Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA

(*virE* locus/Ti plasmid/T-DNA transfer/sequence-nonspecific DNA-binding proteins)

CHRISTINE GIETL*†, ZDENA KOUKOLÍKOVÁ-NICOLA, AND BARBARA HOHN

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

Communicated by Diter von Wettstein, June 22, 1987 (received for review May 26, 1987)

ABSTRACT Crude protein extracts of induced and uninduced octopine wild-type strain of *Agrobacterium tumefaciens*, as well as several mutants of the virulence loci *virA*, *-B*, *-G*, *-C*, *-D*, and *-E*, were probed with single- and double-stranded synthetic oligodeoxynucleotides of different sequence and length in an electrophoretic retardation assay. Four complexes involving sequence-nonspecific, single-stranded-DNA-binding proteins were recognized. One inducible complex is determined by the *virE* locus, two Ti-plasmid-dependent complexes are constitutively expressed, and a fourth one is controlled by chromosomal genes. The protein–DNA complexes were characterized by sucrose density gradient centrifugation and by determination of the length of single-stranded DNA required for their formation. It is hypothesized that the single-stranded-DNA-binding proteins are involved in the production of T-DNA intermediates or have a carrier or protective function during T-DNA transfer.

During infection of a plant, *Agrobacterium tumefaciens* transfers a portion of the Ti-plasmid DNA, the T-DNA, to plant cells (1, 2). Genes of the T-DNA are responsible for tumor induction in the plant, but not for the gene transfer itself (3). Trans-acting functions mediating the transfer of T-DNA are specified in the bacterium by the virulence region of the Ti plasmid comprising the loci *virA*, *virB*, *virG*, *virC*, *virD*, and *virE* (4–6). In addition, constitutively expressed chromosomal loci are necessary for the attachment of the bacterium to the plant cell (7). The transcriptional organization of the Ti-plasmid *vir* loci has been determined (8). Transcription of most *vir* loci is induced by plant-synthesized phenolic compounds such as acetosyringone (9). *virA* is proposed to encode a transport protein for the plant signal molecule; this step leads to the intracellular activation of the *virG* protein, which positively controls transcription of other *vir* genes (10–12). T-DNA in *Agrobacterium* is flanked by 25-base-pair imperfect direct repeats (the border sequences) that are required for the transfer (1, 2). A sequence called “overdrive,” close to the right border repeats of octopine plasmids, enhances T-DNA transfer efficiency (13, 14). A site-specific, *virD*-encoded (15, 16) endonuclease is induced and cleaves within the border sequences. Thereupon the T-DNA is mobilized from the bacterium to the plant in either a double-stranded (17) or single-stranded (18) form. The function of the *virB*, *-C*, and *-E* loci is unknown. However, mutants in the loci *virA*, *-B*, *-G*, and *-D* are avirulent on all plant species, whereas mutants in *virC* and *-E* induce tumor formation to a variable extent on some plant species, but not on others (8, 19).

Acetosyringone induces transcription of the *vir* loci in agrobacteria, even if these had no contact with plant cells, and thus provides a convenient approach to study the func-

tion of the *vir* gene products. It was of interest to explore whether acetosyringone induces in agrobacteria DNA-binding proteins that could be involved in T-DNA transfer to plant cells. Extracts of uninduced and induced agrobacteria strains were tested with ³²P-labeled synthetic oligonucleotides for the presence of DNA-binding proteins in a polyacrylamide gel retardation assay. Several constitutively synthesized and one inducible sequence-nonspecific single-stranded-DNA (ssDNA)-binding proteins were found. The inducible complex is absent in mutants of the *virA* and *-G* loci, in which induction of transcription of the entire *vir* region is abolished, as well as in *virE* mutants.

