 1982) supplemented with the appropriate antibiotics (ampicillin, 50 μg/ml, and tetracycline, 15 μg/ml).

Constructions with *E. coli* plasmids. pVIR250 is described in Figure 1; it was constructed for overexpression of a *trpE-virG* fusion protein. pVIR273 harbors in pINIIA2 the 3′-terminal part of *virB* and the complete *virG* gene on a 2.5-kilobase-pair *Kpn*I-*Eco*RI subfragment of *Kpn*I 11 in the *vir* region of pTiC58. The fragment was excised from pVIR70.58 (Alt-Mörbe *et al.* 1986) with *Xba*I-*Eco*RI (utilizing an *Xba*I site in the vector polylinker) and cloned into the vector pINIIA2 (Nakamura and Inouyé 1982) that was digested with the same enzymes. The cloning procedure assures that the vector promoters read into the cloned fragment in the direction of *virG* transcription.

Constructions with the broad host range vector pCP13. All of the constructions were performed in *E. coli* strain S17-1. pVIR269.1 contains the complete *galK* gene cassette including the promoter and the $lacI^q$ repressor gene. The *E. coli* plasmid pFD105 (Alt-Mörbe *et al.* 1986) was cut with *Sma*I and religated; this removed the *Hin*dIII site in the polylinker. The new plasmid (pFD106.1) was opened at its single *Hin*dIII site and ligated into pCP13 digested with *Hin*dIII. pVIR280.2 contains the *galK* structural gene without the promoter; the gene was cloned as a *Bam*HI fragment into pCP13. pVIR271 contains in pCP13 a transcriptional fusion of the *virD* promoter with a promoterless *galK* protein coding region; the construction is described in Figure 1.

Conjugative crosses. pVIR269.1, pVIR271, and pVIR280.2 were conjugated from *E. coli* S17-1 into *Agrobacterium* APF2 or C58C1(pGV2260). Donors (0.2–0.5 A_{600}/ml) and recipients (0.1 A_{600}/ml) were harvested and washed with 0.9% NaCl. Aliquots of 0.1 A_{600} from each were resuspended together in 50 μl of 0.9% aqueous NaCl and spread on nitrocellulose filters (Schleicher & Schuell, Dassel, West Germany, 0.45 μm pore size) on YEB agar without antibiotics. After 4–6 hr at 28° C, bacteria were suspended in 1 ml of 0.9% NaCl, and aliquots of 1 to 100 μl were plated on YEB agar containing rifampicin, carbenicillin, and tetracycline. Colonies were usually used after 36 hr at 28° C.

Purification of *trpE-virG* fusion protein and raising of antiserum. A 2-ml culture of *E. coli* C600 containing pVIR250 was grown overnight and then used to inoculate 300 ml of M9 medium supplemented with 1% casamino acids. When the culture reached 0.3 A_{600} (after about 2.5 hr), expression of the fusion protein was induced by adding 0.3 ml of β-indoleacrylic acid (10 mg/ml in ethanol). Cells were harvested after an additional 5 hr, resuspended in 10 ml of buffer (25 mM Tris-HCl, pH 8, 50 mM D-glucose, 10 mM EDTA), and lysed for 15 min at room temperature by adding 2.3 ml of lysozyme (10 mg/ml in TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8]). Incubation was continued for 5 min after adding 2.3 ml of EDTA (0.5 M, pH 8) and for 15 min on ice with Triton X-100 at a final concentration of 0.1%. Insoluble material (including the fusion protein) was pelleted by centrifugation (15 min at 15,000 × *g*), washed twice with TE buffer containing 0.1% Triton X-100, and resuspended in 2 ml of TE buffer.

