tein that recognizes the E2 upstream element a cellular protein, and if so, is it present in uninfected cells? If the protein is present before and after the action of E1A, then how is it different as a result of the action of the E1A protein?

REFERENCES AND NOTES

1. A. J. Berk *et al.*, *Cell* **17**, 935 (1979).
2. N. Jones and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3665 (1979).
3. J. R. Nevins, *Cell* **26**, 213 (1981).
4. ———, *ibid.* **29**, 913 (1982).
5. H.-T. Kao and J. R. Nevins, *Mol. Cell. Biol.* **3**, 2058 (1983).
6. R. Stein and E. B. Ziff, *ibid.* **4**, 2792 (1984).
7. R. Elkaim, C. Goding, C. Kedinger, *Nucleic Acids Res.* **11**, 7105 (1983).
8. M. J. Imperiale and J. R. Nevins, *Mol. Cell. Biol.* **4**, 875 (1984).
9. M. J. Imperiale, R. P. Hart, J. R. Nevins, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 381 (1985).
10. R. E. Kingston, R. J. Kaufman, P. A. Sharp, *Mol. Cell. Biol.* **4**, 1970 (1984).
11. S. C. S. Murthy, G. P. Bhhat, B. Thimmappaya, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2230 (1985).
12. D. S. Zajchowski, H. Boeuf, C. Kedinger, *EMBO J.* **4**, 1293 (1985).
13. C. Wu, *Nature (London)* **309**, 229 (1984).
14. L. T. Feldman, M. J. Imperiale, J. R. Nevins, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4952 (1982).
15. M. J. Imperiale, L. T. Feldman, J. R. Nevins, *Cell* **35**, 127 (1983).
16. J. C. Alwine, *Mol. Cell. Biol.* **5**, 1034 (1985).
17. M. J. Imperiale, H.-T. Kao, L. T. Feldman, J. R. Nevins, S. Strickland, *ibid.* **4**, 867 (1984).
18. M. R. Green, R. Treisman, T. Maniatis, *Cell* **35**, 137 (1983).
19. R. B. Gaynor, D. Hillman, A. J. Berk, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1193 (1984).
20. R. B. Gaynor and A. J. Berk, *Cell* **33**, 683 (1983).
21. S. L. McKnight, R. C. Kingsbury, A. Spence, M. Smith, *ibid.* **37**, 253 (1984).
22. G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984).
23. A. Ephrussi, G. M. Church, S. Tonegawa, W. Gilbert, *Science* **227**, 134 (1985).
24. W. S. Dynan and R. Tjian, *Cell* **32**, 669 (1983).
25. ———, *ibid.* **35**, 79 (1983).
26. M. G. Katze, H. Persson, L. Philipson, *Mol. Cell. Biol.* **1**, 807 (1981).
27. F. R. Cross and J. E. Darnell, *J. Virol.* **45**, 683 (1983).
28. H.-T. Kao, O. Capasso, N. Heintz, J. R. Nevins, *Mol. Cell. Biol.* **5**, 628 (1985).

29. Supported by a fellowship from the N.S.E.R.C. (Canada) (to I.K.), by a fellowship from the Deutsche Forschungsgemeinschaft (to R.R.), by NIH research career development award CA00666 (to J.R.N.), and by NIH grant GM26765.

5 August 1985; accepted 6 December 1985

Adrenal-Mediated Endogenous Metabolites Inhibit Puberty in Female Mice

MILOS NOVOTNY, BOZENA JEMIOLO,* SCOTT HARVEY, DONALD WIESLER, ANNA MARCHLEWSKA-KOJ*

While assessing a potential role of adrenal glands in the production of the hitherto unidentified puberty-delaying pheromone of female mice, the urinary volatile profiles of normal and adrenalectomized animals were quantitatively compared. Six components, whose concentrations were depressed after adrenalectomy, were identified: 2-heptanone, *trans*-5-hepten-2-one, *trans*-4-hepten-2-one, *n*-pentyl acetate, *cis*-2-penten-1-yl acetate, and 2,5-dimethylpyrazine. When these laboratory-synthesized chemicals were added (in their natural concentrations) to either previously inactive urine from adrenalectomized females or plain water, the biological activity was fully restored.

