

Attempts to Detect Deoxyribonucleic Acid from *Agrobacterium tumefaciens* and Bacteriophage PS8 in Crown Gall Tumors by Complementary Ribonucleic Acid/Deoxyribonucleic Acid-Filter Hybridization

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Labeled ribonucleic acid (RNA) complementary to *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA (cRNA) were used in a systematic study of the sensitivity of cRNA/deoxyribonucleic acid (DNA)-filter hybridization for detection of small amounts of phage or bacterial DNA immobilized on filters. *A. tumefaciens* cRNA of specific activity 10^6 to 2×10^6 counts per min per μg reacted to a significant extent when the DNA-filter contained 1% *A. tumefaciens* DNA in a salmon DNA background, but 0.1% *A. tumefaciens* DNA was not detectable. PS8 phage cRNA of the same specific activity reacted to a significant extent when the DNA-filter contained as little as 0.01% PS8 DNA in a salmon DNA background. Both kinds of cRNA were found to bind to tobacco crown gall tumor DNA-filters. Similar reaction was found with control normal callus DNA-filters but not with tobacco seedling DNA-filters. The "hybrids" formed by cRNA with normal callus and tumor DNA-filters had low thermal stability. Attempts to purify the tumor and normal callus DNA prior to immobilization on the filter resulted in elimination of this spurious binding. No evidence was found for bacterial or phage DNA in crown gall tumor DNA.

Crown gall, a neoplastic disease affecting many kinds of dicotyledonous plants, is caused by inoculating *Agrobacterium tumefaciens* into a wound site. Autonomous growth of the axenic tumor tissue is manifest as the ability to grow in vitro without addition of auxin or cytokinin, hormones required by normal plant tissue for growth in culture. The nature of the interaction between *A. tumefaciens* and plant tissue has not been elucidated, but the fact that tumor cells maintain their transformed phenotype in culture for many years suggests that they may have undergone permanent genetic alteration. Deoxyribonucleic acid (DNA) from *A. tumefaciens* and its bacteriophage PS8 have been suggested as mediators of this change (10-12, 14, 15).

Attempts to detect *A. tumefaciens* DNA or bacteriophage PS8 DNA in the DNA isolated from axenic tobacco tumor tissue have yielded conflicting results by different hybridization techniques. Labeled complementary ribonucleic acid (cRNA) transcribed from *A. tumefaciens* DNA is reported to hybridize with crown gall tumor DNA immobilized on cellulose nitrate filters (11) or in solution (12). Normal

tobacco leaf DNA gave no significant reaction (11, 12), whereas normal tobacco callus DNA gave 10 to 18% as much as tumor DNA (11). Labeled *A. tumefaciens* DNA is also reported to hybridize with tumor DNA-filters (10, 14), whereas normal tobacco callus DNA-filters give about half as much reaction (14) or no reaction (10). Labeled cRNA transcribed from PS8 bacteriophage is also reported to hybridize with filter-bound DNA isolated from crown gall tumor tissue which was incited by a bacterial strain free from phage PS8 by several criteria including nucleic acid hybridization (11). In contrast, by DNA renaturation kinetic analysis neither bacterial nor phage DNA was detected in crown gall tumor DNA isolated in this laboratory (M.-D. Chilton, T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester, submitted for publication) under conditions shown capable of detecting 0.01% bacterial and 0.001% phage DNA. The latter technique is only capable of detecting a significant fraction ($\geq 5\%$) of the bacterial or bacteriophage genome, whether in single or multiple copies, whereas the techniques of cRNA/DNA-filter hybridization and DNA/DNA-filter hy-

bridization could detect relatively few genes if they are present in many copies in tumor DNA. Since it is possible that only a minor portion of the bacterial or phage genome is present in tumor cells, a suggestion made by Schilperoort et al. (11), we have attempted to detect these foreign DNA sequences in tumor DNA by DNA-filter hybridization techniques. The results of our cRNA hybridization experiments are presented here.