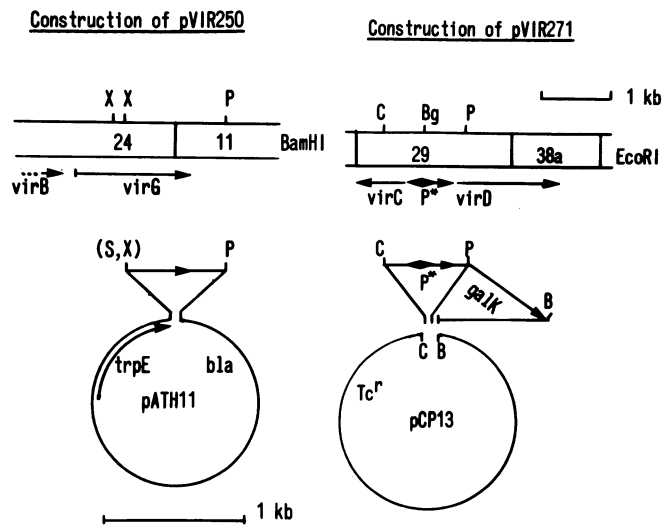

Fig. 1. Left: pVIR250, a construction for overexpression of a *trpE-virG* fusion protein. Top: relevant sites in the octopine *virG* region (Melchers *et al.* 1986). A 0.7-kilobase (kb) *Xho*I-*Pst*I fragment containing about 50% of the 3′ part of the octopine *virG* coding region was inserted into the vector pATH11 digested with *Sal*I and *Pst*I. The plasmid pVIR250 (bottom; pATH11 indicates the vector that is not drawn to scale) expresses an inducible fusion protein of 53 kDa that contains 37 kDa from *trpE* and 16 kDa from *virG*. X, *Xho*I; P, *Pst*I; S, *Sal*I; in parentheses, sites lost by the cloning procedure; and bla, gene for ampicillin resistance. Right: pVIR271, a transcriptional fusion of the *virD* promoter with a promoterless *galK* coding region. Top: restriction map of the *virCD* promoter region in pTiC58 (Tait and Kado 1988). In the first step of the construction, the 1.2-kb *Cla*I-*Pst*I fragment containing both promoters was cloned into pCP13 digested with these enzymes. In the second step, the promoterless coding region was inserted as a *Pst*I-*Bam*HI fragment into the intermediate plasmid digested with *Pst*I and *Bam*HI. The directional cloning strategy assures that the *virD* promoter in pVIR271 reads into *galK* (bottom). Tc^r, tetracycline resistance; C, *Cla*I; Bg, *Bgl*II; P, *Pst*I; and B, *Bam*HI. Bottom: The vector part (pCP13) is not drawn to scale.

Portions of 0.25 ml were mixed with 2 ml of sample buffer containing 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol; proteins were solubilized by boiling for 10 min and separated by preparative SDS-PAGE. The fusion protein, representing the predominant protein, was further purified according to published procedures that also describe the techniques for raising antiserum in rabbits (Alt-Mörbe *et al.* 1988).

Induction of *vir* region genes in *Agrobacterium*. Twenty microliters of cells from dimethylsulphoxide stock cultures (stored at −70° C) was inoculated in 1 ml of YEB medium (pH 7.2, without antibiotics), and precultures grown overnight at 28° C were used for induction in SIM (simplified induction medium). SIM complete contains 2% sucrose, 0.2 mM acetosyringone, and 20 mM sodium citrate, pH 5.5, and induction was performed at 25° C. These conditions combine the factors essential for induction of *virD* (sucrose, a plant signal, acidic pH, and suitable temperature; Alt-Mörbe *et al.* 1988). In experiments requiring centrifugation of the cells (switch-off experiments), SIM was supplemented with 5 mg/ml of peptone (SIM^+). This modification was necessary to improve the sedimentation properties of the bacteria. Previous experiments have shown that peptone in this concentration or any of the other proteinaceous components of YEB medium have no significant effect on the induction or expression of *virD* in SIM (Alt-Mörbe *et al.* 1988). Other variations are noted elsewhere in the text.

Extract and assay for galactokinase. Bacterial pellets (corresponding to 1 A_{600}, stored at −20° C) were suspended in 0.8 ml of lysis buffer (50 mM Tris-HCl, pH 8, 1 mM dithiothreitol [DTT], 0.1% EDTA), mixed with 0.2 ml of lysozyme (stock solution, 2.5 mg/ml), and incubated for 30 min at 37° C. After an additional 5-min incubation period with 20 μl of DNaseI (1 mg/ml in 0.5 M $MgCl_2$), insoluble material was removed by centrifugation for 5 min at 10,000 revolutions per minute.

The assays contained 40 μl of Mixture II (see below) and 60 μl of enzyme. Several dilutions (up to 1:30 in buffer: 0.1 M Tris-HCl, pH 7.8, 1 mM DTT, 1 mM EDTA, 0.1 mg/ml of bovine serum albumin) of the bacterial extracts were assayed in each experiment. This was necessary because product formation was linear only in the range of 1,500 to 4,000 cpm under standard incubation conditions, and because enzyme activity varied strongly with the induction of the *vir* region. Mixture II (for 10 samples) contained 100 μl 1 M Tris-HCl, pH 7.8, 100 μl 15 mM ATP, 50 μl 0.2 M DTT, 20 μl 0.2 M $MgCl_2$, 80 μl 40 mM NaF, and 50 μl Mixture I ([^{14}C]galactose, 61 μCi/μmol, Amersham Buchler, Braunschweig, West Germany) diluted with unlabeled galactose to a final concentration of 30 mM and a specific activity of 0.5 μCi/μmol; the solution was filtered through Whatman DE81 paper before use.

After incubation for 45 min at 37° C, duplicate 30-μl portions were spotted on Whatman DE81 filters that were then soaked in 80% ethanol, washed three times in 2 liters of water, dehydrated in ethanol, dried, and counted. One unit of enzyme activity represents the formation of 1 nmol of galactose-1-phosphate per minute

Protein synthesis *in vivo*. C58C1(pGV3850) cells grown in standard YEB medium were collected by centrifugation and resuspended to 0.2 A_{600}/ml either in SIM^+ complete (2% sucrose) or in SIM^+ without sucrose. After various incubation times (up to 20 hr) on a rotary shaker, portions of 2 ml were pulse-labeled for 0.5 hr with 10 μCi of L-[^{35}S]methionine (1,400 Ci/mmole). The pelleted cells were lysed by boiling in 150 μl of Tris buffer (pH 6.8) containing 2% SDS. The results were evaluated in three ways: 1) 10 μl was counted directly (equal total uptake into the cells), 2) 10 μl was used to determine the amount of radioactivity precipitated with 10% trichloroacetic acid (equal incorporation into protein), and 3) 60 μl was analyzed by PAGE in the presence of SDS (equal pattern of proteins synthesized *de novo*) (Alt-Mörbe *et al.* 1988).