BOTH SOCIAL AND ENVIRONMENTAL factors may accelerate or delay the timing of sexual maturation in the female house mouse, *Mus musculus*. The onset of puberty in juvenile female mice is modified by urinary chemical cues (priming pheromones) originating from both males and females. Treatment of juvenile females with the urine of normal males (*1–3*), or its high molecular weight fraction (*4*), causes puberty acceleration, whereas treatment of juvenile females with the excreted urine of intact females that had been housed together for at least 10 days (*5*) substantially delays puberty (*6*). The efficiency of this female-to-female puberty-delaying urinary factor depends on the density and duration of the grouping of the female urine donors (*5, 7, 8*) but not on the donor's age (*7, 9*). Social contact, while necessary for pheromone production, does not appear to enhance the delay of sexual development in juvenile mice (*10*). Changes in gonadal activity, in adrenal gland size, and in the secretion of adrenal hormones, and decreased reproductive performance appear to result from social stress in high-density populations of various rodent species (*11–13*). Studies of Drickamer and co-workers indicate that adrenalectomy (*14*) but not ovariectomy (*15*) abolishes the biological activity of excreted urine to delay puberty in juvenile mice.

On the basis of the suggestion of Christian (*11–13*) and Drickamer and co-workers (*14, 15*) that the puberty-inhibiting effect may be associated with the adrenal function, we designed the series of experiments in which (i) chemical differences between the urinary excretion of normal and adrenalectomized female animals were quantitatively observed; (ii) the urinary volatile substances that differed consistently between the two sample types were subsequently identified and synthesized for both structural verification and biological testing; and (iii) these synthetic pheromone candidates were tested both in mixtures and individually to determine whether their action parallels that of the natural urinary stimuli. These results

Table 1. Mean (in days) of the first vaginal estrus (± SEM) for young female mice painted daily on the external nares with various stimuli or water (control). Means marked with an asterisk are significantly different from means without an asterisk ($P < 0.001$); $F(\text{df } 8267) = 27.15$, $P < 0.005$ (Duncan's new multiple range test).

Treatment	*n*	Mean day to reach first estrus
April		
Water (control)	32	30.1 (0.3)
Urine from intact females caged:		
Singly	32	30.5 (0.2)
Group	32	33.9*(0.4)
October		
Water (control)	30	31.1 (0.3)
Urine from ovariectomized females caged:		
Singly	30	31.3 (0.4)
Group	30	33.8*(0.5)
September		
Water (control)	30	29.6 (0.3)
Urine from adrenalectomized females caged:		
Singly	30	30.5 (0.3)
Group	30	30.4 (0.3)

Department of Chemistry, Indiana University, Bloomington 47405.

*On leave from the Institute of Zoology, Jagiellonian University, 30-060 Krakow, Poland.

furnish independent verification of the experiments of Drickamer and co-workers.

ICR/Alb mice were purchased from Ward's Natural Science Establishment (Rochester, NY). Intact and adrenalectomized females (surgery performed by the supplier), 80 to 100 days old, were used as urine donors. Singly caged and group-caged urine donors (ten animals per cage) were housed for acclimatization for at least 3 weeks prior to urine collection. The adrenalectomized animals were supplemented with water containing 0.9 percent sodium chloride and 1.0 percent sucrose (*16*). Urine samples from adult, ovariectomized females (50 to 70 days of age at the time of surgery) were used for comparative experiments. These samples were collected 50 days after surgery (recovery period plus acclimatization). For each experimental group, we collected and combined urine from eight to ten females by holding a mouse over a glass vial and gently squeezing the abdomen and flanks. Immediately after collection, samples were stored at −4°C until analyzed or otherwise tested. Use of controls in the bioassay procedure assured adequate checks of biological activity.