MATERIALS AND METHODS

Agrobacteria. Strains carrying mutant octopine-type Ti plasmids were kindly provided by S. E. Stachel and E. W. Nester (8). These plasmids contain promoterless *lacZ* insertions in the different virulence loci of the pTiA6 plasmid. The fully virulent *pinF* mutant 219 was used as a wild type and produced upon induction with acetosyringone 1200 units of β -galactosidase per bacterium, corresponding to a 50-fold induction, in agreement with the results of Stachel *et al.* (6, 20). The following virulence mutants were used: *virA*, pTi240 and pTi226; *virB*, pTi244; *virG*, pTi19 and pTi321; *virC*, pTi364 and pTi365; *virD*, pTi306, pTi307, and pTi348; *virE*, pTi358 and pTi341; for map positions see ref. 8. The strain A136 cured of the Ti plasmid (3) was employed to check for chromosome-encoded proteins.

Agrobacteria were grown overnight at 28°C and 200 rpm in YEB medium (1 g of yeast extract, 5 g of beef extract, 5 g of peptone, 5 g of sucrose, and 0.5 g of MgSO₄·7H₂O per liter) with carbenicillin (100 μ g/ml). An aliquot was diluted into TY medium (5 g of tryptone and 3 g of yeast extract per liter) and grown to early logarithmic phase. To induce the virulence genes, 80 μ M acetosyringone was added and the culture was grown for 14 hr (9). Induction was confirmed by β -galactosidase test (6, 20).

Protein Extracts. Cells of 40-ml cultures were harvested by centrifugation at 12,300 $\times g$ for 10 min at 4°C and resuspended in 2 ml of extraction medium containing 25 mM Hepes (pH 7.5), 5% (vol/vol) glycerol, 50 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 3.33 μ M phenylmethylsulfonyl fluoride, 3.33 μ M L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), and 3.33 μ M L-1-tosylamido-5-aminopentyl chloromethyl ketone (TLCK). After disintegration of the cells in a French pressure cell the homogenate was centrifuged at 31,480 $\times g$ for 1 hr at 4°C. The supernatant was collected and stored at –80°C in aliquots.

Oligodeoxyribonucleotides. Eighteen oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA)

Abbreviation: ssDNA, single-stranded DNA.

*Present address: Institute of Botany, Technical University of Munich, Arcisstrasse 21, D-8000 München 2, F.R.G.

†To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis. Twelve of these are presented in Fig. 1, aligned with the appropriate sequence of the octopine Ti plasmid (14, 21), the nopaline Ti plasmid (22), and the pUC linker (23). An oligonucleotide of the upper and the lower strand was made. Restriction sites were added at the ends for cloning purposes. Double-stranded probes with "sticky ends" were made by mixing equimolar amounts of the corresponding upper (up) and lower (lo) strands, incubating at 80°C for 5 min, and annealing at room temperature for 30 min. Blunt-ending was done with the Klenow fragment of DNA polymerase; ³²P-labeling of the probes was catalyzed by bacteriophage T4 polynucleotide kinase (24). The other six oligonucleotides tested comprised right border sequence plus overdrive (73 bases), right border sequence plus left neighbor (71 bases), a 45-base oligonucleotide covering the lower strand of the overdrive but extending further to the left than oligonucleotide 1, and a 67-base oligonucleotide of the lower strand of the right border, but extending to the left and right of oligonucleotide 2.

Gel Retardation Assay. Typically, 250 fmol of oligonucleotide probe (50,000 cpm) were incubated in a reaction mixture (15 μ l) containing 3 μ g of poly(dI-dC)-poly(dI-dC) (Pharmacia) and 10 μ l of crude protein extract (described above) for 30 min at room temperature. After complex formation, 10 μ l of 10% glycerol/0.1% bromophenol blue was added. The complexes were resolved by electrophoresis in a high-ionic-strength nondenaturing 11% polyacrylamide gel (acrylamide/*N,N'*-methylenebisacrylamide weight ratio of 44:0.8) containing 0.375 M Tris-HCl (pH 8.8), using 40 mM Tris/glycine (pH 8.5) as the running buffer (25). Preelectrophoresis (1 hr) and electrophoresis (3 hr) were carried out at room temperature at 20 V/cm. The gel was autoradiographed at -70°C with two intensifying screens, typically overnight. For molecular weight determination, the reaction mixtures were loaded on top of a 3-ml continuous 5–25% sucrose gradient in 0.375 M Tris-HCl (pH 8.8) and centrifuged in a SW60 Ti Beckman rotor at 40,000 rpm and 4°C for 14 hr. Fractions (100 μ l) were collected from the top of the gradient and analyzed by gel electrophoresis.

