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Quantitative Estimation of *Agrobacterium tumefaciens* DNA in Crown Gall Tumor Cells

(DNA·DNA hybridization/*Vinca rosea*/callus culture)

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ABSTRACT **Several reports suggest that *Agrobacterium tumefaciens* nucleic acids can induce transformation of the cells of susceptible host plants and that bacteria-free tissue cultures of transformed cells contain *A. tumefaciens* DNA, RNA, antigens, or bacteriophages. We assayed *Vinca rosea* tumor DNA for base sequence homologies with *A. tumefaciens* DNA by DNA·DNA solution enrichment and DNA·DNA filter saturation hybridization techniques. No homologies were found by either method. The filter saturation hybridization technique included model filters containing known percentages of bacterial DNA mixed with *V. rosea* leaf DNA. Using this sensitive technique, we found that no more than 0.02% of the crown gall tumor genome could be homologous to *A. tumefaciens* DNA. This upper estimate of homology corresponds to 0.2 bacterial genome equivalent per diploid tumor cell.**

Crown gall is a neoplastic disease of plants initiated by the bacterium *Agrobacterium tumefaciens* Conn. Transformed cells isolated from crown gall tumors are easily cultured *in vitro* free of the initiating bacterium. When these cultured cells are introduced into a suitable host plant, they proliferate into a neoplasm (1) and can transform additional cells of the host plant (2). Although the molecular processes leading to transformation have not been elucidated, crown gall formation appears to parallel transformation of mammalian cells by DNA tumor viruses (3–7). Several reports suggest that bacterial nucleic acids are capable of inducing transformation (8–11), and indicate that bacteria-free tumor tissues contain *A. tumefaciens* DNA (12–15), RNA (16), antigens (17–19), and bacteriophages (20–22). However, tumor induction by purified nucleic acids has not been generally reproducible, and phages are not always found in tumor tissues (15) and they will not induce tumors (23–25). Moreover, the evidence for the presence of bacterial DNA and RNA in tumor tissues is not rigorous; the nucleic acid reassociation studies omitted analyses of the hybrid thermal stabilities and, thus, the fidelity of base pairing in those experiments is unknown.

Since the presence or absence of bacterial DNA in tumor cells is important to models explaining crown gall tumor formation, we analyzed *Vinca rosea* L. crown gall tumor DNA for the presence of *A. tumefaciens* DNA by two forms of DNA·DNA hybridization assays. We estimate that less than 0.02% of the tumor DNA is homologous to *A. tumefaciens* DNA. This represents about one-third of the genome of this bacterium, corresponding to less than 0.2 bacterial genome equivalent per tumor cell.

MATERIALS AND METHODS

Tissue Culture. Crown gall tumor callus cultures of *V. rosea*, initiated by *A. tumefaciens* B6, were obtained from Dr. R. Manasse, Boyce Thompson Institute, Yonkers, N.Y. Normal callus cultures were obtained from Dr. A. C. Braun, The Rockefeller University, New York, N.Y. Cultures were grown on agar media (26, 27). The tumor cultures were capable of growth on media lacking cytokinins and auxins, while normal cultures were not. Samples of callus cultures were periodically incubated in medium 523 broth (9) to verify that they remained free of bacteria.

Purification of DNA Samples. (*1*) *Leaf nuclear DNA. V. rosea* leaves (1 kg), grown under standard greenhouse conditions, were homogenized in 50 mM Tris·HCl (pH 7.8), 10 mM $CaCl_2$. The extract was filtered and incubated in 2% Triton X-100 for 10 min at 23°. Nuclei were collected by centrifugation, resuspended in the buffer, incubated again in 2% Triton X-100 (10 min), concentrated by centrifugation, resuspended in 50 ml of the buffer, and incubated in 4% (w/v) sodium dodecyl sulfate for 3 hr at 23°. $NaClO_4$ was added to 1 M, and the crude DNA was deproteinized by extraction with chloroform. The DNA was precipitated with cold 95% ethanol, spooled onto a glass rod, and then dissolved in 10 mM NaCl. The DNA was treated with pancreatic RNase (50 μg/ml, 30 min, 37°) and then with Pronase (50 μg/ml, 90 min, 37°). Sodium dodecyl sulfate was added to 0.5%, and the incubation was continued for 30 min. NaCl was added to 0.5 M, and the DNA was extracted with neutralized, water-saturated phenol and then with chloroform. The DNA was again precipitated with ethanol and dissolved in 10 mM NaCl. Some preparations containing residual pigments were treated with 0.5% acid-washed Norit A in 20 mM Tris·HCl, pH 7.8.

