MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4298–4303
0270-7306/89/104298-06$02.00/0

Vol. 9, No. 10

Extensive Changes in DNA Methylation Patterns Accompany Activation of a Silent T-DNA *ipt* Gene in *Agrobacterium tumefaciens*-Transformed Plant Cells

MANORAMA C. JOHN AND RICHARD M. AMASINO*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706-0569

Received 20 March 1989/Accepted 11 July 1989

We crossed a male-sterile, *Agrobacterium*-transformed *Nicotiana tabacum* plant that contains a silent, hypermethylated T-DNA *ipt* oncogene with a normal tobacco plant and found that the methylated state of the *ipt* gene was stably inherited through meiosis in the offspring. However, when tissues of these plants were placed in cell culture, the *ipt* gene was spontaneously reactivated in a very small fraction of the cells; if 5-azacytidine was added to the culture medium, *ipt* gene reactivation occurred at high frequency. We analyzed the pattern of DNA methylation in a region spanning the *ipt* gene in a line that does not express the *ipt* gene, in five derivatives of this line that reexpressed the *ipt* gene either spontaneously or after 5-azacytidine treatment, and in tissues of a sibling of this line that reexpressed *ipt* spontaneously. We found that the *ipt* locus was highly methylated in the unexpressed state but that spontaneous or 5-azacytidine-induced reexpression always resulted in extensive demethylation of a region including 5′ upstream, coding, and 3′ downstream regions of the *ipt* gene. The role of DNA methylation in gene regulation in this system is discussed.

Methylation of cytosine residues at the 5 position is a postreplication modification of DNA that occurs in many eucaryotic and procaryotic organisms. In animals, DNA methylation occurs at CpG dinucleotides, and there is much evidence that methylation of DNA is involved in the regulation of gene expression (8). For example, there is often an inverse correlation between the level of cytosine methylation within a gene and transcriptional activity (23). Nonexpressed, methylated genes are often activated by treating cells with the methylation inhibitor 5-azacytidine (13). When genes are methylated in vitro and then integrated into the mammalian genome, these methylated genes adopt an inactive chromatin conformation and are not expressed (14). In vitro methylation of discrete regions of genes before cell transformation indicates that methylation of specific sites is often sufficient to prevent gene expression (7, 30). In many cases, methylation of specific regions in the 5′ noncoding portion of the gene abolishes expression, but in certain cases methylation of the coding region is also inhibitory (7, 15). Methylation may also inhibit the binding of specific proteins to discrete DNA sequence elements in the promoter of a gene (5, 28).

The genomes of many plants are methylated at both CpG dinucleotides and CpNpG trinucleotides (N = A, C, or T), and more than 30% of the C residues may be methylated in certain plant species (11). Several lines of evidence suggest that methylation is involved in regulation of plant gene expression. For example, the levels of ribosomal gene methylation change during plant development (27). The level of methylation of T-DNA genes in crown gall tumor cells and maize transposable elements and storage protein genes is inversely correlated with gene expression (2, 6, 9, 12, 21, 25). Furthermore, nonexpressed T-DNA genes can be activated by treating transformed cells with 5-azacytidine (2, 12, 21). However, few studies have been done in plant systems on the site specificity of DNA methylation and the mechanisms of inhibition of gene expression by DNA methylation. A recent report has shown that methylation reduces the affinity of a maize DNA-binding protein for its target site (10).

The goal of this study was to further characterize the association between DNA methylation and gene expression in plants. We have analyzed the changes in DNA methylation that are linked to changes in T-DNA gene expression in crown gall tumor cells. Crown gall is a tumorous disease of plants caused by the transfer of a portion of the *Agrobacterium tumefaciens* Ti plasmid (the T-DNA) to the genome of plant cells (20). The T-DNA contains plant oncogenes that encode enzymes of plant growth factor biosynthesis (29). The enzyme encoded by one of the oncogenes, *ipt*, is an isopentenyl transferase that catalyzes the first step in the biosynthesis of cytokinins (1, 4). We have isolated a crown gall tumor cell line in which the *ipt* gene is not expressed and is hypermethylated (2). For this study, we obtained revertants of this cell line in which *ipt* gene expression had reactivated either spontaneously or after 5-azacytidine treatment. We present an analysis of the changes in *ipt* gene methylation patterns that accompany this reactivation. Surprisingly, all revertants are extensively demethylated in the region surrounding the *ipt* gene. Thus, the demethylation that accompanies reactivation of this gene exhibits no detectable site specificity in this cell line.