Our bioassay procedure was similar to that described by Drickamer and co-workers (*14, 15, 17*). Pregnant female mice were isolated into individual cages a few days before parturition and checked daily for newborns. Pups from each litter were counted and sexed. Each litter was reduced to ten pups, two or three of which were males to eliminate size and sex composition differences in a litter (*18*). The young mice were weaned 21 days after birth and were immediately assigned to a treatment group and cage according to a random sequence. The females were housed two to three per cage and painted daily on their external nares with 0.02 ml of water (control) or with excreted urine from adult females (experimental groups). Each test mouse was examined daily from day 21 until the occurrence of vaginal perforation. Starting on the day of vaginal opening, vaginal smears were taken until the occurrence of the first vaginal estrus. We examined the vaginal smears immediately under a light microscope and recorded the stage of estrous cycle, using the criteria of Rugh (*19*) and Vandenbergh (*2*). All mice were weighed prior to testing; for each experiment, a one-way analysis of variance was used to compare the body weight. There were no significant differences in weaning body across the various treatment groups.

All mice were housed in plastic cages (12 by 28 by 17 cm) and maintained at 21° ± 0.2°C, 50 to 70 percent humidity, and a 12-hour light/12-hour dark daily regime. Unlimited amounts of Purina Mouse Chow (Ralston Purina Company, St. Louis, MO) and water were supplied throughout each experiment. Bedding was changed weekly.

Vandenbergh *et al.* (*20*) reported a seasonal variation in uterine weight for young female house mice. A seasonal variation in the ability to accelerate or delay puberty was also reported by Drickamer (*21*). In view of these observations, we considered the data obtained from this study in relation to season. The experimental tests were carried out during fall and spring (Table 1) and fall to spring and summer (Table 2).

Females painted daily with the excreted urine from intact or ovariectomized donors, housed in groups, matured significantly later than young females treated with water or with excreted urine from singly caged intact or ovariectomized females. Likewise, the excreted urine from singly or group-caged adrenalectomized donors did not cause statistically significant puberty delay (Table 1).

Following the suggestion that the adrenals (*14*) but not the ovary tissue (*15*) control the puberty-delaying pheromone, we analyzed urinary samples of normal and adrenalectomized animals by gas-phase analytical techniques (*22*). According to current knowledge of the chemical nature of mammalian messengers, either relatively small (volatile) or large molecules may act as pheromones. Our main reason for using gas-phase analytical techniques was that in a recent study by Coppola and Vandenbergh (*23*) the activity of the puberty-delaying chemosignal was lost after exposure to air at room temperature; these results suggested that the pheromone was either an air-sensitive or a volatile substance. Another reason is the success we have had using gas-phase analytical methodology in our other pheromone investigations in mice (*24–26*).

The volatiles were first sparged from 1-ml urinary samples at room temperature with purified helium gas (100 ml/min) and adsorbed on a precolumn packed with Tenax GC porous polymer (*27*). The sample was subsequently desorbed in the heated injec-