(*2*) *Vinca rosea callus DNA.* Callus tissues (tumor or normal) were harvested, mixed with an equal volume of 50 mM borate, 10 mM EDTA buffer, pH 9 and then homogenized in an Omnimixer (Sorvall) for 5 min at top speed at 0°. Sodium 1,5-naphthalene disulfonic acid (0.05%), deoxycholate (0.25%), and sodium dodecyl sulfate (0.5%) were added, and the mixture was shaken overnight at 23°. NaCl (0.2 M) was added, and the homogenate was then extracted with neutralized, water-saturated phenol. The nucleic acids were precipitated from the aqueous phase with cold 95% ethanol collected by centrifugation (1550 × *g*, 10 min), and redissolved in 10 mM NaCl. The DNA solution was next incubated with pancreatic RNase (50 μg/ml, 30 min, 37°) and then with Pronase (50 μg/ml, 90 min, 37°). The DNA was then adsorbed to hydroxylapatite (Biorad) columns (0.5 mg of DNA per g of hydroxylapatite) at 60° in 0.1 M phosphate buffer (equimolar mono- and dibasic sodium phosphate buffer, pH 6.8). The columns were washed with 0.12 M phosphate buffer until the absorbancy at 260 nm was less than 0.05; the DNA was

eluted with 0.4 M phosphate buffer. The final step in the purification was isopycnic centrifugation in CsCl density gradients (28).

(3) *Other DNAs.* DNA was extracted from *A. tumefaciens* B6 and *Erwinia rubrifaciens* cells grown into late logarithmic phase in liquid medium 523 by the method of Kado *et al.* (9). *Micrococcus lysodeikticus* cells were grown to late logarithmic phase in medium 523 (9), harvested by centrifugation (10,000 × *g*, 10 min), resuspended in 0.15 M NaCl, 0.1 M EDTA, and treated with lysozyme (1 mg/ml, 20 min, 37°). After 3 cycles of freezing and thawing, the suspension was diluted 4-fold with 0.15 M NaCl, 0.1 M EDTA. Sodium dodecyl sulfate was added to a final concentration of 1%, and the DNA solution was stirred gently at 23° for 2 hr. Calf-thymus DNA was purchased from Sigma Chemical Corp., St. Louis, Mo. The final stages in the purification of all four types of DNA were ethanol precipitation and treatments with ribonuclease, Pronase, phenol, and chloroform, as described above for leaf nuclear DNA.

Preparation of Radioactive DNA. A. tumefaciens [^{32}P]DNA was extracted as described above from cells grown into late logarithmic phase in CD medium [5 g of glucose, 2.5 g of KNO_3, 2 g of enzyme-hydrolyzed casein, 0.05 g of $MgSO_4 \cdot 7H_2O$, 17 mCi of carrier-free neutralized [^{32}P]phosphoric acid (Schwarz Bioresearch, Orangeburg, N.Y.) per 500 ml]. The DNA was sheared by ultrasonication to pieces 500–600 nucleotides long [determined by alkaline band sedimentation (29)], adsorbed to and eluted from hydroxylapatite columns as described above, passed through a nitrocellulose filter, and dialyzed against distilled H_2O. The DNA was then treated with 0.1 M NaOH (4 hr, 37°) and dialyzed extensively against distilled H_2O. When necessary, the DNA was concentrated by lyophilization.

