Variations in Plasmid DNA Sequences Present in Crown Gall Tumour Lines

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INTRODUCTION

Extracellular infections of wounded dicotyledonous plants by the Gram-negative bacterium *Agrobacterium tumefaciens* (Smith & Townsend) Conn result in the transformation of normal plant cells to tumour cells. These crown gall tumour cells will continue to grow autonomously in the absence of the bacterium (Braun, 1943). Virulent strains of *A. tumefaciens* contain a large plasmid (120×10^6 molecular weight; Zaenen *et al.*, 1974). The presence of this plasmid is correlated with the virulence of the bacterium (Van Larebeke *et al.*, 1974). Bacteria which have lost the plasmid are avirulent and regain virulence when the plasmid is reintroduced into them (Watson *et al.*, 1975). One strain of crown gall tumour cells, *Vinca rosea* V_{A6}, has been shown to contain DNA sequences complementary to more than 50% of the bacterial plasmid DNA (Matthysse & Stump, 1976). In order to determine whether this tumour strain is unique, two other bacteria-free strains of crown gall tumours have been examined for the presence of DNA sequences complementary to plasmid DNA.

METHODS

Media. *Agrobacterium tumefaciens* strains BP and B6 were grown in minimal salts medium containing (g l⁻¹): NH₄Cl, 5; NH₄NO₃, 1; Na₂SO₄, 2; K₂HPO₄, 3; KH₂PO₄, 1; MgSO₄·7H₂O, 0.1; with 0.2% (w/v) glucose and 50 to 100 µCi [methyl-³H]thymidine ml⁻¹ (Clowes & Hayes, 1968). *Vinca rosea* crown gall tumour tissue culture cells were grown on unsupplemented White's medium (Wood & Braun, 1961). Normal *V. rosea* tissue culture cells were grown on White's medium supplemented with (mg l⁻¹): α-naphthalene acetic acid (NAA), 1; kinetin, 0.5; asparagine, 200; glutamine, 200; meso-inositol, 100; AMP, 100; GMP, 100 (Wood & Braun, 1961). Normal tobacco was grown on Murashige & Skoog's (1962) medium supplemented with 5 mg NAA l⁻¹ and 1 mg kinetin l⁻¹. *Bryophyllum* tissue cultures were grown on Miller's medium (1961) containing 1 g citric acid l⁻¹ and 2 mg NAA l⁻¹.

Organisms. *Agrobacterium tumefaciens* strain BP and *V. rosea* normal strain NV and crown gall tissue culture strain V_{BP} were obtained from Professor A. Braun. Tissue cultures of normal *Nicotiana tabacum* were obtained from P. Wyman. *Agrobacterium tumefaciens* strain B6 and *Bryophyllum* crown gall tissue culture strain B_{B6} were obtained from Professor C. Miller.

Isolation of DNA. Plant cell DNA was extracted by the method of Kirby (1957) as previously described (Matthysse & Stump, 1976). Purified DNA preparations in 0.1 × SSC (saline sodium citrate, where 1 × SSC contains 0.15 M-NaCl and 0.015 M-trisodium citrate) had E_{260}/E_{280} ratios between 1.75 and 1.95. No RNA was detectable by examination of the supernatant from the base hydrolysis (0.3 M-KOH, 18 h, 37 °C) of 100 µg DNA ml⁻¹ for E_{260} .

Bacterial plasmid DNA was prepared as previously described (Matthysse & Stump, 1976) or by CsCl-ethidium bromide equilibrium density gradient centrifugation as described by Klein *et al.* (1975). Each of the strains used appears, from restriction endonuclease digestion patterns, to contain only one type of plasmid (Mandelkorn & Matthysse, unpublished observation). The specific activity of the tritiated bacterial plasmid DNA ranged from 2×10^5 to 1×10^6 c.p.m. µg⁻¹. Before use in hybridization reactions, both the plant and plasmid DNAs were degraded to a molecular weight of approximately 2×10^5 by a limited depurination reaction (McConaughy & McCarthy, 1967). The DNA was dissolved in 0.1 M-sodium acetate pH 4.2 and heated at 70 °C for 33 min. The pH of the solution was then adjusted to 12 with NaOH and the solution was heated at 50 °C for 10 min. It was then dialysed against 1.5 × SSC.

Hybridization reactions. DNA hybridization reactions were carried out in $1.5 \times \text{SSC}$ in sealed glass capillary tubes at 67°C . The DNA was denatured by heating at 100°C for 15 min. Total radioactive DNA was measured by trichloroacetic acid (TCA) precipitation at 4°C using bovine serum albumin as a carrier, followed by filtration on Whatman GF/C filters which were washed with 10% (v/v) TCA and ethanol, dried, and counted in a liquid scintillation counter. Double-stranded radioactive DNA was measured by incubating the sample ($20\ \mu\text{g}$ or less) with 2 units of *Neurospora crassa* endonuclease (Miles Laboratories, Elkhart, Indiana, U.S.A.) in $1.5 \times \text{SSC}$ at 37°C for 30 min to degrade single-stranded DNA. The remaining double-stranded DNA was precipitated by TCA, collected by filtration on GF/C filters and counted.