The cRNA hybridization technique as applied here is 10 to 100-fold less sensitive than our DNA renaturation kinetic analysis experiments for finding whole bacterial or phage genomes in model mixtures. An unexpected difficulty was encountered with DNA isolated from both normal and crown gall plant callus tissue. Spurious binding of labeled cRNA to plant tissue culture DNA-filters was observed when the DNA was isolated by the procedure of Schilperoort et al. (11, 12) and partially freed from polysaccharides by cetyltrimethylammonium bromide precipitation (6). Ribonuclease (RNase)-resistant "duplexes" of low thermal stability were obtained. After batch adsorption and elution from hydroxylapatite, the same tumor and normal callus DNA preparations failed to give any significant reaction with either phage or bacterial cRNA. Contrary to the findings of Schilperoort et al. (11), we are unable to detect any bacterial or phage DNA sequences in the DNA of the five crown gall tumor lines examined. Possible explanations for this discrepancy are discussed.

MATERIALS AND METHODS

Cultivation of tumor and normal callus tissue.

Tumor lines and normal callus from *Nicotiana tabacum* var. *Xanthi*, n. c. were isolated and cultivated as described previously (7). Tumor lines have been named for the strain of *A. tumefaciens* used to incite them. Sources of bacterial strains are listed elsewhere (7).

DNA isolation. DNA was isolated from crown gall tumor tissue and normal callus by the method of Schilperoort et al. (11, 12), using frozen total tumor or callus tissue. Cetyltrimethylammonium bromide (CTAB) precipitation (6) was used to free the DNA from large amounts of contaminating polysaccharide. Three additional treatments used as indicated in the text were:

(i) **Banding in CsCl.** DNA was centrifuged to equilibrium in a preparative CsCl gradient ($\rho = 1.700$, volume = 5 ml, Spinco 40 rotor, 70 h, 33,000 rpm, 22 C). Fractions were collected after puncturing the bottom of the tube, and refractive index and optical density were measured at 260 nm. From the refractive index gradient, the position in the CsCl gradient where *A. tumefaciens* DNA ($\rho = 1.718$) would band was determined, and this region through the plant

DNA region (A_{260} peak, $\rho = 1.693$) was pooled. Phage DNA ($\rho = 1.714$) would be included in the pooled gradient fractions. In a reconstruction experiment, it was shown that phage DNA added to tumor DNA could be recovered quantitatively by this method (Table 2).

(ii) **Filtration.** Native tumor DNA (100 μ g) in 2 ml of 5 \times SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate) was filtered under aspirator vacuum through a 25-mm cellulose nitrate membrane filter (Schleicher and Schuell, type B6). A similar procedure has been used in some earlier crown gall DNA-*A. tumefaciens* cRNA hybridization experiments (12) but not in others (11).

(iii) **Hydroxylapatite chromatography.** Native DNA (100 μ g) in 2 ml of 0.15 M PB (PB is an equimolar mixture of mono- and dibasic sodium phosphates) was loaded onto a 0.5-ml column of BioRad HTP hydroxylapatite at 70 C and the column was washed with 2 to 4 ml of 0.15 M PB. The eluate was discarded, and DNA was eluted from the column with two 1-ml washes of 0.3 M PB. Recovery of diphenylamine-positive material was 85%.

DNA was isolated from *A. tumefaciens* by the procedure of Schilperoort et al. (11, 12). Salmon DNA was isolated from fresh sperm by the method of Whiteley et al. (16). PS8 phage DNA was isolated from purified phage by phenol deproteinization.

Preparation of cRNA. *Escherichia coli* RNA polymerase was a gift from E. Davie and R. Discipio. The enzyme, which contains sigma factor, was purified by a modification of the procedure of Chamberlain and Berg (4), including protamine sulfate and ammonium sulfate precipitation and chromatography on columns of DE-52 and hydroxylapatite. For cRNA synthesis, the 1-ml reaction mixture consisted of 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 7.9, 0.2 M KCl, 25 mM MgCl₂, 0.2 mM dithiothreitol, 10⁻³ M ribonucleotide triphosphates including 400 nmol of [³H]GTP (New England Nuclear Corp., 5 Ci/mM), 80 μ g of DNA template, and 0.1 ml of enzyme (4.2 mg of protein per ml, initial concentration; approximately one-third pure). The mixture was incubated for 90 min at 37 C, and reaction was terminated by heating at 80 C for 5 min. Template DNA was digested with deoxyribonuclease (DNase) (Worthington DPFF, 10 μ g/ml) for 20 min at 37 C. Sodium dodecyl sulfate (0.1% final concentration) was added and the RNA was deproteinized by two extractions with equilibrated phenol (pH 7). RNA was purified by gel filtration (Sephadex G-75, 1- by 20-cm column). Specific activities ranged from 1.2 \times 10⁶ to 2.1 \times 10⁶ counts per min per μ g.

