T-DNA of pTi-15955 from *Agrobacterium tumefaciens* is transcribed into a minimum of seven polyadenylated RNAs in a sunflower crown gall tumor

Norimoto Murai and John D. Kemp

Department of Plant Pathology, University of Wisconsin, and Plant Disease Resistance Research Unit, ARS, USDA, Madison, WI 53706, USA

Received 6 November 1981; Revised and Accepted 27 January 1982

ABSTRACT

Northern blot hybridization analysis of polysomal polyadenylated RNA isolated from sunflower crown gall tumor PSCG-15955 demonstrated that a minimum of seven RNAs were transcribed from T-DNA of pTi-15955 from *Agrobacterium tumefaciens*. The sizes of the T-DNA transcripts were 1.8, 1.6, 1.5, 1.1, 1.0 kilo bases (kb) and two transcripts of 0.8 kb long. The relative abundance of these polyadenylated RNAs varied greatly, the 1.0 kb RNA being the most abundant and the 1.6 kb RNA being the least abundant. Assignment of map locations of the seven polyadenylated RNAs indicated that the conserved region of T-DNA which may play a central role in tumorigenesis contained four RNAs of 1.8, 1.1, 0.8(a) and a portion of 0.8(b) kb long.

INTRODUCTION

Agrobacterium tumefaciens strains harboring a large tumor-inducing (Ti) plasmid incite crown gall tumors in gymnosperms and dicotyledonous angiosperms of higher plants (1,2,3). Tumor cells are characterized by the presence of a segment (T-DNA) of the inciting Ti plasmids (4), the ability to grow in culture in the absence of plant growth hormones (cytokinin and auxin) (5), and the accumulation of crown gall-specific compounds, octopine, nopaline, agropine, or agrocinopine (6). During early stages of tumor induction, T-DNA of Ti plasmid is transferred from the inciting bacterium to the host plant cell by a mechanism yet to be specified (7), and becomes covalently linked to plant nuclear, probably chromosomal, DNA (8,9). The size of the T-DNA in the transformed crown gall cell varies from 12 to 23 kilo base pairs (kbp) depending on the type of Ti plasmid and species of plant cell (7). An approximately 9 kbp segment of the T-DNA, which shares extensive homology among most Ti plasmids examined, is termed the conserved region (10). Integration of T-DNA appears not to be restricted to a unique site but can occur at any of several sites on the plant DNA, some involving repetitive DNA sequences (9,11,12).

The exact role that T-DNA plays in the induction and maintenance of crown gall tumors is beginning to unfold. It appears that T-DNA may carry several structural genes for functional proteins. One such structural gene coding for the crown gall-specific enzyme, octopine synthase, is located on a central segment of the T-DNA to the right of the conserved region and is expressed as an active enzyme only after being transferred to the host cell (13,14). Insertion of transposons into the conserved region of T-DNA often leads to bacterial mutants causing tumors with altered morphologies (15, 16). This observation indicates that the conserved region of T-DNA is necessary for the control of tumor morphology, probably by regulating the production of plant growth hormones.

Octopine-type Ti plasmids share extensive homology with one another (17,18) and are virtually identical through the T-DNA region (7). Further, the organization of the conserved region and central segment (restriction fragments BamHI-17 and EcoRI-E) of T-DNA are the same in octopine crown gall tumors (Figure 1). The right hand segment (fragments EcoRI-C and -D) of T-DNA, on the other hand, can vary. For example, it is absent in the T-DNA of A6 tobacco tumor (7), present contiguously with the central segment in the T-DNA of PSCG-15955 (Ursic, D., and Kemp, J. D., manuscript in preparation) and present in 10 to 20 copies excess in the T-DNA of E9 and probably E1 tobacco tumors (7). For E9 tumor tissue (19), Gelvin *et al.*, (20) reported that 100 times as much RNA was hybridized to the right segment of T-DNA as to the remaining part (fragments SmaI-16a and BamHI-17) of the T-DNA. A different transcription pattern of T-DNA was observed in A6 tobacco and PSCG-15955 and-B6 tumor lines (21,22). The conserved region of T-DNA was as actively transcribed as the central and right segments of T-DNA the latter cases.

The present work examined transcription of the T-DNA of pTi-15955 in the sunflower tumor PSCG-15955. It determined the sizes, relative abundances and map locations of T-DNA specific transcripts by Northern blot hybridization analysis of polysomal polyadenylated RNA.