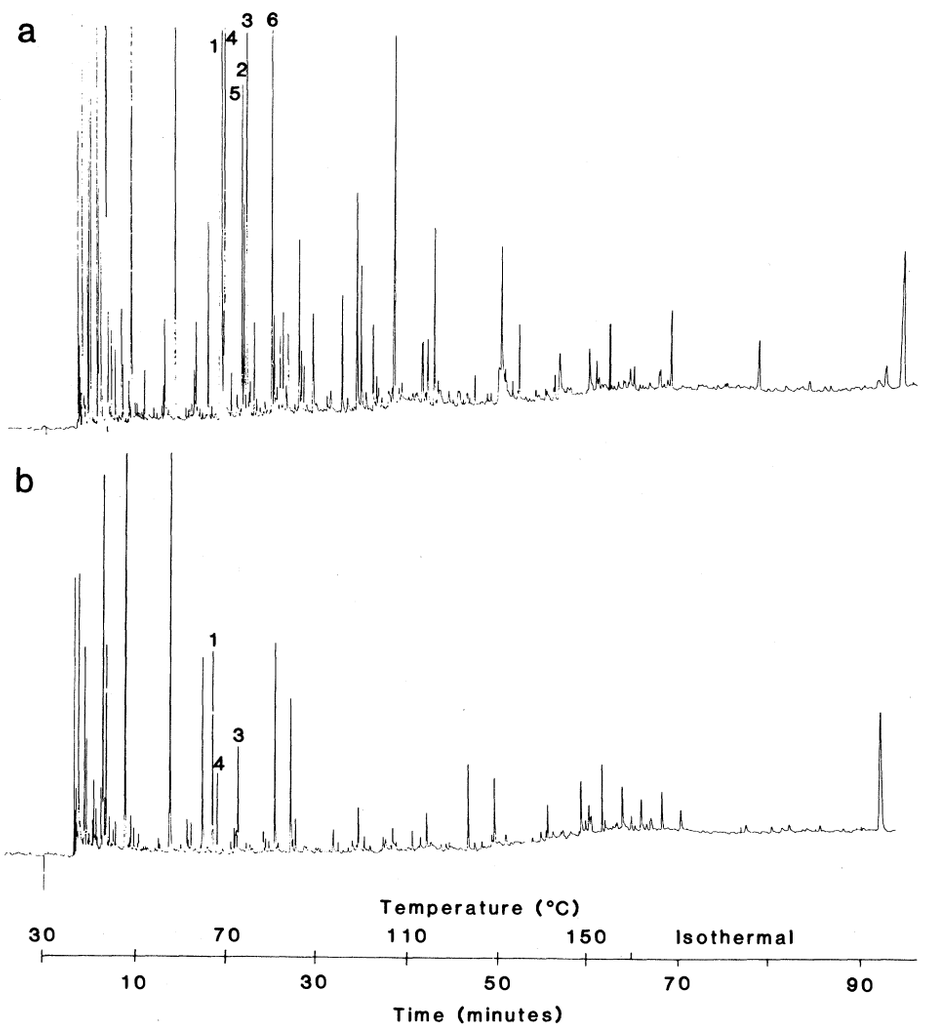
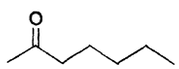


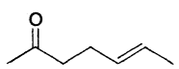
Fig. 1. High-resolution gas-phase chromatograms of urinary samples from (a) normal and (b) adrenalectomized female mice.

tion port (220° to 240°C) of a gas chromatograph and retrapped into a cooled section of a glass capillary column (*24*). Subsequent temperature-programmed analysis yielded a high-resolution chromatogram. A comparison of typical urinary volatile profiles obtained from the two sample types is shown in Fig. 1. Inspection of numerous urinary samples revealed that, although the urines of adrenalectomized animals tend to be more "dilute" and qualitative variations among them may exist, only chromatographic peaks 1 through 6 are consistently depressed after adrenalectomy. A combination of capillary gas chromatography and mass spectrometry (Hewlett-Packard model 5710 A) was used to identify components 1 through 6 (*28*), the structures of which are as follows:



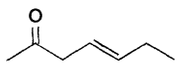
2-heptanone

1



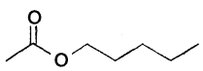
trans-5-hepten-2-one

2



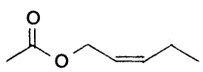
trans-4-hepten-2-one

3



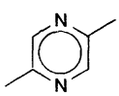
n-pentyl acetate

4



cis-2-penten-1-yl acetate

5



2,5-dimethylpyrazine

6

Conventional organic synthesis procedures were used to obtain components 1 through 6 for authentication as well as biological tests.

Although the urines from either singly caged or group-caged adrenalectomized females did not elicit the puberty-delay effect (see Table 1), spiking the urines of adrenalectomized animals with components 1 through 6 at the levels approximating their natural content (*29*) led to a full recovery of biological activity (Table 2). Components 1 through 6 belong to three structurally distinct classes of compounds: (i) ketones, (ii) acetate esters, and (iii) pyrazine. We examined the effect of the addition of the three classes of compounds individually to the urine of adrenalectomized female mice to determine whether the biological response is due to a single chemical. Statistically significant effects appear to be due to the pyrazine derivative alone and to the acetates, or to both types of compounds together, but not to the ketones alone (Table 2). A continuous use of controls (treatment with water) has minimized variations in the first day of estrus throughout a prolonged overall testing period. The significant differences between the control groups are emphasized by seasonal variation in the time needed for young female mice to reach puberty (*20*).