MATERIALS AND METHODS

Isolation and culture of cell lines. The derivation of a clonal *Nicotiana tabacum* crown gall tumor line that formed normal-appearing plants and contained a transcriptionally silent T-DNA insert has been described (2). One of these plants (plant C) was fertilized with pollen from nontransformed tobacco (variety Xanthi), and the resulting seeds were germinated in sterile culture on agar-solidified MS (Murashige and Skoog) medium (19) without phytohormones (MSO). With one exception (CX1), all of the seedlings developed into normal-appearing plants. Sectors of one of the seedlings

* Corresponding author.

(CX1) that contacted the medium spontaneously (i.e., without 5-azacytidine treatment) reinitiated tumorous growth on MSO medium shortly after germination. In the pool of seedlings that developed normally, the inheritance of the nonexpressed T-DNA insert was identified by placing leaf slices on solid MS medium containing 2 mg of α-naphthalene acetic acid per liter, 0.2 mg of benzyladenine per liter, and 5 μM 5-azacytidine (Sigma Chemical Co., St. Louis, Mo.) for 5 days and then transferring the tissue to solid MSO medium. Leaf pieces that reinitiated tumorous growth on phytohormone-free medium after this treatment were scored as T-DNA-containing progeny (2). The presence of T-DNA in certain lines was further confirmed by DNA analysis as described below. One seedling from this group that contained a silent T-DNA insert (CX2) was chosen for further analysis, and tumorous revertants of this line were obtained in two ways: (i) by 5-azacytidine treatment as described above and (ii) by culturing large numbers of leaf pieces on MSO medium and selecting for spontaneous hormone-independent growth. For nucleic acid analysis, cell lines of the tumorous revertants were cultured on solid MSO medium, where they developed as unorganized and teratomous tumors, and leaf tissue of plant CX2 was maintained as a phenocopy of the teratomous cell lines by culture on MS medium containing 2 mg of benzyladenine per liter. All cultures were incubated at 27°C with continuous light.

DNA and RNA analysis. DNA and RNA were isolated from plant tissues and purified as previously described (24). For DNA (Southern) blot analysis, 3 μg of each DNA sample was first digested with 12 to 15 U of *Bam*HI, ethanol precipitated, redissolved, and then digested with 15 to 30 U of a cytosine methylation-sensitive restriction enzyme. Restriction enzymes were supplied by New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used in the buffer recommended by the supplier supplemented with 2 mM spermidine. To ensure that the restriction enzyme digestions proceeded to completion, a portion of each reaction, containing 0.2 μg of plant DNA and 1 to 2 U of enzyme, was incubated with 0.2 μg of supercoiled pUC8 (26). Complete digestion of plasmid DNA, which was monitored by agarose gel electrophoresis and ethidium bromide staining as described previously (18), was used as a criterion for determining that plant DNA was completely digested. The remainder of the digested sample was electrophoresed in TBE buffer (18) through a 1.0% or 1.5% agarose gel for 1 h at 2 V/cm and then for six h at 5 V/cm. After staining in 0.5 μg of ethidum bromide per ml, the gels were photographed and soaked in 0.25 M HCl until the bromophenol tracking dye turned yellow. The gels were then immediately transferred to 0.4 M NaOH for 10 to 20 min. DNA was transferred from the gels onto nylon membranes (Biotrace; Gelman Sciences, Inc., Ann Arbor, Mich.) by overnight capillary blotting in 20 mM NaOH–1 mM EDTA. All Southern blot analyses were repeated at least once with independent preparations of DNA.