MATERIALS AND METHODS

Isolation of Polyadenylated RNA

The tissue culture line PSCG-15955 of sunflower (*Helianthus annuus* cv. Mammoth Russian) crown gall was isolated from a primary crown gall tumor incited by *Agrobacterium tumefaciens* (E.F. Sm. and Town.) Conn. strain 15955. Habituated sunflower stem section culture HSSS and PSCG-15955 were maintained as described (23). PSCG-15955 synthesizes octopine synthase, accumulates

octopine, and contains the T-DNA of pTi-15955 (Ursic, D., and Kemp, J. D., manuscript in preparation).

Polysomal RNA was prepared from polysomes isolated from three-week-old callus by a modification (13) of the method of Sun *et al.* (24). Polyadenylated RNA (Poly(A)(+)-RNA) was purified from polysomal RNA by two passages through a column of oligo(dT)-cellulose (Collaborative Research, Inc.) (25).

RNA Fractionation and Transfer to Nitrocellulose

Poly(A)(+)-RNA and rRNA standards were denatured by glyoxal and dimethyl sulfoxide as described by McMaster and Carmichael (26). A 1.5 (w/v) agarose gel (20 x 14 x 0.6 cm) containing 10 mM sodium phosphate, pH 7.0, was prepared on a horizontal glass plate with a well size of 5 x 1 x 6 mm. Samples of denatured RNA (20 to 50 µg per lane) were applied to the gel and fractionated by electrophoresis at 40 mA for 6 to 8 hr. Generally, five to seven identical lanes of RNA were simultaneously fractionated. The size of mRNA was determined by comparison with the electrophoretic mobilities of rRNA molecules of pea (25S, Mr 1.20×10^6 ; 18S, Mr 0.68×10^6) and *Escherichia coli* (23S, Mr 1.03×10^6 ; 16S, Mr 0.55×10^6) (26).

Fractionated RNAs were transferred to a nitrocellulose sheet (Schleicher and Schuell) by blotting for 20 hr as described by Thomas (27). The nitrocellulose sheet was cut into longitudinal strips so that each strip contained one lane of the fractionated RNAs. The strips were baked at 80°C for 2 hr with reduced pressure to immobilize the RNAs.

Recombinant Plasmid DNA and Isolation of DNA Fragments

A physical map of the T-DNA region of pTi-15955 was constructed by using restriction endonucleases EcoRI, BamHI, HindIII, SmaI, HpaI, and BstEII (Figure 1). Some T-DNA fragments were cloned into *E. coli* plasmid pBR322 and these recombinant clones were designated as p101, p102, *etc.* as indicated. Plasmid DNA was isolated from *E. coli* by a combination of a cleared lysate procedure (28) and a polyethylene-glycol precipitation procedure (29). DNA fragments were isolated from agarose gels by either a ground-glass absorption procedure (30) or a filter-paper absorption procedure (31). All experiments involving recombinant DNA were conducted under P1/EK1 containment in accordance with the revised NIH Guidelines (1980).

Labeling DNA and Hybridization

Plasmid DNA and DNA fragments (1 µg each) were labeled with deoxyadenosine 5-(α - 32 P)triphosphate (Amersham, 600 Ci/mmol) by using a Nick Translation Kit (Amersham). Specific activity of plasmid DNA ranged from 2 to 10×10^7 cpm/µg and that of isolated DNA fragments from 1 to 5×10^7

cpm/ μ g.

Individual nitrocellulose strips containing 20 to 50 μ g of the immobilized RNAs were pretreated at 42°C overnight and hybridized with 1 μ g of labeled DNA at 42°C for 20 hr as described by Wahl *et al.* (32). Dextran sulfate was omitted from the hybridization solution because it caused higher radioactive background. Nitrocellulose strips were washed four times for 60 min each at room temperature by shaking on a gyratory shaker in 300 mM sodium chloride/30 mM sodium citrate/0.1 (w/v) sodium dodecyl sulfate. The strips were then washed twice for 30 min each at 60°C in 15 mM sodium chloride/1.5 mM sodium citrate/0.1 (w/v) sodium dodecyl sulfate. Washed and dried nitrocellulose strips were exposed at -80°C to Kodak X-Omat AR films in a Kodak X-Omatic cassette with two intensifying screens for the duration specified in the figure legends.