Other methods. Protein expression in *E. coli* minicells, immunoprecipitations, and western blots were performed as previously described (Alt-Mörbe *et al.* 1988). Standard DNA manipulation techniques followed the procedures detailed in Maniatis *et al.* (1982).

RESULTS

***virG*: Protein levels in noninduced cells and regulation of induction.** Figure 1 (left) shows the construction of a gene fusion overexpressing a *trpE-virG* fusion protein that contains half of the *virG* coding region from the octopine plasmid pTiAch5. The protein was purified and used to raise antiserum.

Figure 2 summarizes the first experiments. pVIR273 (containing the 3′-terminal part of *virB* and the complete *virG* coding region from pTiC58) expresses several polypeptides in *E. coli* minicells, but only one protein of the size expected for the *virG* gene product (29–30 kDa; Melchers *et al.* 1986; Powell *et al.* 1987; Winans *et al.* 1986) is recognized by the antiserum. No immunoreactive polypeptide is detected in cells harboring the vector plasmid pINIIA2. Western blots of *Agrobacterium* cell extracts show that the protein is present in induced cells containing either pGV2260 (octopine type) or pGV3850 (nopaline type). Figure 2C also shows that the octopine plasmid gave a much weaker signal than does pGV3850. This was observed consistently in more detailed experiments and indicates that the amount of *virG*-encoded protein expressed from the two plasmids is different. The antiserum was raised against the octopine plasmid *virG*-encoded protein; therefore, poor cross-reaction with a heterologous protein is excluded as the explanation for this result.

No *virG*-encoded protein is detectable in YEB-grown cells containing the octopine plasmid pGV2260 or the nopaline plasmid pGV3850 (only shown for the latter in Fig. 2C), with standard times for color development in the western blots. Much longer exposure times reveal a polypeptide of 29–30 kDa as well as other nonspecific background bands, and therefore, identification as a *virG* product is ambiguous. When YEB is supplemented with the inducing signal acetosyringone and culture conditions are adjusted to pH 5.5 at a temperature of 25° C (also inducing factors), an induction comparable to that in SIM complete is observed (Fig. 3F, compare lanes 5 and 6). This indicates that the lack or very low level of expression

in standard YEB medium (pH 7.2) is not due to the presence of beef extract, yeast extract, or peptone. The plasmid-free strain APF2 contains no detectable protein reacting with the antiserum against the *virG*-encoded protein (Fig. 2C).

The results indicate that the antiserum against the *trpE-virG* fusion protein specifically recognizes the protein encoded by *virG* of octopine and nopaline Ti plasmids. Noninduced *Agrobacterium* cells containing pGV2260 or pGV3850 contain very little if any of the protein.

Figure 3 summarizes western blot experiments analyzing the conditions necessary for induction of *virG* with the nopaline plasmid pGV3850 (the same results were obtained in experiments with the octopine plasmid pGV2260). In the presence of all inducing factors (SIM complete), the time course shows that the protein is clearly visible 1 hr after the onset of induction (Fig. 3A), indicating that this gene is induced more rapidly than is *virD2* which requires 2 hr for detectable protein levels (Alt-Mörbe *et al.* 1988). In other experiments, three of the inducing factors are kept at optimal conditions, and the fourth is varied. Induction is temperature-dependent with an optimum temperature being between 15° C and 25° C (Fig. 3B); this is in the same range as was determined for *virD2* (Alt-Mörbe *et al.* 1988). Expression is stimulated by acidic pH and by acetosyringone (Figs. 3C and 3D). In the presence of the plant signal, the optimum pH is between 5.5 and 6; higher pH values reduce induction, but the *virG*-encoded protein is clearly detectable at pH 7.2 (Fig. 3D). This is in contrast to *virD2*, which is not expressed at this pH (Alt-Mörbe *et al.* 1988). Analysis of pH dependence in the absence of acetosyringone (Fig. 3C) reveals a similar profile, except that the overall amount of *virG*-encoded protein is lower under these conditions.