Table 2. Mean (in days) of the first vaginal estrus (± SEM) for young female mice painted daily on the external nares with excreted urine from adrenalectomized females mixed with compounds 1 through 6 or water (control). Those means not marked with the same superscript letter (a, b, c, d, or e) are significantly different at the $P = 0.005$ level: Duncan's new multiple range test, $F(\text{df } 10,386) = 21.95$; $P < 0.005$.

Treatment	*n*	Mean day to reach first estrus
	March	
Water (control)	30	28.8^{b} (0.3)
Compounds 1, 2, and 3 (ketones)	30	$29.8^{b,c}$(0.3)
	February	
Water (control)	30	$29.8^{b,c}$(0.3)
Compounds 4 and 5 (acetate esters)	30	$31.3^{d,e}$(0.3)
Compound 6 (pyrazine)	30	32.2^{e} (0.3)
	March	
Water (control)	30	$29.8^{b,c}$(0.2)
Compounds 4, 5, and 6	30	31.5^{e} (0.3)
	June	
Water (control)	29	27.4^{a} (0.5)
Water plus compounds 1 through 6	29	$30.1^{c,d}$(0.5)
	November	
Water (control)	65	28.5^{b} (0.3)
Compounds 1 through 6	64	32.2^{e} (0.3)

Perhaps the most striking result from the experiments summarized in Table 2 is the fact that the synthetic mixture of these six compounds, in water solution in the concentrations mimicking their natural levels in the mouse urine (*29*), is biologically active. We have recently described that two synthetic male mouse urinary components, 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole, induce aggression in other males (*25*) and preferential sniffing in females (*26*); however, the substances were behaviorally active only when presented in the overall context of urinary odors but not in pure water.

Puberty delay by urinary cues from female *Mus musculus* has been widely documented under laboratory conditions; similar phenomena appear to operate in other rodent species as well (*12, 30*). The results of Massey and Vandenbergh (*8*), obtained from studies of the urines of wild mice living in feral populations (isolated "highway islands") of different population density, support the notion of a broader ecological significance of the puberty-delaying signal. Perhaps the substances identified in this work could be used in rodent pest control.

Thus far, we have been unable to demonstrate any significant quantitative chemical differences between the urinary volatiles of singly caged and group-caged female mice. Although an association between the puberty-delaying factor and the adrenal function, as observed by Drickamer and McIntosh (*14*), has been corroborated and supported by chemical evidence in the present study, the question remains unanswered whether the adrenally mediated stimuli are synonymous with or different from the naturally occurring pheromone or pheromones.

REFERENCES AND NOTES

1. J. G. Vandenbergh, *Endocrinology* **81**, 345 (1967).
2. ———, *ibid.* **84**, 658 (1969).
3. F. H. Bronson and C. Desjardins, *ibid.* **94**, 1658 (1974).
4. J. G. Vandenbergh, J. Finlayson, W. J. Dobrogosz, S. S. Dulls, T. A. Kost, *Biol. Reprod.* **15**, 260 (1976).
5. L. C. Drickamer, *J. Reprod. Fertil.* **69**, 723 (1983).
6. ———, *Dev. Psychobiol.* **15**, 433 (1982); *Anim. Behav.* **30**, 456 (1982).
7. ———, *Dev. Psychobiol.* **7**, 257 (1974).
8. A. Massey and J. G. Vandenbergh, *Science* **209**, 821 (1980).
9. J. J. Cowley and D. R. Wise, *Anim. Behav.* **20**, 499 (1972); D. R. Colby and J. G. Vandenbergh, *Biol. Reprod.* **11**, 268 (1974).
10. L. C. Drickamer, in *Environmental Factors in Mammal Reproduction*, D. Gilmore and B. Cook, Eds. (University Park Press, Baltimore, 1981), pp. 100–111.
11. J. J. Christian, *Coll. Int. C.N.R.S.* **173**, 289 (1968).
12. ———, *Biol. Reprod.* **4**, 248 (1971).
13. ——— and D. E. Davis, *Science* **146**, 1550 (1964).
14. L. C. Drickamer and T. K. McIntosh, *Horm. Behav.* **14**, 146 (1980).
15. ———, E. A. Rose, *ibid.* **11**, 131 (1978).
16. P. C. Will and M. E. Barnett, *Lab. Anim. Sci.* **33** (No. 2), 172 (1983).
17. T. K. McIntosh and L. C. Drickamer, *Anim. Behav.* **25**, 999 (1977).
18. L. C. Drickamer, *J. Reprod. Fertil.* **46**, 369 (1976).
19. R. Rugh, *The Mouse, Its Reproduction and Development* (Burgess, Minneapolis, 1968).