RESULTS

The T-DNA of *A. tumefaciens* pTi-15955 was present in the sunflower crown gall callus PSCG-15955 (Ursic, D., and Kemp, J. D., manuscript in preparation) and transcribed to complementary RNAs (21). The T-DNA is approximately 21 kbp long, extending contiguously from HindIII-d to EcoRI-D fragment (Figure 1). Poly(A)(+)-RNA was purified from polysome preparations isolated from actively growing tumor callus. It served as highly active mRNA for an *in vitro* translation system of rabbit reticulocyte lysates (13). Poly(A)(+) RNA was denatured with glyoxal and dimethyl sulfoxide and fractionated by electrophoresis on a 1.5 (w/v) agarose gel. When visualized by staining with ethidium bromide, the RNA preparation represented heterogeneous RNA with molecular weights ranging from 1.20×10^6 to 2.5×10^4 . These preparations did not contain detectable levels of rRNA. The fractionated RNAs were transferred to nitrocellulose strips and hybridized to T-DNA fragments labeled to a high specific activity with deoxyadenosine 5'-(α - 32 P) triphosphate.

At least seven poly(A)(+)-RNAs were detected reproducibly when the entire T-DNA region of pTi-15955 was examined for T-DNA specific transcripts by using six recombinant plasmids, *i.e.*, p101, p202, p203, p303, p403, and p501 (Figure 2). Arrows in the figures indicate the position of RNA bands observed reproducibly. Some films were purposely overexposed to bring out the less abundant RNA species. BamHI-8 fragment (p101) hybridized weakly to two RNA species 1.8 and 1.1 kilo bases (kb) long and strongly to the third RNA 0.8(a) kb long (Figure 2, lane 1). Both EcoRI-B (p202) and BamHI-17

Figure 1. Restriction endonuclease map (linear version) of the T-DNA region of pTI-15955 and map locations of seven poly(A)(+)-RNAs isolated from the sunflower crown gall tumor PSCG-15955. The shaded areas in the physical map represent DNA fragments used for Northern blot hybridization analysis of poly(A)(+) RNAs. Solid bars with number below represent the size (bases long) of poly(A)(+) RNAs and lines running through solid bars define the map locations of transcripts. CS indicates the location of octopine synthase gene.

(p203) fragments were homologous to another three RNA species of 1.5, 1.0 and 0.8(b) kb in length (Figure 2, lanes 2 and 3). The 1.5 kb RNA was also partially complementary to EcoRI-E fragment (p303) (Figure 2, lane 4). EcoRI-C fragment (p403) hybridized very weakly to a 1.6 kb RNA (Figure 2, lane 5). No RNA was detected by hybridization to EcoRI-D fragment (p501) (Figure 2, lane 6). Assuming that the amount of radioactivity detected in each RNA band is approximately proportional to the quantity of RNA, the relative abundance of the seven RNA species varied greatly. The 1.0 kb RNA was the most abundant and the two 0.8 kb RNA, 0.8(a) and 0.8(b), were more abundant than the remaining four RNAs, among which the 1.6 kb RNA was the least abundant. These RNA species are probably T-DNA specific transcripts since poly(A)(+) RNA isolated from habituated sunflower callus free from the T-DNA showed no apparent homology to either BamHI-8 (p101) (Figure 2, lane 7), BamHI-17 (p203) (Figure 2, lane 8), or EcoRI-C fragments (p403) (Figure 2, lane 9).

The relative map locations of the seven RNA transcripts were assigned by using DNA fragments that facilitate the division of the entire T-DNA into 15 map sections, as indicated in Figure 1. The 1.1 kb poly(A)(+) RNA that hybridized to BamHI-8 (Figure 3, lane 1) was also homologous to HindIII-d

Figure 2. Autoradiography of sunflower crown gall poly(A)(+) RNAs (lanes 1-6) and habituated callus poly(A)(+) RNAs (lanes 7-9) analyzed by Northern blot hybridization to six different T-DNA fragments that cover the entire T-DNA region of pTi-15955.

Lane 1, BamHI-8 (p101) (2.1); lane 2, EcoRI-B (p202) (2.1); lane 3, BamHI-17 (p203) (.7); lane 4, EcoRI-E (p303) (2.4); lane 5, EcoRI-C (p403) (4.0); lane 6, EcoRI-D (p501) (2.0); lane 7, BamHI-8 (p101) (2.1); lane 8, BamHI-17 (p203) (1.7); lane 9, EcoRI-D (p403) (4.0).