Preparation of DNA filters. DNA filters were prepared by the procedure of McCarthy and McConaughy (9), except that the DNA was denatured by addition of 0.1 volume of 1 M NaOH, incubation at 22 C for 5 min, and neutralization with 0.1 volume of 1 M NaH₂PO₄ solution. Model filters were constructed by using mixtures of salmon DNA and phage or bacterial DNA as follows: 500 μ g of phage or bacterial DNA for the 100% filter, 50 μ g of phage or bacterial DNA plus 450 μ g of salmon DNA for the 10% filter, etc. Denatured DNA was adjusted to 5 \times SSC

and loaded onto 50-mm Schleicher and Schuell B6 membrane filters. After washing and treating with Denhardt's preincubation mixture (5), 6-mm circles were punched from the large filter (9). The amount of DNA per small circle was measured by diphenylamine determination (3) run directly on the DNA filter; the presence of the filter caused no interfering color in this assay.

Hybridization of cRNA to DNA filters. Filter-bound DNA (7 to 17 μg) was incubated in 0.2 ml of $2\times$ SSC containing 0.1 to 0.2 μg of [^3H]cRNA for 18 to 20 h at 67 C. Input radioactivity ranged from 120,000 to 300,000 counts/min. Filters were washed three times with $2\times$ SSC at 67 C and were then treated 60 min at 22 C with RNase (10 μg of RNase A and 3 μg of RNase T1 per ml, previously heated 10 min at 80 C in 0.01 M acetate, pH 5.5). Filters were then washed twice in $2\times$ SSC at 22 C, air dried, and counted in toluene-Liquifluor (Nuclear Chicago Corp.). Radioactivity bound was normalized to percent of input radioactivity bound per 10 μg of DNA initially bound to the filter.

Determination of thermal stability of duplexes. Filters were removed from the toluene-Liquifluor solution in which they had been counted and were washed in toluene to remove scintillants. Dry filters were incubated in SSC (1 ml) for 5 min, and then in fresh portions of SSC (1 ml) for 5 min at 60 C, 65 C, etc., up to 100 C. Radioactivity eluted at each temperature was determined by counting the eluted in "Triton cocktail" (Triton X-100 [Rohm and Hass]; toluene-Liquifluor, 1:2 vol/vol).

RESULTS

To determine the sensitivity of our cRNA hybridization technique for detecting small amounts of phage or bacterial DNA in eukaryotic DNA, highly labeled cRNA was allowed to hybridize with a series of model filters containing decreasing percentages of homologous DNA. The upper portion of Table 1 shows that 0.01% phage DNA gives 0.2% binding, significantly above the salmon DNA background (0.06% binding). The PS8-cRNA duplexes melt sharply with a T_m of 82 to 84 C (Fig. 1) in all cases except that the 0.01% model filter melting profile shows the presence of a significant fraction of lower melting nonspecific "duplexes". Even so, a component with high thermal stability is clearly visible in the melting profile. As expected, PS8 DNA can be detected easily in DNA from lysogenic *A. tumefaciens* strains B6-806(PS8) and B6Sch (Table 1, bottom). These bacterial DNA preparations appear to have more than 1% phage DNA in them (1 copy of the phage genome per bacterial cell) judging by the 1% phage model filter binding. This may be due to a low level of spontaneous induction of lysogenic bacteria. Nonlysogenic bacterial strains 15955 and B6-806 show no significant reaction with PS8-cRNA. The low level of binding to B6-806 DNA filters is insignificant