20. J. G. Vandenbergh, J. M. Whitsett, J. R. Lombardi, *J. Reprod. Fertil.* **43**, 515 (1975).
21. L. C. Drickamer, *ibid.* **72**, 55 (1984).
22. M. Novotny, M. L. Lee, K. D. Bartle, *Chromatographia* **7**, 333 (1974).
23. D. M. Coppola and J. G. Vandenbergh, *J. Reprod. Fertil.* **73**, 517 (1985).
24. F. J. Schwende, D. Wiesler, M. Novotny, *Experientia* **40**, 213 (1984); F. J. Schwende, J. W. Jorgenson, M. Novotny, *J. Chem. Ecol.* **10**, 1603 (1984).
25. M. Novotny, S. Harvey, B. Jemiolo, J. Alberts, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2059 (1985).
26. B. Jemiolo, J. Alberts, S. Sochinski-Wiggins, S. Harvey, M. Novotny, *Anim. Behav.* **33**, 1114 (1985).
27. R. van Wijk, *J. Chromatogr. Sci.* **8**, 418 (1970).
28. Components 1 through 6 are relatively simple organic compounds. Their structures were readily suggested by comparison with the available libraries of mass spectra or through interpretation of simple fragmentation patterns. Authentic chemicals were further analyzed to verify coincidence of both mass spectra and retention indices in high-resolution capillary gas chromatography. Although compounds 1, 4, and 6 are readily available from commercial sources, compounds 2, 3, and 5 were synthesized in our laboratory through simple procedures. A perfect match between mass spectra and chromatographic migration was obtained for all six components.
29. The six synthetic compounds were added to either urine from adrenalectomized females or water, corresponding to concentrations of 2.5, 0.28, 0.39, 0.50, 1.1, and 0.25 ppm, respectively.
30. J. R. Clark, *Proc. R. Soc. London Ser. B* **144**, 68 (1955); J. J. Christian, *Ecology* **37**, 258 (1956); C. R.Terman, *ibid.* **49**, 1169 (1968); L. L. Getz, D. Dluzen, J. L.McDermott, *Behav. Process.* **8**, 59 (1983).

28 October 1985; accepted 19 November 1985

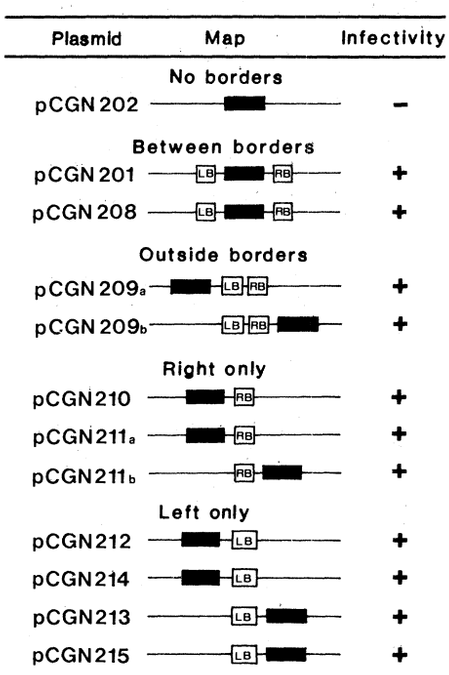
Transfer of *Agrobacterium* DNA to Plants Requires a T-DNA Border But Not the *vir*E Locus