For RNA (Northern) blot analysis, 10 μg of each RNA sample was denatured by the addition of 3 volumes of 70% formamide–20% formaldehyde (vol/vol) in 1× morpholinopropanesulfonic acid running buffer (pH 7.0) (18), followed by heating to 60°C for 10 min. The samples were then mixed with 1/10 volume of loading buffer (2% sodium dodecyl sulfate, 20% Ficoll, [Pharmacia Fine Chemicals, Piscataway, N.J.], 20% glycerol, 0.25% bromophenol blue) and electrophoresed through 1.0% agarose-formaldehyde gels as described previously (24). The RNA was transferred from the gels directly to nylon membranes by overnight capillary blotting in 10 mM sodium citrate–1 mM EDTA (pH 7.0) that had been pretreated with 0.05% (vol/vol) diethylpyrocarbonate.

The membrane-bound nucleic acids were hybridized to uniformly ^{32}P-labeled RNA probes as previously described (24).

RESULTS

Phenotypes of transformed cell lines. The starting material for this study was a normal-appearing plant (plant C) that carries a hypermethylated, transcriptionally inactive copy of T-DNA. This plant was derived as previously described from a cloned crown gall tumor line originally transformed with the T-DNA of an octopine-type Ti plasmid (2). Plant C and other plants derived from this tumor line were male sterile and exhibited other developmental abnormalities common to plants regenerated from cell lines that have been in culture for long periods of time. Therefore, to test the stability and inheritance of the transcriptionally inactive state of the T-DNA insert, it was necessary to fertilize plant C with pollen from a nontransformed tobacco plant. One hundred seeds from this cross were germinated in sterile culture. Of these, 99 formed normal-appearing plants, and 1 (CX1) exhibited tumorous development in regions of shoot tissues contacted the growth medium (Fig. 1A). However, the upper portions of this plant retained a normal phenotype (Fig. 1A) and did not express T-DNA (data not shown). We determined the inheritance pattern of the silent T-DNA copy in the normal-appearing plants by placing leaf pieces on medium containing phytohormones (to stimulate cell division) and 5-azacytidine (to demethylate the genome) and then transferring the leaf pieces to medium without phytohormones or 5-azacytidine (2). Leaf tissue that continued to proliferate on medium lacking phytohormones after this treatment contained a reactivated copy of T-DNA (Fig. 1B), and tissue lacking T-DNA failed to proliferate on this medium after 5-azacytidine treatment. By this criterion, 55% of the plants inherited a silent T-DNA copy. Southern blot analysis of a number of these plants confirmed that this assay for the presence of silent T-DNA was accurate; T-DNA was always present in plant tissues that reverted after 5-azacytidine treatment and never present in tissues that did not revert (data not shown). This Mendelian inheritance pattern was not surprising, since plant C contained one T-DNA copy per diploid genome (2). Of greater interest was the observation that the transcriptional inactivity of the insert was stably maintained through meiosis and could be activated only by 5-azacytidine treatment or cell culture (see below).

Two approaches were used to obtain tumorous revertants of one of the normal-appearing plants (plant CX2). First, leaf pieces were subjected to 5-azacytidine treatment as described above. After this treatment, the frequency of reversion to phytohormone-independent growth was too high to estimate, since revertant tissue developed as a confluent ring around the perimeter of the leaf piece (Fig. 1B). Two revertants obtained in this manner, referred to as CX2 azacytidine revertants 1 and 2 (CX2AR1 and CX2AR2), were chosen for further study. In the second approach, leaf pieces were plated directly onto phytohormone-free medium. From 1,000 leaf pieces that were approximately 1 by 2 cm, we obtained three sectors of phytohormone-independent tumorous tissue (e.g., Fig. 1C); in all cases, these sectors developed in the region of wound healing at the perimeter of the leaf piece. By estimating the number of cells at the perimeter of the leaf pieces, which appeared to be the