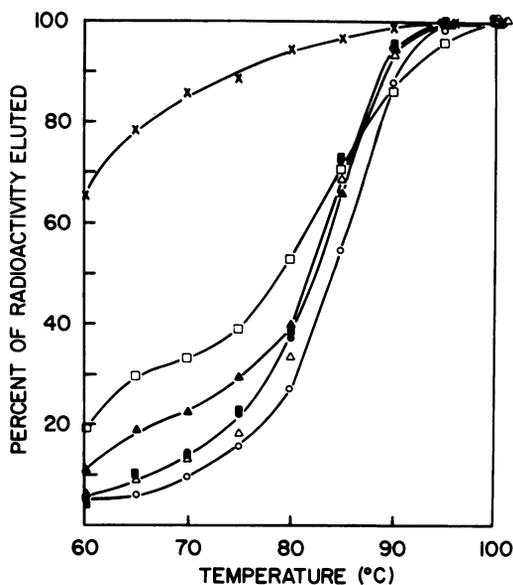


FIG. 1. Thermal stability of PS8 cRNA-DNA duplexes. [^3H]RNA complementary to PS8 DNA (1.2×10^6 counts per min per μg) was hybridized to filter-bound DNA from PS8, *A. tumefaciens*, and crown gall tumors, as well as artificial mixtures of PS8 DNA and salmon DNA. Duplexes were melted in SSC. Symbols: \circ , 100% PS8; \bullet , 10% PS8; Δ , 1% PS8; \blacktriangle , 0.1% PS8; \square , 0.01% PS8; \blacksquare , *A. tumefaciens* strain B6 Sch; \times , crown gall tumor strain B6 Sch.

because the duplexes have a T_m of <60 C (Table 1).

Complementary RNA from A6 bacterial DNA template gives significant cross-reaction with distantly related (8) 15955 and B6 bacterial DNA filters (Table 1, bottom), compared with homologous A6 bacterial DNA filters (Table 1, 100% A6 model filter). Assuming that any DNA specific to tumor induction will be among the DNA sequences held in common by all three strains, this cRNA can thus be used as a probe in attempts to detect *A. tumefaciens* DNA in these other lines of crown gall tumors. The heteroduplexes formed between A6 cRNA and putative bacterial DNA sequences in B6-strain-induced tumor DNA would be expected to have low thermal stability, however. Heteroduplexes between A6 cRNA and B6-806 bacterial DNA have $T_m = 65$ C (Table 1).

The limits of sensitivity for detection of bacterial DNA, judging by the model filter data, are about 1% (Table 1, Fig. 2). A reaction of marginal significance can be seen with the 0.1% model filter, which shows 0.04% binding, compared with 0.02% for salmon DNA. A component of high thermal stability is also visible in the thermal dissociation profile.

TABLE 1. Hybridization of PS8 cRNA and *A. tumefaciens* A6 cRNA to filter-bound DNA from PS8 bacteriophage, *A. tumefaciens*, and crown gall tumors, normal callus, and seedlings

Filter-bound DNA	% of Input cRNA bound		T_m of duplexes	
	PS8 cRNA	A6 cRNA	PS8 cRNA	A6 cRNA
100% PS8	50.1		84	
10% PS8, 90% salmon	7.1		82	
1% PS8, 99% salmon	4.7		82	
0.1% PS8, 99.9% salmon	0.6		82	
0.01% PS8, 99.99% salmon	0.2		79	
100% A6		10.1		85
10% A6, 90% salmon		0.33		85
1% A6, 99% salmon		0.06		82
0.1% A6, 99.9% salmon		0.04		76
Salmon	0.06	0.02		
Tobacco seedling	0.04	0.05		
Normal tobacco callus	3.5	0.22	<60	<60
Crown gall tumor 15955	3.3	0.16	<60	<60
Crown gall tumor A6	3.1	0.10	<60	<60
Crown gall tumor B6-806 (PS8)	3.8	0.13	<60	<60
Crown gall tumor B6M	3.2	0.13	<60	<60
Crown gall tumor B6 Sch	0.14	0.07	<60	<60
<i>A. tumefaciens</i> 15955	0.05	3.2		85
<i>A. tumefaciens</i> B6-806	0.28	3.3	<60	65
<i>A. tumefaciens</i> B6-806 (PS8)	22.3		83	
<i>A. tumefaciens</i> B6 Sch	9.6		82	

We next attempted to determine whether bacterial and PS8 phage DNA could be detected in crown gall tumor DNA immobilized on filters. These efforts led us to discover an unexpected pitfall in preparation of DNA filters by using DNA isolated from plant tissue culture materials.

Filter-bound DNA from tobacco seedlings binds very little cRNA compared to salmon DNA filters (background) (Table 1), showing that cross-reaction between phage or bacterial cRNA and tobacco DNA sequences is minimal under our reaction conditions. However, DNA isolated from normal tobacco callus binds significant amounts of each of the cRNA preparations. These "duplexes" differ from homologous duplexes in that they exhibit extremely low thermal stability. Similar results were obtained when these cRNAs were hybridized with crown gall tumor DNA filters: significant binding occurred with four of the five tumor DNA isolates tested, but in no case were high melting duplexes obtained (Table 1, Fig. 1 and 2). The extent of this nonspecific binding is much greater for PS8 cRNA than for A6 cRNA; the explanation for this seeming specificity remains

obscure. The binding data would suggest that 1% phage and bacterial DNA were present in most of the tumors, but the melting profiles and the similar binding for normal callus DNA filters make this conclusion untenable.