The numbers in parenthesis represent $\text{cpm} \times 10^5$, the radioactivity of DNA probes applied to each hybridization. Strips containing lanes 2 (lower half) and 3 were exposed to X-ray film for 3 hr, those containing lanes 1 and 2 (upper half) for 7 hr, those containing lanes 4, 5 and 6 for 22 hr and the rest for 72 hr.

(p102) (Figure 3, lane 2) and SmaI-17 (Figure 3, lane 5), but not to SmaI16a fragment (Figure 3, lane 4). Since the left terminus of T-DNA in the PSCG-15955 tumor lies within HindIII-d fragment, the 1.1 kb RNA is probably transcribed from the right side of map section 1. The 0.8(a) kb RNA hybridized to HindIII-d (p102) (Figure 3, lane 2), SmaI-16a (Figure 3, lane 4) and to SmaI-17 fragment (Figure 3, lane 5). This indicates that the map location of this RNA lies mostly in map section 2 and slightly extends into map section 1. The discrete RNA 1.8 kb long was detected by HindIII-e (p103) (Figure 3, lane 3) but not by SmaI-16a (Figure 3, lane 4) or EcoRI-B (p202) fragments (Figure 2, lane 2). The coding region for the RNA should be located mostly in map section 4 and should extend to one or both sides of the adjacent map sections.

The relative map locations of the abundant 0.8(b) and 1.0 kb poly(A)(+) RNA homologous to both EcoRI-B (p202) and BamHI-17 (p203) fragments were determined by using subfragments of BamHI-17 generated by either SmaI or

Figure 3. Autoradiography of tumor poly(A)(+)RNAs analyzed by Northern blot hybridization to six different T-DNA fragments from the conserved region of T-DNA.

Lane 1, BamHI-8 (p101) (6.4); lane 2, HindIII-d (p102) (2.7)

lane 3, HindIII-e (p103) (3.1); lane 4, SmaI-16a (4.7)

lane 5, SmaI-17 (4.7)

Strips containing lanes 1 (upper half) and 5 were exposed for 120 hr, those containing lane 4 for 12 hr, and the rest for 72 hr.

BstEII restriction endonucleases. The 0.8(b) kb RNA hybridized to only map section 7 (Figure 4, lanes 3 and 5), whereas the 1.0 kb RNA hybridized to all subfragments corresponding to map sections 7, 8, and 9 (Figure 4, lanes 2, 3, 4, and 5). This indicates that the map location of 0.8(b) kb RNA lies within map section 7 and possibly 6, and that of 1.0 kb RNA lies in map section 8 and extends into both map sections 7 and 9.

The 1.5 kb poly(A)(+)RNA was shown to be the mRNA for octopine synthase by identifying the *in vitro* translation products of this mRNA (13). Hybridization of the mRNA to BamHI-17 (p203) was more intense than that to EcoRI-B (p202) or -E (p303) (Figure 2, lanes 2, 3, and 4). The mRNA was not detected by hybridization to either BamHI digests of EcoRI-E fragment (map section 11) (Figure 4, lane 7) or subfragments of BamHI-17 corresponding to map sections 7 and 8 (Figure 4, lanes 3 and 5). This mRNA should be transcribed from DNA corresponding to the right side of map section 9 and the entire map section 10.

The low level of radioactivity detected for the 1.6 kb poly(A)(+)RNA made it difficult to assign it to a map location to the right (map section 14) and/ or left sides (map section 12) of EcoRI-C fragment.

Figure 4. Autoradiography of tumor poly(A)(+)RNAs analyzed by Northern blot hybridization to seven different T-DNA fragments from the central segment of T-DNA.

Lane 1, BamHI-17 (p203) (5.0);

lanes 2 and 3, the right (1.0) and left parts (1.6), respectively, of BamHI-17 digested with SmaI.

lanes 4 and 5, the right (1.5) and left parts (1.7), respectively, of BamHI-17 digested with BstEII.

lane 6, EcoRI-E (p303) (4.2)

lane 7, subfragment of EcoRI-E digested with BamHI (1.0)

Strips containing the lower halves of lanes 1 and 2 and the entire lane 5 were exposed for 3 hr, those containing the upper halves of lanes 1 and 2 for 12 hr and the rest for 112 hr.