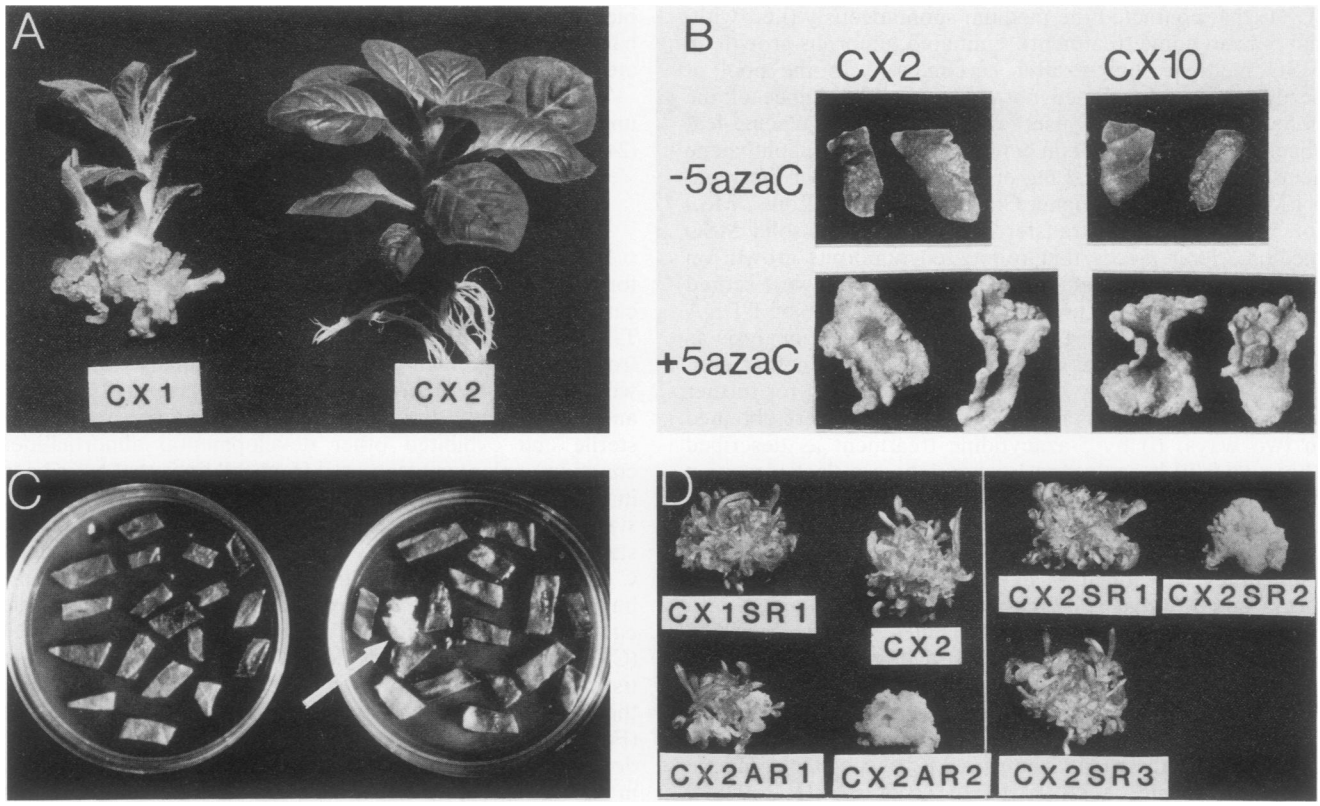

FIG. 1. Phenotypes and culture conditions of the transformed lines. (A) Eight-week-old CX1 and CX2 plants that were grown from stem cuttings in sterile culture on phytohormone-free medium. (B) 5-Azacytidine-induced reversion to tumorous growth. Leaf pieces of plants CX2 and CX10 were cultured with or without 5-azacytidine for 5 days and then transferred to medium without phytohormones as described in Materials and Methods. Leaf pieces are shown 15 days after 5-azacytidine treatment. (C) Example of spontaneous reversion. Leaf pieces of plant CX2 were plated on phytohormone-free medium and, at low frequency, colonies of tumorous revertant tissue were observed developing from the perimeter of the leaf piece (arrow). (D) Phenotypes of the cell lines used for nucleic acid analysis. Line CX2 was cultured on medium containing 0.5 mg of the cytokinin benzyladenine per liter; all other lines were cultured on phytohormone-free medium.

only cells subject to reversion, we estimate the reversion frequency to be less than 10^{-6}. All three spontaneous revertants, CX2 spontaneous revertants 1, 2, and 3 (CX2SR1, CX2SR2, and CX2SR3), were further characterized.