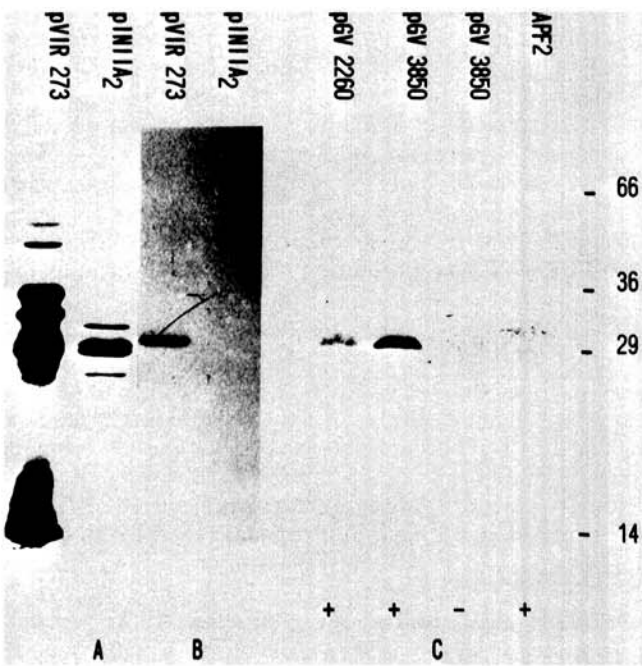

Fig. 2. *virG*-specific antiserum: Immunological detection of *virG*-encoded protein. **A**, In *Escherichia coli*, radioactive proteins synthesized in minicells with pVIR273 (*virG*) and pINIIA2 (vector control): **B**, The corresponding immunoprecipitates. **C**, In *Agrobacterium*, western blots of cells containing the octopine Ti plasmid pGV2260, the nopaline Ti plasmid pGV3850, or no plasmid (APF2). + = induction with simplified induction medium (SIM) complete at 25° C for 6 hr; − = noninduced (YEB medium, pH 7.2, at 28° C). At right: molecular mass markers in kDa.

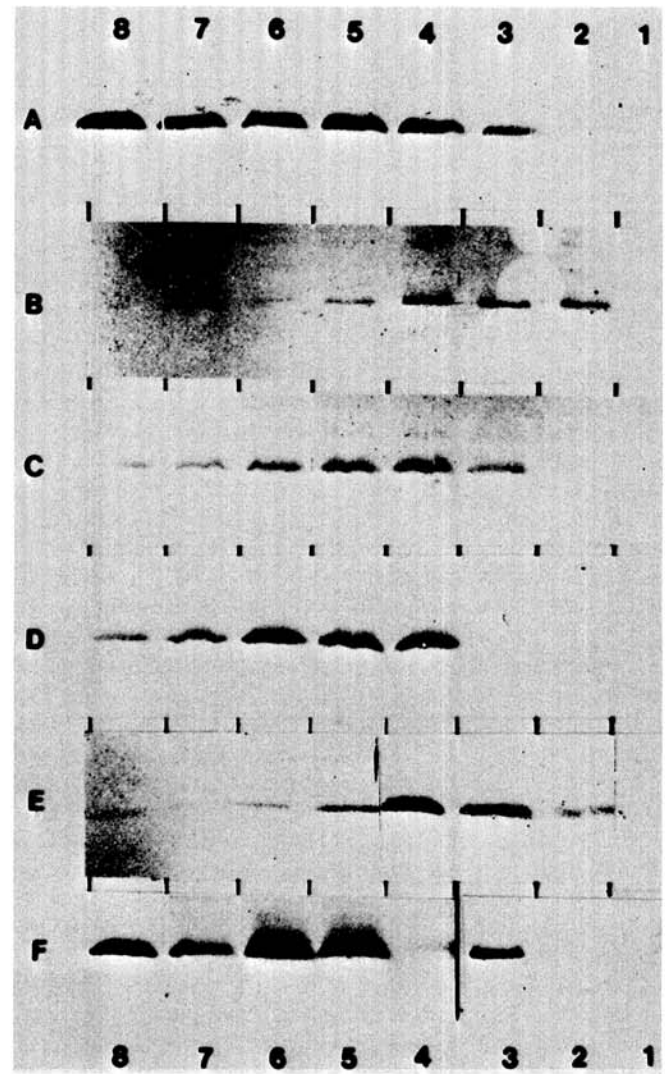

Fig. 3. Western blot analysis of *virG* induction in *Agrobacterium* containing the nopaline plasmid pGV3850. Only the parts of the blots containing the *virG*-encoded protein are shown. If not stated otherwise, the experiments were performed with simplified induction medium (SIM) complete at 25° C. In the series **B** to **F**, the cells were harvested 3 hr after the onset of induction. **A**, Time dependence in SIM complete (1 = 0 hr, 2 = 0.5 hr, 3 = 1 hr, 4 = 2 hr, 5 = 4 hr, 6 = 6 hr, 7 = 8 hr, and 8 = 21 hr). **B**, Temperature dependence (1 = 6° C, 2 = 15° C, 3 = 20° C, 4 = 25° C, 5 = 28° C, 6 = 30° C, 7 = 32° C, and 8 = 37° C). **C** and **D**, pH dependence in the absence (**C**) and presence (**D**) of acetosyringone (1 = pH 4.8, 2 = pH 5.0, 3 = pH 5.25, 4 = pH 5.5, 5 = pH 5.75, 6 = pH 6.0, 7 = pH 6.5, and 8 = pH 7.2). **E**, Sucrose dependence (1 = 0%, 2 = 0.5%, 3 = 1%, 4 = 2%, 5 = 3%, 6 = 5%, 7 = 7%, and 8 = 10%). **F**, 1 = SIM complete plus 5 mM potassium acetate; 2 = SIM complete plus 1 mM potassium acetate; 3 = SIM complete without additions; 4 = YEB at pH 7.2 without acetosyringone, 25° C; 5 = YEB at pH 5.5 plus 0.1 mM acetosyringone, 25° C; 6 = SIM complete at pH 5.5 plus 0.1 mM acetosyringone, 25° C; 7 = SIM complete at pH 5.5 without acetosyringone, 25° C; and 8 = SIM complete at pH 5.5 plus 0.1 mM acetosyringone plus 25 mM potassium phosphate (pH 5.5), 25° C.