DISCUSSION

Northern blot hybridization analysis of poly(A)(+)-RNA isolated from the PSCG-15955 tumor demonstrated that at least seven RNAs were transcribed from the T-DNA of *A. tumefaciens* pTi-15955. Assignment of map locations of the T-DNA specific transcripts showed that the conserved region of T-DNA was actively transcribed into at least four RNAs. Active transcription of the conserved region of T-DNA was also demonstrated by Southern blot hybridization analysis in some previous reports (21,22), but not in others (19, 20). Our results on the transcription pattern of T-DNA differ from the previous observations (19, 20, 21) in that transcription of the right segment of T-DNA was very intense in previous reports, but barely detectable in the present work. These apparent intense transcription may be due to hybridization of this region to nuclear RNA. The low level of transcription observed in our work may then be accounted for by the assumption that only a small fraction of the nuclear RNA that hybridizes to the right segment of T-DNA is processed

into mature polysomal RNA. It was reported, however, that both nuclear RNA and polysomal poly(A)(+)-RNA hybridized to similar extent to the conserved region and central segment of T-DNA (22).

Our results imply that the T-DNA of pTi-15955 may carry a minimum of seven structural genes for functional proteins. The poly(A)(+)-RNA preparations used for hybridization analysis were originally isolated from polysome, and served as highly active mRNA for an in vitro translation system of rabbit reticulocyte lysates (13). The 1.5 kb mRNA for octopine synthase was previously purified from total poly(A)(+)-RNAs by hybridization to EcoRI-B, -E or BamHI-17 fragments and identified by comparison of its in vitro translation products with the authentic enzyme of 40,000 molecular weight (13). When RNAs homologous to EcoRI-B or BamHI-17 fragments were translated in vitro into polypeptides which were then immunoprecipitated with antibody against octopine synthase, polypeptides of molecular weight 25,000, 23,000, and 19,000 were coprecipitated with octopine synthase (13). The 1.0 kb RNA was the most abundant among the seven T-DNA transcripts, whereas the 25,000 polypeptide was the most abundant among the in vitro translation products of RNAs hybridized to EcoRI-B or BamHI-17 fragment. On the basis of the above observations and the coding capacity of these two RNAs, it is proposed that the 1.0 kb RNA codes for the 25,000 polypeptide, whereas the 0.8(b) kb RNA codes for either the 23,000 or the 19,000 polypeptide. The functions of these polypeptides remains to be specified.

A. tumefaciens mutants causing altered tumor morphology were obtained by insertion of transposons into the right end of the conserved region of T-DNA from the octopine-type Ti plasmids (15,16). Mutants with either the Tn904 insertion at the left end of HpaI-13 or the Tn5 insertion in HpaI-14 fragment gave rise to small tumors with excessive adventitious root formation on Kalanchoe daigremontiana. The Tn5 insertion at two different sites of HpaI-14 fragment resulted in mutants causing tumors with shoots proliferating on Kalanchoe stems (16). Our results indicate that the map location of 0.8(b) kb poly(A)(+)RNA lies in map sections 6 and 7 including HpaI-14 fragment. These observations imply that the map location of 0.8(b) kb mRNA may be in close vicinity to the HpaI-14 fragment. It is proposed that this RNA codes for a polypeptide that regulates in some way the production of plant growth hormones. Insertion of transposons into the putative coding sequence for the mRNA may eliminate mRNA production, whereas insertion into the 5 or 3 -end of the coding sequences of mRNA may amplify mRNA synthesis.

The map location of 1.6 kb poly(A)(+)RNA lies within EcoRI-C fragment.

Replacement of HindIII-f fragment with Tn5 DNA segment by using a broad host range plasmid pRK290 system (33,34) resulted in a bacterial mutant harboring the engineered pTi-15955 (Kemp, J. D., and Sutton, D. W., unpublished). Tumors caused by this mutant did not contain detectable level of agropine, whereas the wild-type PSCG-15955 callus produced agropine. This observation implies that the map location of the 1.6 kb RNA may be in close vicinity to HindIII-f fragment and that this RNA may code for agropine production.