The rate of renaturation of a known concentration of radioactive bacterial plasmid DNA was determined in the presence and absence of non-radioactive DNA from crown gall tumour cells. The concentration of radioactive bacterial plasmid DNA in these reaction mixtures was small, in order that the point at which the change in slope of the reactions occurred would be as distinct as possible. The rate of renaturation of the plasmid DNA alone was unaffected by the addition of $300\ \mu\text{g}$ normal tobacco DNA ml^{-1} or $800\ \mu\text{g}$ normal *V. rosea* DNA ml^{-1} . Under the conditions used $1\ \mu\text{g}$ plasmid DNA ml^{-1} renatures with a slope of $0.01\ \text{h}^{-1}$ ($K_2 = 0.02\ \text{ml}\ \mu\text{g}^{-1}\ \text{h}^{-1}$) (Matthyse & Stump, 1976). The rate of renaturation of the bacterial plasmid DNA sequences in the mixtures of plasmid DNA with tumour cell DNA is determined by the concentration of DNA sequences complementary to plasmid DNA in the tumour since the radioactive bacterial plasmid DNA is present at too low a concentration to renature at a significant rate by itself.

The data were analysed using the method of Sharp, Pettersson & Sambrook (1974) and the equation (Gallimore, Sharp & Sambrook, 1974):

$$\frac{C_0}{C} = \frac{K_2 C_0 (1+r)t}{2} + 1$$

where C_0 and C refer to the concentrations of total and single-stranded radioactive plasmid DNA respectively, K_2 is the second order rate constant, t is time, and r is the ratio of non-radioactive complementary DNA sequences to the radioactive sequences. If only a fraction of the radioactive DNA sequences is contained in the added non-radioactive DNA, then the rate of renaturation will be increased only until these sequences have renatured (Gallimore *et al.*, 1974). The value of C_0/C at which the slope of C_0/C against time changes will be the inverse of one minus the fraction of the radioactive DNA sequences present in the non-radioactive DNA. Thus, if 30% of the plasmid sequences are present in the tumour cells the slope of C_0/C versus t will change at $C_0/C = 1/1 - 0.3 = 1.43$.

RESULTS AND DISCUSSION

The results of hybridization reactions between ^3H -labelled plasmid DNA derived from two strains of *A. tumefaciens* (B6 and BP) and crown gall tumour cell DNA from tumours induced by these strains (*Bryophyllum* B_{B6} and *V. rosea* v_{BP} , respectively) are shown in Fig. 1. Each of the tumour lines appears to contain some DNA sequences complementary to bacterial plasmid DNA. The slope of the renaturation curves of the two tumour lines indicates that between 0.1 and 0.2% of the tumour cell DNA from each of the lines studied was complementary to plasmid DNA. [It is difficult to convert this number into an amount of plasmid DNA per diploid plant cell genome as many of these tissue culture cells are aneuploid (Matthyse, unpublished observations).] However, the percentage of the total plasmid DNA sequences present varies with the tumour line: B_{B6} appears to contain about 40%, and v_{BP} about 15%. This is in contrast to the crown gall tumour line previously studied, *V. rosea* v_{A6} , which appeared to contain more than 50% of the bacterial plasmid DNA sequences (Matthyse & Stump, 1976).

The variation in the percentage of the plasmid DNA sequences present in the tumour lines does not appear to correlate with either the species of plant or the length of time the tumour has been in tissue culture. The two lines of *V. rosea* studied differ, v_{A6} having more than 50% (Matthyse & Stump, 1976) and v_{BP} having only about 15% of the plasmid DNA sequences present. The strains studied vary greatly in the length of time since they were originally transformed and placed in tissue culture. The *Vinca* strains have been in culture since about 1960 (A. Braun, personal communication); and the *Bryophyllum* strain has been in culture less than 1 year (C. Miller, personal communication). Thus the differing amounts of plasmid DNA present do not appear to represent a gradual loss of plasmid DNA sequences with time in tissue culture.

Fig. 1. Renaturation of ^3H -labelled *A. tumefaciens* plasmid DNA in the presence and absence of crown gall tumour cell DNA. (a) Renaturation of $0.04 \mu\text{g}$ *A. tumefaciens* B6 plasmid DNA ml^{-1} : ●, alone; ○, in the presence of $800 \mu\text{g}$ *Bryophyllum* B₆₆ tumour DNA ml^{-1} . (b) Renaturation of $0.04 \mu\text{g}$ *A. tumefaciens* BP plasmid DNA ml^{-1} : ●, alone; ○, in the presence of $300 \mu\text{g}$ *V. rosea* V₆₆₆ tumour DNA ml^{-1} .

This situation may be similar to adenovirus-induced tumours in animal cell cultures in which the incorporation of only a small fraction of the virus genome (about 5%) is required for tumour formation (Graham, van der Eb & Heijneker, 1974). However, different cell lines transformed by adenovirus-2 contain amounts of virus DNA varying between 14 and 49% of the total virus DNA (Gallimore *et al.*, 1974; Sharp *et al.*, 1974). Thus more than the minimum amount of virus DNA necessary for transformation is maintained in some of these cell lines.

Similarly only a small portion (15% or less) of the *Agrobacterium* plasmid may be required for transformation, but some cell lines may maintain more than this minimum fragment of the plasmid DNA.

The evidence presented above supports the hypothesis that *A. tumefaciens* induces tumours by transferring plasmid nucleic acid from the bacterium to the plant cells. Apparently the maintenance (and possibly transfer) of the entire *A. tumefaciens* plasmid is not necessary for the maintenance of the crown gall tumour.

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