^{3}H- and ^{32}P-labeled *V. rosea* callus DNAs were extracted as described above from callus cultures grown for 3–4 weeks on agar medium containing either 0.2 mCi of [*methyl*-^{3}H]thymidine (20 Ci/mmole, Schwarz Bioresearch) or 0.5 mCi of neutralized [^{32}P] phosphoric acid per 15 ml of agar.

Preparation of DNA Filters. DNA filters were prepared by a modification of the method of McCarthy and McConaughy (30). The DNA samples were treated with 0.1 M NaOH for 4 hr at 37° and then dialyzed extensively against distilled H_2O. DNA aliquots in 1 mM phosphate buffer were then placed in boiling water for 10 min, cooled quickly in ice water, and diluted. The denatured DNA was adjusted to 0.75 M NaCl–0.075 M Na_3 citrate, pH 7.0, and loaded by gravity flow onto rinsed nitrocellulose filters (0.45 μm pore size, 44 mm effective diameter, Sartorius, Brinkmann Instr., Westbury, N.Y.). The loaded filters were washed with 50 ml of 0.75 M NaCl–0.075 M Na_3 citrate, dried at 23°, and then at 80° under reduced pressure for 8 hr. Each filter was cut into 6-mm diameter circles for use in the hybridization experiments.

All filters were first incubated overnight at the incubation temperature in a modified Denhardt's medium (31) containing 0.75 M NaCl–0.075 M Na_3 citrate, 50% formamide, 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone, and 0.02% bovine-serum albumin.

Filter Hybridization. Hybridizations were carried out at 46.5° in a final volume of 0.6 ml containing 50% formamide, 0.75 M NaCl, 0.075 M Na_3 citrate, denatured (by treatment at 100° for 10 min), sheared, radioactive *A. tumefaciens* DNA, and filters were loaded with various types of DNA. After 22 hr, the reactions were stopped by removing the filters from solution, washing them twice in 0.75 M NaCl–0.75 M Na_3 citrate and 50% formamide at 46.5°, and by rinsing four times in 0.75 M NaCl–0.075 M Na_3 citrate at 23°. The filters were dried, placed individually in vials containing 5 ml of scintillation fluid [(4 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene)] and counted in a Beckman liquid scintillation spectrometer, model LS-233. After they were counted, the filters were removed from the scintillation vials, dried, and assayed for DNA by the method of Burton (32). Control experiments showed that no DNA was lost from the filters during the counting procedure. Hybrid formation was calculated as μg of radioactive DNA bound per μg of nonradioactive DNA on each filter and expressed as a percentage.

Measurement of Hybrid Thermal Stability. Filter-bound DNA was hybridized with *A. tumefaciens* [^{32}P]DNA as described above. Rinsed filters were then incubated at various temperatures (each filter was incubated at only one temperature) for 15 min in 0.75 M NaCl–0.075 M Na_3 citrate, 50% formamide. The filters were rinsed at the incubation temperature, dried, and finally assayed for radioactivity and DNA content.

RESULTS

Solution Hybridization Analyses. Nucleic acid hybridization data have supported the concept that *A. tumefaciens* DNA persists in crown gall tumor cells (12–15). We conducted two types of solution DNA·DNA reassociation experiments to test this hypothesis. In both cases tumor DNA preparations were enriched for bacterial nucleotide sequences and then analyzed. In the first type of experiment, sheared, denatured tumor [^{3}H]DNA (60 μg/ml, 15,000 cpm/μg) was allowed to reassociate in the presence of a high concentration of sheared, denatured, nonradioactive leaf nuclear DNA (1 mg/ml) for 20 hr at 63° in 0.12 M phosphate buffer. We expected that reiterated plant sequences would reassociate under these conditions (C_0t = 200 where C_0t of 1 is equivalent to 83 μg/hr per ml) (33). On the other hand, previous filter hybridization experiments (13, 14) indicated that any bacterial sequences would be present at such low concentrations (about 0.1% or C_0t = 0.012) that they would remain single-stranded. After