RESULTS

Sequence-Nonspecific ssDNA-Binding Proteins. Radioactively labeled probes covering different parts of the se-

quences shown in Fig. 1 were chosen to search for DNA-binding proteins. Oligonucleotides with a length of 34 or 36 bases, representing either the upper or lower strands of the overdrive, the right octopine T_L border, or its "left neighbor" were used to probe for protein binding with the two strands separately as well as with the annealed double strands, having 4 bases protruding at the 5' ends or blunt ends. Both the left neighbor and a pUC-linker sequence were employed to test for sequence specificity. Protein extracts from acetosyringone-induced and uninduced agrobacteria were tested in a gel retardation assay, in which DNA bound to protein migrates slower than free DNA (25, 26). Poly(dI-dC) was used to prevent nonspecific binding of proteins (27). Fig. 2 presents a typical experiment in which protein extracts were probed with the right border sequence. Extracts from the uninduced wild-type strain formed three complexes (B, B', and C), while a fourth complex (A) appeared consistently only with proteins from induced bacteria. DNA probes incubated with extraction medium alone (Fig. 2, rightmost lane) were not retarded (band F) and complexes were absent if proteinase K (4 mg/ml) was added during the assay (see Fig. 6, lane 1). Complex C was formed in varying amounts with constitutively synthesized protein encoded by the chromosome, since it was also present in extracts of agrobacteria cured of the Ti plasmid (Fig. 3, rightmost lane). The four proteins retard the upper as well as the lower strand and apparently the duplex with the protruding ends but only to a very minor extent the blunt-ended double-stranded probe (Fig. 2). The four ssDNA-binding proteins are nucleotide sequence-nonspecific: each formed complexes with several of the 18 probes tested.

The role of *vir* loci in determining the induced synthesis of the ssDNA-binding protein in complex A was studied by probing strains mutated in the six *vir* loci with the different oligonucleotides. Complex A was produced by extracts from *virB*, *-C*, and *-D* mutants, although to a lesser extent when *virD* mutants were employed (Figs. 2 and 3). Extracts of *virA*, *-G*, and *E* mutants lack this DNA-binding protein. Other *virE* mutants (described in ref. 3) were also tested, with the same result (data not shown). Since in addition to mutants in the *virA* and *-G* loci (whose expression is required for induction of the other *vir* loci), those in the *virE* locus failed to form

FIG. 1. Single-stranded synthetic oligodeoxynucleotides used for assays of DNA-binding protein are aligned with the relevant sequences of the octopine Ti-plasmid DNA, the nopaline right border, and the pUC-linker control.

FIG. 2. Gel retardation analysis of complexes formed with 32 P-labeled oligonucleotide no. 2 (right border, octopine T-DNA; Fig. 1). A, DNA-protein complex formed in *Agrobacterium* upon induction with acetosyringone; B and B', constitutively formed DNA-protein complexes in *Agrobacterium* carrying the Ti plasmid; C, constitutively formed DNA-protein complexes in *Agrobacterium* cured of Ti plasmid; F, free DNA; O, origin; -, uninduced; +, induced; wt, wild type (strain 219); *virE* and *virD*, mutants in virulence E and D loci (strain pTi358 and pTi307).

complex A, we conclude that the *virE* region determines the formation of this ssDNA-binding protein.

Complex A exhibits considerable variation in electrophoretic mobility, in contrast to the complexes B, B', and C (Figs. 2 and 3). As the migration behavior was independent of variations in temperature during electrophoresis, DNA concentration per assay in the range 2.5–10 fmol, and age of the bacterial extracts stored at -80°C , a trivial explanation is excluded.