To determine whether tumor and normal callus DNA filters were capable of entering into hybridization reactions and giving duplexes of expected thermal stability, each type of tumor DNA filter, normal callus DNA filters, and tobacco seedling DNA filters were compared in reactions with sheared denatured ^3H -labeled tobacco seedling DNA. All filters bound a similar amount of radioactivity (20 to 33% of 65,000 counts/min input) after reaction in $2\times$ SSC at 67 C for 18 h, and the T_m of the DNA duplexes formed in each case was 73 C, as expected for tobacco redundant DNA duplexes. This rules out the possibility that tumor DNA-filter hybrids exhibit low T_m because of DNA coming off the filter during the early stages of the melting curve determination.

In an effort to remove putative impurities from tumor and normal callus DNA preparations, three additional treatments were tried: banding in CsCl, prefiltration through cellulose nitrate filters, and hydroxylapatite batch ad-

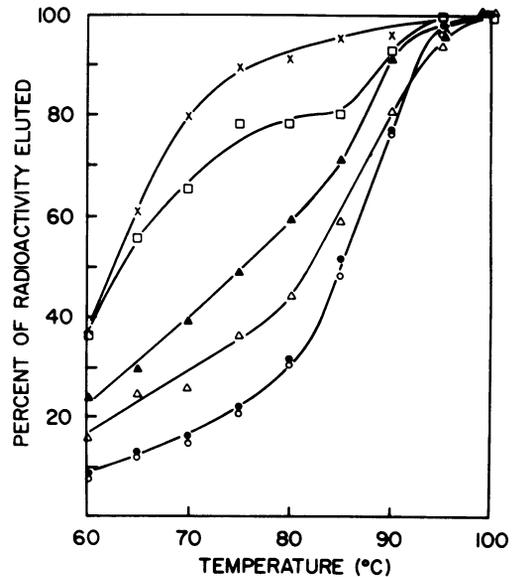


FIG. 2. Thermal stability of *A. tumefaciens* A6 cRNA-DNA duplexes. [^3H]RNA complementary to DNA from *A. tumefaciens* A6 was hybridized to filter-bound DNA from *A. tumefaciens* strains and crown gall tumors, as well as artificial mixtures of *A. tumefaciens* and salmon DNA. Duplexes were melted in SSC. Symbols: \circ , 100% A6 DNA; \bullet , 10% A6 DNA; Δ , 1% A6 DNA; \blacktriangle , 0.1% A6; \square , 0.01% A6; \times , crown gall tumor strain A6.

TABLE 2. Effect of DNA purity on hybridization between PS8 or *A. tumefaciens* cRNA and crown gall tumor DNA

Filter-bound DNA	Method of purification	% Input bound per 10 µg of DNA	
		PS8 cRNA	A6 cRNA
Salmon	Standard	0.05	0.02
Salmon + 0.1% PS8	Standard	0.4	
Salmon + 0.1% PS8	Standard + NCF ^a	0.4	
Normal tobacco callus	Standard	2.9	0.22
Normal tobacco callus	Standard + CsCl ^b	3.7	0.17
Normal tobacco callus	Standard + HAP ^c	0.04	0.04
Crown gall tumor 15955	Standard	1.8	0.16
Crown gall tumor 15955	Standard + CsCl	1.2	0.14
Crown gall tumor 15955	Standard + HAP	0.04	0.04
Crown gall tumor 15955 + 0.1% PS8	Standard + CsCl	1.7	
Crown gall tumor 15955 + 0.1% PS8	Standard + HAP	0.7	
Crown gall tumor A6	Standard	1.0	0.1
Crown gall tumor A6	Standard + HAP	0.05	0.05
Crown gall tumor A6	Standard + NCF	0.08	0.1
Crown gall tumor B6M	Standard	0.8	0.11
Crown gall tumor B6M	Standard + HAP	0.05	0.06
Crown gall tumor B6M	Standard + NCF	0.05	0.08
Crown gall tumor B6-806 (PS8)	Standard	1.3	0.13
Crown gall tumor B6-806 (PS8)	Standard + HAP	0.05	0.05

^a NCF, Nitrocellulose filtration (see Materials and Methods).