In the experiments described below, we analyzed the pattern of methylation at the *ipt* locus and the amount of *ipt* mRNA in the lines that spontaneously reinitiated T-DNA expression (CX1, CX2SR1, CX2SR2, and CX2SR3), two lines in which reexpression occurred after 5-azacytidine treatment (CX2AR1 and CX2AR2), and one line in which T-DNA remained silent (CX2). The lines in which T-DNA expression had reinitiated were independently maintained as shoot-forming (teratomous) or unorganized cultures on phytohormone-free medium. (The different phenotypes of these lines most likely resulted from differences in the level of expression of T-DNA oncogenes involved in auxin biosynthesis [2].) CX2 tissues was maintained as a phenocopy of the teratomous cell lines on medium containing cytokinin. The phenotypes of the lines under these culture conditions are illustrated in Fig. 1D.

***ipt* expression and tumor phenotype.** To determine whether expression of the *ipt* gene contributed to the tumorous phenotype (i.e., phytohormone-independent growth) of the revertant lines, we analyzed steady-state levels of *ipt* mRNA in various cell lines by blot hybridization. All of the tumorous lines contained detectable levels of *ipt* mRNA, whereas no *ipt* mRNA was detectable in the phytohormone-requiring tissue of plant CX2 (Fig. 2). As observed previously with related cell lines (2), many of these tumorous cell lines (specifically the teratomous cell lines) did not contain detectable levels of other T-DNA transcripts (data not shown).

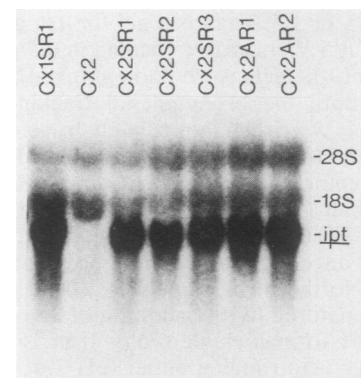

FIG. 2. Northern blot analysis of the steady-state levels of *ipt* mRNA. Total RNA (10 μg) from each line was size fractionated by denaturing gel electrophoresis, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled antisense RNA probe complementary to the *ipt* transcript (probe 2; Fig. 3). Positions of the *ipt* transcript and the 28S and 18S rRNAs, to which some slight cross-hybridization occurred, are indicated.

Thus, expression of the *ipt* locus alone is sufficient to allow phytohormone-independent growth and is always associated with the tumorous phenotype. Furthermore, both modes of reversion (spontaneous and 5-azacytidine induced) gave rise to similar levels of *ipt* gene expression.

DNA methylation patterns at the *ipt* locus in transcriptionally active and inactive cell lines. We compared the pattern of DNA methylation within and flanking the *ipt* gene in the line containing a silent T-DNA insert (CX2) with the DNA methylation patterns found in lines that expressed *ipt* either spontaneously or after 5-azacytidine treatment. For this analysis, DNA was first cleaved to completion with the restriction enzyme *Bam*HI, which splits the *ipt* region into two fragments (Fig. 3D). The samples were then digested with the methylation-sensitive enzyme *Dde*I, *Hha*I, *Hpa*II, *Msp*I, or *Pst*I. The restricted DNA samples were size fractionated by agarose gel electrophoresis, blotted onto nylon membranes, and hybridized to radiolabeled probes complementary to 5′ (probe 1) or 3′ (probe 2) regions of the *ipt* locus (see Fig. 3D for restriction map and probe locations). The sites at which methylation-sensitive enzymes cleaved the DNA within the two *Bam*HI fragments were revealed by the pattern of low-molecular-weight fragments that hybridized to the probes. Cleavage at one of these methylation-sensitive enzyme sites demonstrates that the site does not contain a methylated cytosine residue at a position inhibitory to the enzyme. Resistance to cleavage at a restriction site indicates that the DNA at that site is modified such that cleavage is blocked, presumably by DNA methylation (see legend to Fig. 3 for locations of cytosine methylation that block cleavage). Examples of the data obtained in this study are presented in Fig. 3A to C. Figures 3A and B show the analysis of the 5′ end of the *ipt* gene with the isoschizomers *Msp*I and *Hpa*II, respectively. These enzymes recognize a single common site in this region. In DNA from line CX2, the 980-base-pair (bp) 5′ *Bam*HI fragment remained intact after cleavage with either enzyme. However, in DNA from the lines that reexpressed the *ipt* gene, this site appeared to be demethylated, since enzyme cleavage resulted in the appearance of an 820-bp fragment (and a 160-bp fragment that escaped detection). In DNA from line CX2SR2, two fragments were present after *Hpa*II cleavage, indicating that approximately 50% of the *Hpa*II sites at this position remained methylated. Figure 3C shows the analysis of the 3′ end of the gene with *Msp*I. In DNA from line CX2, the 3′ *Bam*HI fragment was partially cleaved at the third *Msp*I site, which was approximately 650 bp beyond the region containing the 3′ end of the mature mRNA. In DNA from lines that reexpressed the *ipt* gene, the first *Msp*I site downstream of the coding region was demethylated in all of the expressing lines except line CX2AR2, in which these downstream sites were partially methylated.