TABLE 1. *Recovery of radioactive tumor and normal V. rosea DNA after enrichment for presumptive A. tumefaciens nucleotide sequences*

Type of enrichment	Source bacterial DNA	% [^{3}H]DNA recovered: Tumor DNA	% [^{3}H]DNA recovered: Normal DNA
First incubation[a] with nonradioactive leaf DNA (C_0t = 200); second with bacterial DNA (C_0t = 60)	*A. tumefaciens*	0.1	0.1
	E. rubrifaciens	0.1	0.07
Incubation[b] with excess bacterial DNA only (C_0t = 72)	*A. tumefaciens*	0.15	0.2
	M. lysodeikticus	0.5	0.5

[a] Incubation at 63° in 0.12 M phosphate buffer.
[b] Incubation at 80° in 0.09 M phosphate buffer.

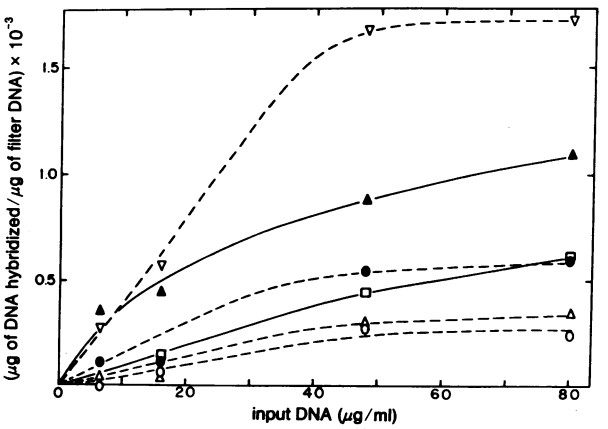

FIG. 1. Filter saturation hybridization of *A. tumefaciens* [^{32}P]DNA to filter-bound plant DNAs. The specific activity of the bacterial [^{32}P]DNA was 2×10^5 cpm/μg, and each filter contained about 6 μg of nonradioactive DNA. Filters contained crown gall tumor DNA (▲), normal callus DNA (□), or leaf nuclear DNA *plus* the following small percentages of *A. tumefaciens* DNA: 0% (○); 0.07% (△); 0.1% (●); 0.5% (▽).

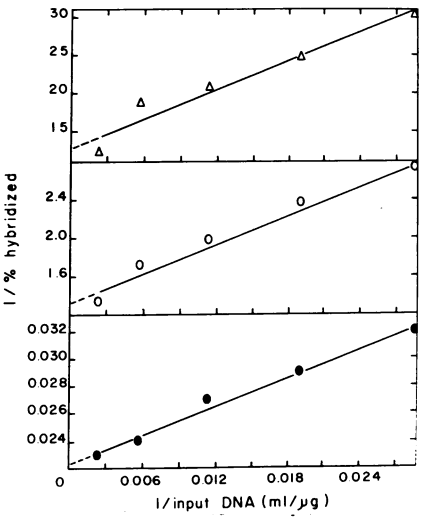

FIG. 2. Double-reciprocal plots of *A. tumefaciens* [^{32}P]DNA hybridization to filter-bound DNAs. Percent hybridization was calculated from the μg of [^{32}P]DNA bound per μg of nonradioactive DNA present on the filters after counting. In all cases background binding to leaf nuclear DNA has been subtracted. In addition to 100% *A. tumefaciens* DNA (●), model filters are shown that contained leaf nuclear DNA plus either 1% (○) or 0.1% (△) *A. tumefaciens* DNA. Each regression line was determined by least squares analysis.