Oligonucleotide Length Specificity. While the gel pattern of ssDNA-protein complexes was independent of the nucleotide sequence offered to the *Agrobacterium* protein extract, it was dependent on the length of the DNA sequence added (Figs. 4 and 5). When the 36-base-long upper strand of the nopaline right border sequence was added, the inducible and constitutive Ti-plasmid-determined bands A, B, and B' were obtained (Fig. 4). On the other hand, the 24-base lower strand of this sequence was not long enough to give a retarded band (Fig. 4). A titration experiment of complex formation is presented in Fig. 5. With a DNA sequence of 20 bases, no complex was present. Probes with lengths of 30 and 34 bases gave rise to the formation of the complexes B and B' with the constitutively expressed binding protein. Oligonucleotides with 36 and more bases gave the DNA complex with the inducible protein determined by the *virE* region. In this experiment the DNA-protein complex C formed by the chromosome-encoded and constitutively expressed protein elicited a prominent band with probes of 67 and more bases (Fig. 5). These longer probes yielded a further complex (D) that is also formed by a constitutively expressed protein.

Absence of retardation with single-stranded probes containing 20–24 bases was observed without exception. The apparent contradiction of this finding by the binding of the proteins to the 4-base sticky ends of the annealed duplex in

FIG. 3. Gel retardation analysis of ssDNA-binding proteins present in acetosyringone-induced wild-type *Agrobacterium* and virulence mutants. A 36-base oligonucleotide synthesized according to the pUC-linker lower strand (no. 6 lo; Fig. 1) was used as a radioactive probe. B, B', and C, constitutively formed DNA-protein complexes; A, acetosyringone-induced DNA-protein complex exhibiting variable electrophoretic mobility; F, free DNA. Leftmost lane: uninduced wild-type control; rightmost lane, strain cured of Ti plasmid.

Fig. 2 might indicate a promotion of strand-separation by these ssDNA-binding proteins. The trend illustrated in Fig. 5—i.e., an increasing number of retardation bands with increasing probe length—was characteristic for all experi-

FIG. 4. Minimal length of single-stranded probe required for DNA-protein complex formation. Binding of the acetosyringone-induced protein in complex A and of constitutively formed proteins in complexes B, B', and C was obtained with the 36-base oligonucleotide from the nopaline right border but not with the 24-base oligonucleotide. F, free DNA; b, bases.

FIG. 5. Titration experiment of DNA-protein complex (A, B, B', C, D) formation with oligonucleotide probes of different length and extracts of induced wild-type *Agrobacterium*. The following probes were used: 20 bases, a mixture of oligonucleotides specifying the amino acid sequence Met-Leu-Met-Lys-Met-Gln-Pro; 30, 34, and 36 bases, see Fig. 1; 45 bases, overdrive plus 11 bases to the left of 1 lo; 67 bases, probe 2 lo extended to the left and right; 71 bases, probe 2 lo plus 4 lo; 73 bases, probe 1 lo plus 2 lo. F, free DNA; b, bases.

ments, but a sharp border for a minimal length required for a certain complex could not be defined. Two examples can be mentioned: whereas probes with 36 bases consistently permitted visualization of complex A, containing the acetosyringone-induced protein, this was frequently also possible with the 34-base overdrive probe but never with the 34-base probe derived from the lower strand of the left neighbor. Complex C appeared under some conditions with 36-base probes (Figs. 2 and 3), but in the experiment of Fig. 5 this required a probe of 67 bases.

Characterization of the DNA-Binding Proteins. Complexes

A, B, and B' were separated on a sucrose density gradient, and fractions were analyzed by nondenaturing polyacrylamide gel electrophoresis. The complexes were formed in a reaction mixture containing a 45-base oligonucleotide covering the overdrive sequence and extracts from either uninduced or induced wild-type *Agrobacterium* and then were loaded on gradients. Molecular weight markers were run in parallel. Fig. 6 presents the analysis of complexes present in induced *agrobacteria*. Newly made starting material is shown in lane 3, while the complexes found in the DNA-binding assay mixture kept at 4°C during gradient centrifugation are displayed in lane 2. The complexes B and B', formed by proteins present also in uninduced Ti-carrying *agrobacteria*, were separated with peaks in fractions 24 and 21, respectively. Complex A, containing the induced protein, was found in fractions 14 and 15. The analogous fractions obtained with extract from uninduced bacteria are shown in the *Inset*. The apparent molecular weights can be estimated as 320,000–360,000 for complex B, 220,000–250,000 for complex B', and $95,000 \pm 15,000$ for complex A. The contribution of the ssDNA (45 bases) is calculated to be $\approx 15,000$, and so the molecular weight of the protein in complex A be $\approx 80,000$. Thus, the protein-nucleic acid complexes behave as defined units both when migrating in an electric field and when sedimenting in an ultracentrifuge.