Even if all other factors are optimal, the induction is completely dependent on the presence of sucrose, 2% is optimal; the induction decreases with higher concentrations (Fig. 3E). The expression of *virG* is inhibited by acetate salts (1 mM is sufficient for complete inhibition; Fig. 3F, compare lanes 1 to 3). Phosphate salts up to 20 mM have no significant effect on induction, but higher concentrations are inhibitory to some extent (Fig. 3F, compare lanes 6 and 8). No significant differences between *virG* and *virD*2 (Alt-Mörbe *et al.* 1988) were detected for these properties.

The results indicate that the regulation of *virG* and *virD* is comparable in some aspects. The expression is strictly dependent on temperature and sucrose. Induction in the presence of the inducing signals is not inhibited by typical components of rich media (for example, beef extract, yeast extract, or peptone of YEB) or by phosphate salts (up to 20 mM), but acetate salts are strongly inhibitory. The differences are twofold: the lag period is shorter with *virG* than with *virD*2, and acetosyringone and acidic pH are stringent requirements for *virD*, but not for *virG* induction.

***virD*: Lag period and preconditioning for initiation of expression.** The construction of a transcriptional fusion of the nopaline *virD* promoter region with a promoterless *galK* cassette (pVIR271) is shown in Figure 1 (right). This reporter gene construct was used after conjugation into C58C1(pGV2260), and galactokinase activity was monitored as a measure of *virD* promoter activity. The use of galactokinase as a reporter enzyme in *Agrobacterium* was established by the following: 1) a promoterless *galK* gene (pVIR280.2) did not lead to galactokinase activity, and this result showed that the vector plasmid pCP13 contained no promoter activity reading into the cloned fragment and that the bacteria themselves do not express galactokinase under the induction conditions; and 2) extracts from *Agrobacterium* cells containing a plasmid with the complete *galK* gene including the promoter (pV1R269.1) were used to establish the extraction and assay conditions for galactokinase activity.

Figure 4 summarizes some of the results of the preconditioning experiments. The cells were exposed either to SIM minus acetosyringone throughout the experiment (negative control) or to SIM complete throughout the experiment (positive control for each of the variables necessary for induction: sucrose, a plant signal, acidic pH, and correct temperature), or cells were exposed for 1.5 hr to a combination of three inducing factors, and the missing factor (acetosyringone, or correct temperature, or sucrose) was adjusted after this preincubation period. The negative control demonstrates that galactokinase activity does not increase under noninduced conditions. The positive control shows that a small but significant increase of *virD* transcription is detectable after 1.5 hr. This is much shorter than described previously (Winans *et al.* 1988), and slightly shorter than measured by the direct demonstration of the *virD*2-encoded protein in western blots (Alt-Mörbe *et al.* 1988), suggesting that the reporter gene assay is more sensitive.

The other curves in Figure 4 (missing factor added after 1.5 hr) show significant increases of *galK* activity only after an additional period of 1 to 1.5 hr, indicating that none of the preincubations with a three-factor combination leads to a significant reduction of the lag period. The slope of the curves later on is comparable to that of the positive control, suggesting that the overall capacity for *virD* induction is not affected by the preincubations. Very similar data were obtained when the cells were first exposed to all factors except for the correct pH (changed from 7.2 to 5.5 after 1.5 hr). The results indicate that all four factors must be present simultaneously for initiation of induction. Because the effect of sucrose could simply be due to a lack of energy available for protein synthesis, we also compared the rates of *de novo* protein synthesis in SIM complete in the presence (2%) and absence of sucrose. The results of *in vivo* pulse-labeling experiments with [^{35}S]methionine with several time points up to 20 hr (not shown) indicate: 1) in the absence of sucrose, the rate of amino acid uptake is reduced by 40–60%; and 2) when