ACKNOWLEDGEMENTS

We thank Ms. Kim Jurens for plasmid and DNA fragment isolation and Mr. Steven Vican for photographs. This work was supported in part by U.S. Department of Agriculture, SEA Competitive Research Grant 5901-0410-80168-0. Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

REFERENCES

1. Smith, E. F., and Townsend, C. O. (1907). *Science* 25,671-673.
2. Zaenen, I., van Lerebeke, N., Tuechy, H., van Montagu, M., and Schell, J. (1974). *J. Mol. Biol.* 86,109-127.
3. De Cleene, M., and De Ley, J. (1976). *Bot. Review* 42,389-466.
4. Chilton, M-D., Drummond, M. H., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P., and Nester, E. W. (1977). *Cell* 11,263-271.
5. Braun, A. C. (1956). *Canc. Res.* 16,53-56.
6. Menage, A., and Morel, G. (1964). *C. R. Hebd. Seances Acad. Sci.* 259,4795-4796.
7. Thomashow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P., and Nester E. W. (1980). *Cell* 19,729-739.
8. Chilton, M-D., Saiki, R. K., Yadav, N., Gordon, M. P., and Quetier, F. (1980). *Proc. Natl. Acad. Sci., USA* 77,4060-4064.
9. Yadav, N. S., Postle, K., Saiki, R. K., Thomashow, M. F., and Chilton, M-D. (1980). *Nature (London)*. 287,458-461.
10. Chilton, M-D., Drummond, M. H., Merlo, D. J., and Sciaky, D. (1978). *Nature (London)* 275,147-149.
11. Thomashow, M. F., Nutter, R., Postle, K., Chilton, M-D., Blattner, F. R., Powell, A., Gordon, M. P., and Nester, E. W. (1980). *Proc. Natl. Acad. Si. USA* 77,6448-6452.
12. Zambryski, P., Holsters, M., Kruger, K., Depicker, A., Schell, J., van Montagu, M., and Goodman, H. M. (1980). *Science* 209,1385-1391.
13. Murai, N., and Kemp, J. D. (1981). *Proc. Natl. Acad. Sci., USA* 78 (in press).
14. Schroder, J., Schroder, G., Huisman, H., Schilperoort, R. A., and Schell, J. (1981). *FEBS Lett.* 129,166-168.
15. Ooms, G., Klapwijk, P. M., Poulis, J. A., and Schilperoort, R. A. (1980). *J. Bacteriol.* 144,82-91.
16. Garfinkel, D. J., and Nester, E. W. (1980) *J. Bacteriol.* 144,732-743.
17. Currier, T. C., and Nester, E. W. (1976). *J. Bacteriol.* 126,157-165.
18. Sciaky, D., Montoya, A. L., and Chilton, M-D. (1978). *Plasmid* 1,238-253.

19. Drummond, M. H., Gordon, M. P., Nester, E. W., and Chilton, M-D. (1977). *Nature* (London) 269,535-536.
20. Gelvin, S. B., Gordon, M. P., Nester, E. W., and Aronson, A. I. (1981). *Plasmid* 6, 17-29.
21. Gurley, W. B., Kemp, J. D., Albert, M. J., Sutton, D. W., and Callis, J. (1979). *Proc. Natl. Acad. Sci., USA* 76,2828-2832.
22. Willmitzer, L., Otten, L., Simons, G., Schmalenbach, W. Schroder, J., Schroder, G. van Mantagu, M., de Vos, G., Schell, J. (1981). *Mol. Gen. Genet.* 182,255-262.
23. Merlo, D. J., and Kemp, J. D. (1976). *Plant Physiol.* 58,100-106.
24. Sun, S-M M., Buchbinder, B. U., and Hall, T. C. (1975). *Plant Physiol.* 56,780-785.
25. Bantle, J. A., Maxwell, I. H., and Hahn, W. E. (1976). *Anal. Biochem.* 72,413-427.
26. McMaster, G. K., and Carmichael, G. G. (1977). *Proc. Natl. Acad. Sci., USA* 74,4835-4838.
27. Thomas, P. S. (1980). *Proc. Natl. Acad. Sci., USA* 77,5201-5205.
28. Clewell, D. B., and Helinski, D. R. (1969). *Proc. Natl. Acad. Sci., USA* 62,1159-1166.
29. Humphreys, G. O., Willshaw, G. A., and Anderson, E. S. (1975) *Biochim. Biophys. Acta* 383,457-463.
30. Vogelstein, B., and Gillespie, D. (1979). *Proc. Natl. Acad. Sci., USA* 76,615-619.
31. Girvitz, S. C., Bacchetti, S., Rainbow, A. J., and Graham, F. L. (1980). *Anal. Biochem.* 106,492-496.
32. Wahl, G. M., Stern, M., and Stark, G. R. (1979). *Proc. Natl. Acad. Sci., USA* 76,3683-3687.
33. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. (1980) *Proc. Natl. Acad. Sci., USA* 77,7347-7351.
34. Matzke, A. J. M., and Chilton, M-D. (1981). *J. Mol. Appl. Genet.* 1,39-49.