DISCUSSION

A gel retardation assay (25–27) uncovered protein-DNA interactions in extracts of *A. tumefaciens*. Four distinct complexes form with ssDNA in extracts of wild-type bacteria. The DNA-protein interactions are of noncovalent nature, as only nonretarded, free DNA is obtained when the complexes are electrophoresed in a polyacrylamide gel containing 0.1% NaDodSO₄ (data not shown). Based on their behavior in our assay, the complex-forming proteins are classified as ssDNA-binding proteins, although proteins such as helicase, primase, RecA protein, and RNA polymerase might behave similarly in such an assay (28). Our results show that the gel retardation assay is a useful technique to study sequence-nonspecific ssDNA-binding proteins. Previously, Jost *et al.* (29) have used the same assay to detect sequence-specific binding to ssDNA.

Complex A is the most interesting one, since its formation is dependent on the induced expression of the virulence region of the Ti plasmid. Whereas the variable migration

FIG. 6. Separation of the DNA-protein complexes by sucrose density gradient centrifugation. Gradient fractions were analyzed by nondenaturing polyacrylamide gel electrophoresis. Molecular weight markers (phosphorylase b, M_r 94,000; bovine serum albumin, M_r 67,000) are indicated by arrows. Additional markers were ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α -lactalbumin (M_r 14,400). Complexes were formed by incubation of the 45-base oligonucleotide covering the overdrive with extract from induced wild-type *Agrobacterium*. Complexes obtained with extract from uninduced bacteria are shown in the *Inset*. Lane 1: no complexes formed in the presence of proteinase K (4 mg/ml). Lane 2: freshly made assay mixture. Lane 3: assay mixture kept at 4°C during gradient centrifugation. Other lanes: sucrose density gradient fractions 11–30. F, free DNA; B and B', constitutively formed complexes; A, induced complex.

behavior of this complex remains to be explained, the dependence of complex formation on the length of the ssDNA (a minimal length of 34–36 bases is required) might reflect the size of the binding site. In the case of the gene 32 protein of bacteriophage T4, it has been estimated to be 5–11 nucleotides, whereas 24–32 nucleotides bind per tetramer of the *Escherichia coli* single-strand-binding (SSB) protein (28).

Complex A was not detected with extracts of *Agrobacterium* strains containing transposon insertions in the *virA*, *virE*, or *virG* locus. Since *virA* and *virG* function as positive regulators of other *vir* genes in response to plant signal molecules (10–12), *virE* must be involved in production of the ssDNA-binding protein in complex A. The *virE* mutants we used contain transposon insertions in *virE2*, which encodes a hydrophilic polypeptide of 60.5 kDa (30). Further experiments are needed to test whether the hydrophilic VirE1 polypeptide of 7.1 kDa (30) also is involved in binding to ssDNA. The ≈80-kDa apparent molecular mass of the protein component in complex A as determined by sucrose gradient centrifugation is compatible with the hypothesis that the VirE1 and VirE2 polypeptides interact directly with the oligonucleotide probe.