the incubation period, the single- and double-stranded DNA molecules were separated by phosphate buffer gradient elution from hydroxylapatite columns at 60°. Fractions containing single-stranded molecules were pooled, dialyzed, incubated at 100° for 5 min, quickly cooled, adjusted to 0.12 M phosphate buffer, and then subjected to a 5-hr incubation at 63° in the presence of 1 mg/ml of sheared, heat-denatured, nonradioactive bacterial DNA. This second incubation period is too short for plant sequences to reassociate. However, any *A. tumefaciens* sequences present originally in the crown gall tissues and labeled with ^{3}H would reassociate with the excess-nonradioactive *A. tumefaciens* DNA and appear in the double-stranded fractions after hydroxylapatite chromatography. In a control experiment, 85% of *A. tumefaciens* [^{32}P]DNA (0.2 μg/ml) added at the first step of this procedure was recovered in the final double-stranded fractions, verifying the expected fractionation of low concentrations of bacterial sequences. However, there was no difference between the recoveries from tumor [^{3}H]DNA and normal callus [^{3}H]DNA (Table 1). Likewise, there was no difference in recovery when the nonradioactive *A. tumefaciens* DNA in the second incubation was replaced by DNA from the unrelated bacterium *E. rubrifaciens*.

If only a small portion of the bacterial genome persists in crown gall tumor cells and if this portion is present as highly reiterated sequences, the bacterial DNA could have reassociated in the first incubation step and thus escaped detection. To test this possibility, a second type of enrichment experiment was performed which exploited thermal denaturation differences between *V. rosea* and *A. tumefaciens* DNA arising from the 20% difference in GC content. Sheared, denatured, tumor [^{3}H]DNA (100 μg/ml, 15,000 cpm/μg) was allowed to reassociate with nonradioactive bacterial DNA (1 mg/ml) at 80° in 0.09 M phosphate buffer for 6 hr, conditions that were too stringent for plant DNA reassociation but allowed 70% of the bacterial DNA to reassociate. The reassociated double-stranded DNAs were separated from single-stranded DNAs by hydroxylapatite chromatography. Reassociation of tumor [^{3}H]DNA in the presence of *A. tumefaciens* DNA was not greater than the reassociation of normal [^{3}H]DNA in the presence of *A. tumefaciens* DNA (Table 1). Since the annealing conditions were very stringent, AT-rich sequences would not have been detected by this method.

Neither of these enrichment experiments would have detected *A. tumefaciens* DNA if it represented less than 0.1% of the tumor DNA. Attempts were made to improve the resolving power of these methods by raising the specific radioactivities of the plant DNAs, but increases in the radioactive dosage or exposure time caused noticeable cell damage.

Filter Hybridization Analyses. The presence of *A. tumefaciens* DNA in tumor DNA was also examined by filter saturation hybridization techniques. Denatured tumor and normal callus DNAs immobilized on nitrocellulose filters were incubated with various concentrations of sheared, denatured *A. tumefaciens* [^{32}P]DNA. Since the *A. tumefaciens* [^{32}P]DNA in solution reassociates with itself in addition to hybridizing with homologous, filter-bound DNA (30, 33), the saturation plateaus underestimate the degree of homology (in the present experiments, the plateau for 100% homology was reached at 44% hybridization). The hybridization system was calibrated by measuring the binding of *A. tumefaciens* [^{32}P]DNA to model filters containing mixtures of *V. rosea* leaf nuclear DNA and *A. tumefaciens* DNA. An example of this type of calibration is shown in Fig. 1. The lower limit of detection of *A. tumefaciens* DNA varied from 0.03% to 0.07%. When the saturation curves, corrected for background binding by subtraction of binding to leaf and calf-thymus DNA, are expressed in double-reciprocal plots, they plot as straight lines (Fig. 2). Since double-reciprocal plots allow all of the data points to be used, they were used to determine saturation values by extrapolation to the ordinate. The extrapolated saturation values obtained from model filters were then used to generate standard curves (Fig. 3),