^b CsCl, Banding in CsCl gradient (see Materials and Methods).

^c HAP, Hydroxylapatite chromatography (see Materials and Methods).

sorption and elution. Control studies (Table 2) with synthetic mixtures of PS8 DNA and salmon or tumor DNA showed that no selective loss of PS8 DNA sequences occurs during any of the three procedures. For each of the tumor DNA preparations and for normal callus DNA, treatment by one or a combination of these methods caused a reduction in the amount of PS8 of *A. tumefaciens* cRNA bound. When DNA eluted from hydroxylapatite was used, no significant duplex formation between phage or bacterial cRNA and DNA from crown gall tumors or normal tobacco callus occurred.

When 0.1% PS8 DNA is added to tumor DNA and the mixture is subjected to CsCl density gradient centrifugation (see Materials and Methods), the recovery DNA mixture after filter binding reacts to a slightly greater extent in the phage cRNA hybridization system than does filterbound tumor DNA alone, similarly banded in CsCl. Tumor plus phage DNA filters bind 1.7% of input radioactivity while tumor DNA filters bind 1.2% (Table 2). The melting profiles of these duplexes (Fig. 3) verify that a high melting component is detectable in tumor plus phage DNA hybrids but not in tumor DNA hybrids. However, when the mixture of 0.1% added phage DNA in tumor DNA is subjected to hydroxylapatite batch elution and immobilized on a filter, 0.7% of input phage cRNA is bound, compared with 0.04% for similarly treated tumor DNA alone. The background reaction is thus decreased dramatically. The melting curve for the hybrids formed with phage plus tumor DNA filters compares very favorably with that of the 0.1% model phage plus salmon DNA filter (Fig. 3). It is noteworthy that the high background tumor plus phage DNA filters

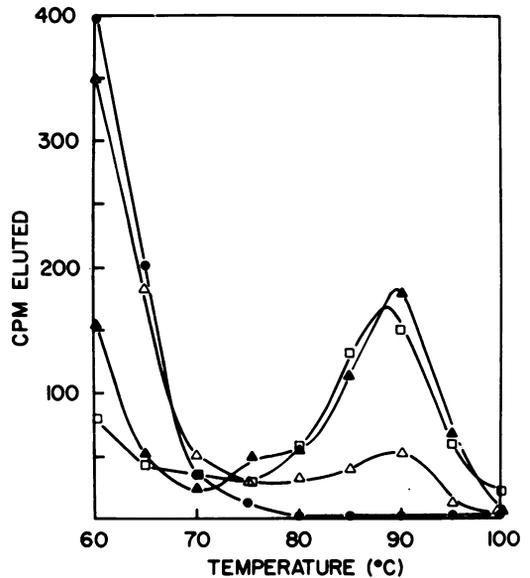


FIG. 3. Effect of DNA purity on thermal stability of PS8 cRNA-DNA duplexes. [³H]RNA complementary to PS8 DNA was hybridized to crown gall tumor DNA or an artificial mixture of tumor and PS8 DNA after successive DNA purification steps. Duplexes were melted in SSC. Symbols: ●, 15955 tumor DNA after banding in CsCl; ▲, 15955 tumor DNA + 0.1% PS8 DNA, banded in CsCl and purified by hydroxylapatite chromatography; □, 0.1% PS8 + salmon DNA.

when incubated with phage cRNA not only exhibit more low melting radioactivity bound but also less high-melting hybrid, as these differentially plotted melting curves reveal. Whether this is due to interference with binding or with melting behavior is uncertain, but it is clear that the putative impurities in tumor DNA tend to mask any signal that might be present.

DISCUSSION

The results of this study place limits on the sensitivity of our cRNA-DNA filter hybridization technique for the detection of bacterial or phage DNA in crown gall tumor DNA. Using cRNA of specific activity 10^6 to 2×10^6 counts per min per μg , as little as 0.01% PS8 phage DNA and 1% *A. tumefaciens* DNA could be detected in synthetic mixtures of these DNAs with salmon DNA. The DNA content of diploid tobacco nuclei is 6×10^{12} daltons (13), and the genome sizes of PS8 and *A. tumefaciens* 15955, determined by renaturation kinetics (2), are 4.1×10^7 and 2×10^9 daltons, respectively (data not shown). Thus, 0.01% phage DNA in crown gall tumor DNA would represent 15 phage genomes per diploid cell, and 1% bacterial DNA would be 30 bacterial genomes per diploid cell. The sensitivity of detection of complete copies of foreign DNA in genomes per tumor cell is thus independent of the genome size of the foreign DNA. A larger foreign genome is more readily detectable because there is more of it per copy, but also concomitantly more difficult to detect because the intrinsic rate of the cRNA-DNA reaction is slower for larger genomes (1).