Sequence-nonspecific ssDNA-binding proteins share a characteristic domain with an alignment of five aromatic and four charged residues separated by rather consistent spacings (31). Protein-DNA stabilization by this domain is thought to involve stacking interactions of the aromatic residues with the DNA bases and electrostatic interactions of the basic side chains with the phosphate groups of the DNA. A look at the 553 amino acid long sequence of the VirE2 polypeptide discloses a large number of aromatic and charged residues. Four consecutive domains for ssDNA binding can be postulated: (i) His-65...Lys-73...Phe-79...Tyr-94...Phe-105...Asp-114...Phe-122...Tyr-129...Lys-145; (ii) Lys-162...Lys-166...Trp-176...Phe-191...Tyr-204...Asp-210...Tyr-218...Tyr-227...Lys-243; (iii) His-250...Arg-254...Tyr-260...Phe-265...Tyr-276...Glu-281...Tyr-296...Trp-300...Arg-308; (iv) Arg-342...His-345...Phe-350...Trp-360...Trp-370...Asp-379...Tyr-391...Phe-397...Arg-412. If these binding sites are operative *in vivo*, it becomes understandable why the host range and the frequencies of tumor formation diminish when the protein is increasingly truncated from the carboxyl terminus (32).

The F factor of *E. coli* and other conjugative plasmids of enterobacteria contain a gene coding for a ssDNA-binding protein, which is induced if the F factor is derepressed for conjugation (33, 34). This gene complements the chromosomal *E. coli* *ssb-1* mutants and restores partially the functions normally performed by ssDNA-binding proteins encoded in the chromosome. Moreover, *in vitro*, the *E. coli* protein can substitute for the bacteriophage T7-coded ssDNA-binding protein with regard to its stimulatory effect on the activity of T7 DNA polymerase (35). It thus seems plausible that constitutively expressed agrobacterial ssDNA-binding proteins (for example, those forming complexes B, B', or C) might partially substitute for the protein missing in the *virE* mutants and cause their attenuated phenotypes (8, 19, 32). By analogy to the F factor, transfer-constitutive Ti plasmids might overexpress such proteins and partially complement *virE* mutants.

T-strands (18) or double-stranded linear T-DNA molecules with single-stranded ends (Z.K.-N. and B.H., unpublished observations) have been proposed as intermediates of the T-DNA transfer from *Agrobacterium* into the plant cell. The sequence-nonspecific ssDNA-binding protein found in complex A and determined by the *virE* locus might function in the production, protection, and/or transport of such intermediates. Indeed, interbacterial complementation of *virE* mutants (36) supports the idea that the hydrophilic *virE* polypeptides

are exported from the bacterial cell and play a role in protection or transport of T-DNA. It will be of interest to see whether the VirE polypeptides expressed in *E. coli* (30) bind ssDNA. Further experiments are required to evaluate the *in vivo* behavior of the described ssDNA-binding proteins.

We thank Drs. J.-P. Jost and W. Filipowitch for discussions during the work and comments on the manuscript. We are indebted to Dr. J.-P. Jost for instruction on his technique of generating continuous sucrose gradients, to Drs. S. Stachel and E. Nester for providing the *vir* mutants, to W. Zürcher and Dr. J. Jiricny for synthesizing oligonucleotides, and to Drs. R. Schneider and E. Winnacker for information on the gel retardation assay. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to C.G.