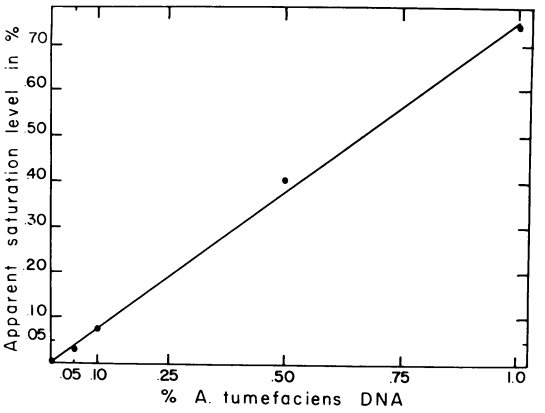

FIG. 3. Calibration of filter hybridization. Apparent saturation levels were determined by extrapolation of regression lines in Fig. 2 for model filters containing small, known amounts of *A. tumefaciens* DNA mixed with large amounts of leaf nuclear DNA.

which were in turn used to relate tumor saturation values to percent homology.

Five preparations of tumor DNA were assayed for base sequence homologies with *A. tumefaciens* DNA by the methods described above. The apparent percent homology after subtraction of normal callus DNA values (which were generally higher than those of leaf nuclear DNA) is summarized in Table 2. Each determination included at least three replicate filters and a calibration curve from model filters. The average apparent homology by this filter saturation method was 0.04%, corresponding to about 0.4 bacterial genome per tumor cell.

It was necessary to assume that *A. tumefaciens* and *V. rosea* DNAs were bound equally during the preparation of model filters because neither ^{3}H- nor ^{14}C-labeled *A. tumefaciens* DNA could be obtained with specific activities high

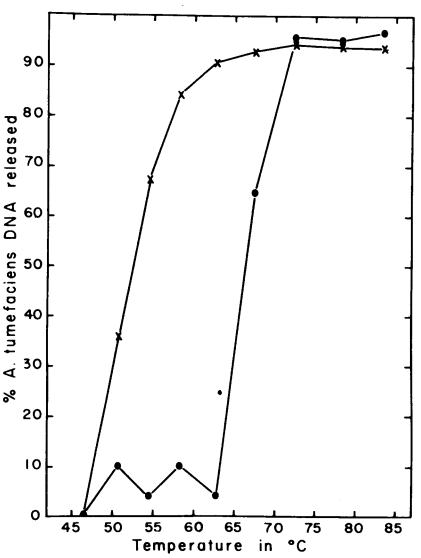

FIG. 4. Thermal elution profile of *A. tumefaciens* [^{32}P]DNA hybridized to filters containing crown gall tumor DNA (×) or a mixture of leaf nuclear DNA and 0.5% *A. tumefaciens* DNA (●). Background binding and elution, determined from filters containing only leaf nuclear DNA, has been subtracted from all values. Percent elution has been corrected for loss of nonradioactive DNA from filters at high temperatures.

enough to directly determine the amount of bacterial DNA present. This assumption seems reasonable since the efficiencies of binding were equal, and the two DNAs bound to filters in an additive way. The ratio of *A. tumefaciens* DNA to leaf nuclear DNA applied to filters was 0.25, 0.67, 1.5 and 4.0. The ratio of DNA actually bound per expected binding if additive was 1.01, 1.02, 0.99, and 1.01, respectively. A calibration of this type also assumes that the leaf nuclear DNA and the crown gall tumor DNA were equally accessible for hybridization. This is probably the case, since we found sheared tumor [^{32}P]DNA bound equally to leaf and tumor DNAs immobilized on filters.

Although the incubation conditions were moderately stringent [23° and 9° below the midpoint melting temperature (T_m) of *A. tumefaciens* DNA and *V. rosea* DNA, respectively], the hybrids measured above could be imperfectly paired duplexes and, thus, still greatly overestimate any true nucleotide sequence homologies between *A. tumefaciens* DNA and crown gall tumor DNA. This possibility was tested by measuring the thermal dissociation profiles of the hybrids (Fig. 4). The steep slope of the hybrid dissociation curve is consistent with the formation of duplexes having a high degree of base pairing fidelity. However, the hybrid dissociation midpoint is 11° lower than bonafide *A. tumefaciens* DNA hybrids and clearly indicates that no hybrids of high base pairing fidelity were detected having the same average base composition as *A. tumefaciens* DNA.