It is difficult to construct model filters to determine the limits of detection for multiple copies of a small portion of a phage or bacterial genome. It seems plausible that 10 copies of 10% of a genome would give binding similar to that of one copy of the whole genome, etc., so long as labeled transcript for the repeated sequence is not exhausted from solution. Since we do not know whether the bacterial and phage DNA are transcribed randomly into cRNA, it is not possible to establish the point at which exhaustion would occur. Since at the threshold of detection we observed only 0.1 to 0.2% of input cRNA binding, of the order of 500 to 1,000 copies of a fraction of either genome should give a proportional signal without such exhaustion. Under our conditions, 30 copies of a whole genome are just detectable, so 300 copies of 10% of the genome of 3,000 copies of 1% of the genome would be required for detection.

Using cRNA of the same specific activity as ours, Schilperoort et al. (11) performed satura-

tion hybridizations with crown gall tumor DNA filters. They interpret their data to show that crown gall tumor DNA contains 1.8% PS8 DNA and 0.9% bacterial DNA, levels which are technically detectable in model filters under our experimental conditions. However, we did not detect any PS8 phage DNA or bacterial DNA in the DNA of five different lines of crown gall tumor cells, including tumor line A6, incited by the same bacterial strain as the tumor Schilperoort et al. (11) investigated. The source of the discrepancy in hybridization results is not clear.

Differences in technique could be responsible for our failure to detect foreign DNA. These differences include: different cRNA preparation (although made by the same method with the same type of *E. coli* RNA polymerase) which might lack transcripts of the important parts of the bacterial and phage genomes; our failure to perform saturation curves using increasing concentration of cRNA (although our input cRNA concentration, 0.5 to 1 $\mu\text{g}/\text{ml}$, is close to the level these investigators found to be saturating: 1 $\mu\text{g}/\text{ml}$ for PS8 cRNA and 3 $\mu\text{g}/\text{ml}$ for bacterial cRNA). Differences in the crown gall tumors themselves could also be invoked.

On the other hand it is possible that the interfering impurities in plant tissue culture DNA which we have encountered might also have caused difficulties in the tumor DNA studied by Schilperoort et al. Although the thermal stability measurements of their tumor DNA-cRNA hybrids give T_m values 8 C below those of homologous hybrids, their normal callus DNA-cRNA hybrids have T_m only 10° below homologous hybrids (N. J. van Sittert, Ph.D. thesis, University of Leiden, 1972). These data fail to substantiate the claim that well-matched hybrids were detected (11).

We would also point out that it is very surprising to detect PS8 phage DNA in A6 tumor cells (11), because the bacterial strain which incited this tumor appears by many criteria to be free from PS8 bacteriophage. A6 bacterial DNA contains no detectable PS8 DNA by renaturation kinetic analysis (unpublished data) and by cRNA hybridization (11).

A hybridization technique which circumvents the problem of selective transcription of the probe genome is the DNA/DNA-filter reaction. Unfortunately, this technique is less sensitive for detection of small amounts of bacterial and phage DNA in model filters (S. K. Farrand, F. C. Eden, A. J. Bendich, M. P. Gordon, and M.-D. Chilton, manuscript in preparation, and also fails, in our hands, to detect PS8 or *A. tumefaciens* DNA in crown gall tumor DNA.

Either of these DNA-filter hybridization methods might have detected multiple copies of a small portion of a bacterial or phage genome, which could have escaped detection by DNA renaturation kinetic analysis. By the latter technique, we have been able to rule out the presence of as much as one bacterial or phage genome per diploid crown gall tumor cell (Chilton et al., op. cit.). From all of our results taken together, we conclude that our crown gall tumor DNA is free from detectable bacterial or phage DNA. Although hybridization experiments can never prove rigorously that none is present, we suggest the need for reevaluating the idea that bacterial or phage DNA integration is the molecular basis of crown gall tumorigenesis.

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