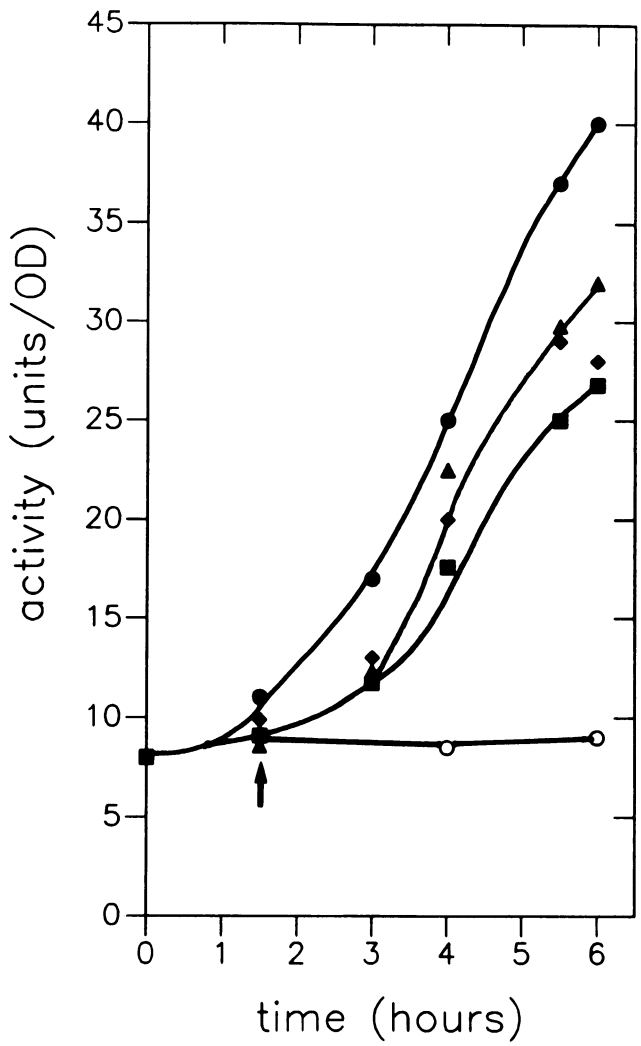

Fig. 4. Preconditioning for induction of *virD*. C58C1(pGV2260) harboring pVIR271 (*virD* promoter and *galK* construct) was transferred at time 0 to SIM (simplified induction medium) lacking one induction factor; the missing factor was added after 1.5 hr, and *virD* induction was monitored by measuring galactokinase activity. Preconditioning media: (■) SIM without acetosyringone, (▲) SIM without sucrose, and (◆) SIM at a high temperature (34° C). Positive control: (●) SIM complete throughout the experiment. Negative control: (○) SIM without acetosyringone throughout the experiment.

this is taken into account, the rate of incorporation from the intracellular pool of radioactive methionine into trichloroacetic acid precipitable material in the absence of sucrose is, throughout the time course, at most 40% lower than in the presence of sucrose. The results indicate that cells kept without sucrose are quite capable of *de novo* protein synthesis for prolonged periods of time.

***virD*: Controls in maintenance of expression.** Figure 5 summarizes experiments investigating the question of whether *virD* expression becomes independent of external factors once it is initiated. Cells containing pGV2260 and the *virD* promoter-reporter plasmid pVIR271 were induced for 11 hr in SIM^+ complete (SIM supplemented with peptone), and the cells were subsequently pelleted and resuspended in SIM^+ complete or in SIM^+ lacking one of the inducing factors. The result with SIM^+ complete shows that the experimental manipulation of the cells by centrifugation has no significant effect on continued *virD* expression. Drastic effects, however, are observed with SIM^+ now lacking acetosyringone or with SIM^+ at a high temperature (34° C): gene expression ceases without noticeable delay, suggesting that these two factors must be present continuously to maintain induction. The removal of sucrose reduced expression slightly during the next 5 hr, and after that no further increase was observed. A raise of pH to 7.0 reduced expression to a lower rate. All of these experiments were also performed after the removal of an induction factor (switch off) at 3 or 8 hr after the start of induction, and the results are the same as described for 11 hr. Taken together, these results suggest that temperature and acetosyringone exert an immediate control on the maintenance of induction, while the effects of sucrose and pH are more complex.

Previous experiments (Alt-Mörbe *et al.* 1988) have shown that low concentrations of acetate completely inhibit the initiation of induction under otherwise optimal conditions; therefore, we also tested whether maintenance of *virD* expression was affected similarly. Figure 6 shows that 5

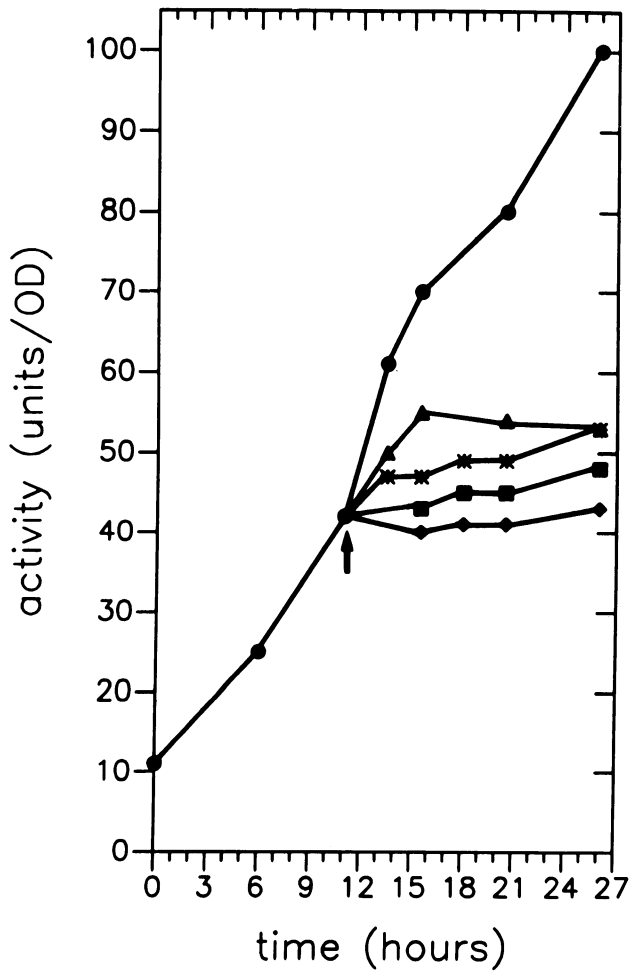

Fig. 5. Continued control of induced *virD* expression. C58C1(pGV2260) harboring pVIR271 was induced at 25° C for 11 hr with simplified induction medium (SIM) complete supplemented with peptone (SIM^+). The cells were subsequently centrifuged and resuspended in SIM^+ complete or SIM^+ lacking one induction factor, and *virD* expression was monitored by measuring galactokinase activity. Composition of the medium after 11 hr: (■) SIM without acetosyringone, (▲) SIM without sucrose, (◆) SIM at a high temperature (34° C), and (*) SIM at pH 7. Positive control for continued induction: (●) SIM complete throughout the experiment.