RICHARD C. GARDNER* AND VIC C. KNAUF

***Agrobacterium tumefaciens* induces tumors in plants by transferring and integrating oncogenes (T-DNA) into the chromosomes of host plant cells. *Agrobacterium* strains were used to transfer complementary DNA copies of a potato spindle tuber viroid (PSTV) to plant cells at a wound site on tomato plant stems. Subsequently, infectious viroid RNA was found in the leaves of these plants, indicating systemic PSTV infection. This process utilized the T-DNA transfer mechanisms of *Agrobacterium* since PSTV infection required most virulence genes (*vir*) as well as one of the DNA sequences that flank either side of the *Agrobacterium* T-DNA. However, transfer still occurred from *vir*E mutants of *Agrobacterium*, strains that fail to induce tumors even though a completely functional T-DNA is present. The *vir*E gene seems to be directly involved in the integration of foreign DNA into plant chromosomes.**

CROWN GALL DISEASE IS MEDIATED by the expression of T-DNA oncogenes introduced into the plant host by *Agrobacterium tumefaciens* (*1*). The T-DNA in the bacterial host resides on a Ti (tumor-inducing) plasmid that contains other genetic loci (*vir*) that are required for virulence (*2*). The boundaries of the portion of the plasmid constituting T-DNA are marked by DNA sequences termed border sequences (*3*). The requirement for border sequences and the *vir* genes has been established by assaying gall formation (*2, 4–8*). However, determining the functional roles of these elements in transformation requires an assay for intermediate steps in the process.

It has been shown (*9*) that *Agrobacterium* with multimeric complementary DNA (cDNA) copies of potato spindle tuber viroid (PSTV) (*10, 11*) in the T-DNA resulted in systemic infections by PSTV when the bacterial strain was inoculated onto tomato plants, a natural host of PSTV. Mock-inoculated tomato plants and tomato plants inoculated with *Agrobacterium* lacking viroid cDNA did not show the presence of viroid or viroid symptoms. Although other explanations are possible, these results suggested that the PSTV cDNA copies were transferred to the plant and subsequently transcribed into infectious RNA or that the T-DNA was transferred as an RNA intermediate containing infectious PSTV genomes. In either case, the infection process would not require T-DNA integration. The data described below indicate that the infection process does indeed require *Agrobacterium* T-DNA transfer and that this PSTV assay may separate the T-DNA transfer and integration events.



To examine the feasibility of this assay, we have used a binary vector system to introduce viroid cDNA into *Agrobacterium* (Fig. 1). Plasmid pCGN201 contains trimeric PSTV DNA located between the left and right borders of the T-DNA. This wide host range plasmid was conjugated into a virulent *A. tumefaciens* strain A722 containing the octopine-type Ti plasmid pTiA6NC (*6*), and into a nontumorigenic strain, PC2760-pAL4404 (*12*), which contains a Ti plasmid that has *vir* loci but no T-DNA. As expected, only the wild-type A722 strain caused tumors on tomato plants. However, all plants inoculated with either strain showed typical symptoms of systemic PSTV infection after 10 to 12 days. The consistent appearance of PSTV symptoms shows that this assay is a sensitive indicator of DNA transfer, since the transfer from *Agrobacterium* must be limited to a fairly small number of cells around the wound site. Infections were verified by hybridization analysis (Fig. 2).

To examine the role of the T-DNA borders in the process of infection, we inserted the PSTV-containing plasmid into wide host range plasmids that contained two bor-

Fig. 1. The role of border sequences in PSTV infection induced by strains of *Agrobacterium*. The structure of the wide host range plasmids containing PSTV trimeric DNA and combinations of the T-DNA borders is shown: the thin line refers to the pRK290 vector; the filled box is the pCGN163a containing the PSTV trimer; open boxes designate borders, drawn in the same orientation as they are present in the T-DNA. Each transconjugant was inoculated on at least six tomato plants (*Lycopersicon esculentum* cv. Rutgers) grown in a growth chamber. Plants were scored for symptoms of systemic PSTV infection (epinasty and rugosity) after 14 to 18 days. The pCGN211a and pCGN211b contain a Hind III fragment from pTiC58 (*1*) containing the right T-DNA border; all other constructs with borders contain the left or right border regions of the T_L-DNA from pTiA6 (*1*). Details of the construction of these plasmids are available from the authors.

Calgene, Inc., 1920 Fifth Street, Davis, CA 95616.

*Present address: Department of Cell Biology, University of Auckland, Private Bag, Auckland, New Zealand.