- Gheysen, G., Dhaese, P., Van Montagu, M. & Schell, J. (1985) in *Advances in Plant Gene Research*, Genetic Flux in Plants, eds. Hohn, B. & Dennis, E. S. (Springer, Wien), Vol. 2, pp. 11–47.
- Koukoliková-Nicola, Z., Albright, L. & Hohn, B. (1987) in *Advances in Plant Gene Research*, Plant DNA Infectious Agents, eds. Hohn, T. & Schell, J. (Springer, Wien), Vol. 4, pp. 109–148.
- Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P. & Nester, E. W. (1981) *Cell* 27, 143–153.
- Klee, H. J., White, F. F., Iyer, V. N., Gordon, M. P. & Nester, E. W. (1983) *J. Bacteriol.* 153, 878–883.
- Hooykaas, P. J. J., Hofker, M., DenDulk-Ras, H. & Schilperoort, R. A. (1984) *Plasmid* 11, 195–205.
- Stachel, S. E., An, G., Flores, C. & Nester, E. W. (1985) *EMBO J.* 4, 891–898.
- Douglas, C. J., Staneloni, R. J., Rubin, R. A. & Nester, E. W. (1985) *J. Bacteriol.* 161, 850–860.
- Stachel, S. E. & Nester, E. W. (1986) *EMBO J.* 5, 1445–1454.
- Stachel, S. E., Messens, E., Van Montagu, M. & Zambryski, P. (1985) *Nature (London)* 318, 624–629.
- Stachel, S. E. & Zambryski, P. C. (1986) *Cell* 46, 325–333.
- Melchers, L. S., Thompson, D. V., Idler, K. B., Schilperoort, R. A. & Hooykaas, P. J. J. (1986) *Nucleic Acids Res.* 14, 9933–9941.
- Winans, S. C., Ebert, P. R., Stachel, S. E., Gordon, M. P. & Nester, E. W. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8278–8282.
- Peralta, E. G. & Ream, L. W. (1985) *Proc. Natl. Acad. Sci. USA* 82, 5112–5116.
- Peralta, E. G., Hellmis, R. & Ream, W. (1986) *EMBO J.* 5, 1137–1142.
- Yanofsky, M. F., Porter, S. G., Young, C., Albright, L. M., Gordon, M. P. & Nester, E. W. (1986) *Cell* 47, 471–477.
- Veluthambi, K., Jayaswal, R. K. & Gelvin, S. B. (1987) *Proc. Natl. Acad. Sci. USA* 84, 1881–1885.
- Koukoliková-Nicola, Z., Shillito, R. D., Hohn, B., Wang, K., Van Montagu, M. & Zambryski, P. (1985) *Nature (London)* 313, 191–196.
- Stachel, S. E., Timmermann, B. & Zambryski, P. (1986) *Nature (London)* 322, 706–712.
- Hille, J., Dekker, M., Luttighuis, H. O., Van Kammen, A. & Zabel, P. (1986) *Mol. Gen. Genet.* 205, 411–416.
- Stachel, S. E., Nester, E. W. & Zambryski, P. (1986) *Proc. Natl. Acad. Sci. USA* 83, 379–383.
- Barker, R. F., Idler, K. B., Thompson, D. V. & Kemp, I. D. (1983) *Plant Mol. Biol.* 2, 335–350.
- Zambryski, P., Depicker, A., Kruger, K. & Goodman, H. (1982) *J. Mol. Appl. Genet.* 1, 361–370.
- Vieira, J. & Messing, J. (1982) *Gene* 19, 259–268.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
- Schneider, R., Gander, I., Müller, U., Mertz, R. & Winnacker, E. L. (1986) *Nucleic Acids Res.* 14, 1303–1317.
- Strauss, F. & Varshevsky, A. (1984) *Cell* 37, 889–901.
- Singh, H., Sen, R., Baltimore, D. & Sharp, P. A. (1986) *Nature (London)* 319, 154–158.
- Chase, J. W. & Williams, K. R. (1986) *Annu. Rev. Biochem.* 55, 103–136.
- Jost, J.-P., Saluz, H.-P., Jiricny, J. & Moncharmont, B. (1987) *UCLA Symp. Mol. Cell. Biol. New Ser.*, in press.
- Winans, S. C., Allenza, P., Stachel, S. E., McBride, K. E. & Nester, E. W. (1987) *Nucleic Acids Res.* 15, 825–837.
- Prasad, B. V. V. & Chiu, W. (1987) *J. Mol. Biol.* 193, 579–584.
- Hirooka, T. & Kado, C. I. (1986) *J. Bacteriol.* 168, 237–243.
- Chase, J. W., Merrill, B. M. & Williams, K. R. (1983) *Proc. Natl. Acad. Sci. USA* 80, 5480–5484.
- Willets, N. & Wilkins, B. (1984) *Microbiol. Rev.* 48, 24–41.
- Kowalczykowski, S. C., Bear, D. G. & von Hippel, P. H. (1981) in *The Enzymes*, ed. Boyer, P. (Academic, New York), Vol. 14, pp. 373–444.
- Otten, L., De Greve, H., Leemans, J., Hain, R., Hooykaas, P. & Schell, J. (1984) *Mol. Gen. Genet.* 195, 159–163.