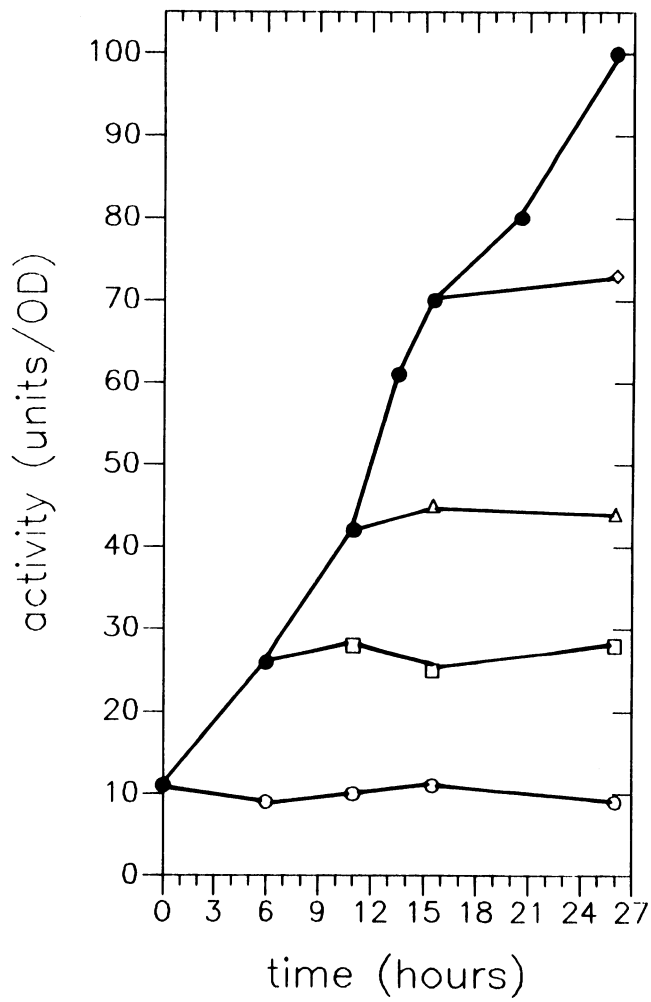

Fig. 6. Inhibition of *virD* expression by acetate. C58C1(pGV2260) harboring pVIR271 was induced in simplified induction medium (SIM) complete at 25° C. Potassium acetate (5 mM, pH 5.5) was added at 0 hr (○), 6 hr (□), 11 hr (△), or 15.5 hr (◇) after the onset of induction, and *virD* expression was monitored by measuring galactokinase activity. (●) = induction without the addition of potassium acetate.

mM potassium acetate is sufficient to inhibit the initiation as well as the maintenance of expression when added at 0, 6, 11, or 15.5 hr after transfer of the cells into SIM complete.

DISCUSSION

Our results indicate that cells grown in YEB at pH 7.2 and 28° C contain very little if any *virG*-encoded protein, as measured by detectable protein on western blots. Beef extract, yeast extract, or peptone as components of the YEB medium do not inhibit expression in the presence of the inducing factors; similar results have been reported by Rogowsky *et al.* (1987). The induction of *virG* requires temperatures below 28° C and sucrose; expression in the presence of these two factors is stimulated by acetosyringone and acidic pH.

The absence or very low level of *virG* expression in noninduced cells is in contrast to most of the published reports analyzing transcriptional regulation, with the exception of Winans *et al.* (1988) who described very low levels of noninduced *virG* transcription. It seems likely that the conditions of the precultures are the main reason for these differences with respect to the extent of "constitutive" expression of *virG*. For example, Stachel and Nester (1986) and Stachel and Zambryski (1986) describe for octopine *virG* significant levels of constitutive transcription and about a 13-fold stimulation by inducing factors. Preculturing was performed at 28° C in AB minimal liquid medium at pH 7.0 and with glucose as a carbon source. It is very likely that this combination of carbon source, pH, and temperature (upper limit of the range allowed for induction) does stimulate some expression of the *virG* gene in the preculture. These authors also described two different transcripts from *virG*: C-RNA ("constitutive," starting just one base before the presumed AUG start codon, present in noninduced and induced cells) and I-RNA ("inducible," 50 nucleotides longer than C-RNA, present only in induced cells).

It would seem useful to reexamine the significance of these two transcripts if in fact the precultures were already partially induced, and in particular because it is not clear whether C-RNA is translated efficiently into protein. Rogowsky *et al.* (1987) reported with both octopine and nopaline plasmids high constitutive transcription and little if any inducibility of *virG*. Standard preculturing was at 25° C in Murashige-Skoog medium, which has pH 5.7 and contains sucrose as a carbon source. Our findings suggest that the precultures were already induced at least partially for *virG*. It may be useful to use the term "constitutive" for *virG* expression only in direct context with the precise culture conditions. The results of Winans *et al.* (1988) suggest that the same consideration may apply to *virA*.