The sensitivity of the filter hybridization assay can be estimated from the thermal dissociation profiles in Fig. 4. If half of the hybrids had the same thermal stability as *A. tumefaciens* DNA, the hybrid curve in Fig. 4 would have been biphasic. Consequently, less than one-half of the hybrid, i.e., less than 0.02% of the tumor DNA, could be homologous to *A. tumefaciens* DNA.

DISCUSSION

We detected no *A. tumefaciens* DNA in preparations of *V. rosea* crown gall tumor DNA by DNA·DNA solution enrichment hybridization and very little, if any, by DNA·DNA filter saturation hybridization. The most sensitive of these methods, filter saturation hybridization, showed that less than 0.02% of the tumor DNA formed hybrid duplexes of high thermal stability with *A. tumefaciens* DNA. If bacterial DNA persists in crown gall tumors, it must average less than 0.2 bacterial genome equivalent per diploid tumor cell. The steep slope of the hybrid thermal dissociation profile may be due to AT-rich regions of *A. tumefaciens* DNA present in tumor DNA. Such a dissociation profile is a necessary,

TABLE 2. *Summary of apparent sequence homologies of A. tumefaciens DNA in crown gall by filter saturation hybridization*

Crown gall DNA preparation	Apparent homology[a] (%)
1	0.02
2	0.07
3	0.00
4	0.04
5	0.09
Average	0.04

[a] Binding to *Vinca rosea* normal callus DNA has been subtracted from all values.

but not a sufficient criterion for base pairing fidelity. A conclusion of homology is tenuous at such low levels of binding and must await more refined experimentation.

Our results clearly contradict the reports of Quétier *et al.* (13) and Srivastava (14), which were interpreted to show homologies as high as 0.1%. Three factors account for this discrepancy. Our hybridization conditions were more stringent than those used in the two earlier investigations (temperatures 20° as compared to 35°–40° below the T_m of *A. tumefaciens*), thus eliminating many imperfectly paired hybrids. Secondly, the earlier reports did not include model filter studies to correct for the fact that DNA·DNA filter saturation plateaus do not reach 100%. Thirdly, neither Quétier *et al.* (13) nor Srivastava (14) conducted thermal melting studies to evaluate the fidelity of hybrid base pairing. Neither the small amounts of hybridization found by us nor that detected by Farrand *et al.* (personal communication) have thermal melting profiles similar to bonafide *A. tumefaciens* DNA hybrids.

Farrand *et al.* also performed DNA·DNA filter saturation experiments and found no nucleotide sequence homologies between tobacco crown gall tumor and *A. tumefaciens* DNA. They were able to reproduce the same level of binding (before calibration with model filters) as Quétier *et al.* (13) and Srivastava (14), but the hybrid thermal stability was low and the melting profile was broad. Although their system would not detect less than 1% homology, as judged by calibration with model filters, another report from the same laboratory (Chilton *et al.*, personal communication) concluded from reassociation kinetics that no homologies were present at levels greater than 0.01%.

Schilperoort's evidence (12, 15) for sequence homologies detectable by hybridization of tumor DNA with RNA complementary to *A. tumefaciens* DNA or PS8 phage DNA has also been challenged recently (34). As in the DNA·DNA experiments discussed above, thermal stability profiles of the RNA·DNA hybrids indicated little fidelity of base pairing. Based on our hybridization experiments, we believe that either there may be no *A. tumefaciens* nucleic acids in bacteria-free crown gall tumors, or that this bacterial nucleic acid exists in quantities below the limits of DNA-hybridization assays used herein. Consequently, we believe it is necessary to re-evaluate the concept that *A. tumefaciens* nucleic acids persist in bacteria-free crown gall tumors.

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