The available data indicate that regulation of *virG* expression is complex. Early reports stressed the role of plant signals (for example Stachel and Nester 1986; Stachel and Zambryski 1986); later reports pointed out that acidic pH (Rogowsky *et al.* 1987), or acidic pH and high osmotic stress (Vernade *et al.* 1988), or acidic pH and phosphate depletion are important factors, and their effects are probably mediated by chromosomal genes (Winans *et al.* 1988). In agreement with the findings of Rogowsky *et al.* (1987), our results give no indication that phosphate depletion plays a role. The induction medium used in our experiments is free of phosphate and thus should impose such stress on the cells, but the addition of less than 20 mM of such salt did not lead to a significant reduction in *virG* induction.

The results described here indicate that acidic pH and acetosyringone are stimulatory, but not essential for *virG* expression; however, correct temperature and availability of sucrose are stringent requirements. For *virG*, both of these factors have not been tested previously. It might seem that the necessity for sucrose simply indicates that the bacteria are starved for the energy which is required for protein synthesis. This, however, is not the case. Pulse-labeling experiments show that *de novo* protein synthesis, albeit at a reduced rate, is maintained for at least 20 hr in the absence of sucrose, suggesting that other regulatory mechanisms are involved. The temperature effect is of particular interest, because a thermosensitive step in tumor induction is well-known. The activity of *virG* is a prerequisite for the induction of the *virD* function. It seems, therefore, quite likely that the previously described dependence of *virD* expression and of *tzs*-encoded cytokinin secretion on temperature and on sucrose (Alt-Mörbe *et al.* 1988) is a consequence of noninduction of *virG*.

Winans *et al.* (1988) proposed a two-step model for induction of the *vir* region: acidic pH induces *virG* expression; and *virA*, activated by plant signals, converts *virG* to the active form that creates a feedback loop of positive autoregulation of *virA* and *virG* induction and activation of the other *vir* genes. This model accommodates the controlling functions of these two genes, and it could also explain the rather slow kinetics of *vir* gene induction (6-hr lag period). Our observations refine several points in this model.

1. Temperature and sucrose availability appear to be the stringent requirements for *virG* induction rather than pH. However, *virD* expression also requires acidic pH and acetosyringone, indicating that the second step of *vir* gene induction is controlled more tightly. One of the consequences of this difference is that *virG*-encoded protein may be present without the concomitant induction of *virD*. This supports the idea that the *virG*-encoded protein must be activated, presumably by acetosyringone-regulated activity of the *virA*-encoded protein, before it can activate the other *vir* genes. This is certainly not the only point of control, because chromosomal genes involved in *virD* regulation have also been identified (Close *et al.* 1987; Tait and Kado 1988).

2. The lag period of *vir* gene expression is shorter than assumed previously (for example Winans *et al.* 1988). The *virG*-encoded protein is clearly detectable 1 hr after the onset of induction, and only a slightly longer lag (1–1.5 hr) is observed for *virD*. Considering the multifactorial regulation of *vir* gene expression, it is of interest that the lag phase cannot be explained by a hypothesis that one or several of the factors are necessary for an adaptation of the cells before they become competent for response

to plant signals; the preconditioning experiments show that all inducing factors must be present simultaneously for the initiation of *virD* induction.

3. A model for a feedback loop of positive autoregulation must deal with the question of whether or not induction leads to an irreversible commitment to a certain physiological state. The first possibility is realized in the well-known bacteriophage lambda system (Ptashne *et al.* 1980), but this does not appear to be the case with the *vir* genes of Ti plasmids. The switch-off experiments show that the expression of *virD* is stopped rapidly after removing acetosyringone or increasing the temperature, and more slowly after removing sucrose or increasing the pH. This is of interest because it shows that expression of the virulence genes may be suppressed or reduced at any time by a change of environmental conditions. Recent experiments by Vernade *et al.* (1988) have shown that raising the pH in the induction medium at 10 or 20 hr after the onset of induction also blocked the expression of *virG* and *virE*, respectively.

Our results indicate that the initiation of *vir* gene expression is under the control of at least four factors and that in the case of *virG*, the factors of temperature and sucrose availability overrule the regulation by acidic pH or acetosyringone. This is not the case with *virD*, which stringently requires the simultaneous presence of all four factors. The mechanisms of these controls are not understood, but they must operate at multiple levels. It is tempting, however, to speculate that at least for the maintenance of *virD* expression, the fast stop response to removing acetosyringone and to increasing temperature is mediated at a common step in a direct signal chain controlling *vir* gene expression. The most likely target seems to be the catalytic activity of *virA*: the protein is localized in the membrane (Leroux *et al.* 1987; Melchers *et al.* 1987) and is thus the most direct sensor for environmental conditions. And, it is thought that *virA* is responsible for activation of the *virG*-encoded protein, as proposed by Stachel and Zambryski (1986).

ACKNOWLEDGMENTS

We thank G. Lurz for his competent technical help, B. Rak for his introduction to the galactokinase assay, and O. Schmidt for providing vector pATH11. This work was supported by Deutsche Forschungsgemeinschaft (SFB206) and Fonds der Chemischen Industrie.

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