We extended this analysis to include all of the sites at the 5′ and 3′ ends of the *ipt* gene that are recognized by the restriction enzymes listed above (Fig. 3E). There was a striking contrast between the DNA methylation pattern at the *ipt* locus in line CX2 and the DNA methylation patterns found in lines that expressed this gene. In line CX2, the *ipt* region was methylated at every site analyzed. However, in 5-azacytidine-induced and spontaneous revertants of CX2, in which *ipt* expression had reinitiated, as well as in tissues of a sibling of CX2, line CX1, that expressed *ipt*, there was extensive demethylation of a region spanning the *ipt* gene. A high density of methylated restriction sites was maintained in the areas surrounding this demethylated region (Fig. 3E and further mapping not illustrated). Thus, the spontaneous and 5-azacytidine-induced reinitiation of *ipt* gene expression was always associated with the extensive demethylation of a region surrounding and including the coding region of the *ipt* gene in these cells.

DISCUSSION

We have crossed a transformed plant containing a highly methylated and transcriptionally silent copy of the T-DNA from the B6 Ti plasmid (2) with a normal (untransformed) plant and found that the silent, methylated state of the T-DNA is stably inherited. However, reversion does occur at a low frequency in cell culture or at a high frequency after 5-azacytidine treatment, resulting in cell lines that reexpress the *ipt* gene on the T-DNA. In this study, we compared the DNA methylation pattern of the silent copy of *ipt* with the DNA methylation patterns of active copies in six revertant lines by assessing the ability of five different cytosine methylation-sensitive restriction enzymes to cleave the DNA in the region of the gene. Our results demonstrate that (i) the *ipt* locus is highly methylated in the line containing a silent T-DNA copy and (ii) extensive demethylation of the *ipt* gene is always associated with spontaneous or 5-azacytidine-induced reversion to *ipt* reexpression.

In this study, analysis of DNA methylation patterns in cell lines that express or do not express the *ipt* gene indicates that expression of this gene is tightly linked to its methylation status. In this and previous (2) studies, we have demonstrated that treatment of cultured cells with the demethylating agent 5-azacytidine results in a high frequency of silent T-DNA gene activation and that gene activation is correlated with gene demethylation. It is possible that a side effect of 5-azacytidine other than its demethylating activity (e.g., DNA damage) was responsible for gene activation and that the observed demethylation of T-DNA genes was not directly linked to gene activation. However, spontaneous reversion to T-DNA expression is a low-frequency event in this system, and it is unlikely that the strict correlation between gene expression and demethylation observed in spontaneous revertants is fortuitous. Therefore, that 5-azacytidine treatment results in a high reversion frequency, and that low-frequency spontaneous reversion is also accompanied by extensive demethylation, is consistent with DNA methylation serving as a primary determinant of *ipt* gene expression in this system. This hypothesis is supported by our observations that in these lines, the kinetics of genomic demethylation and *ipt* mRNA appearance are similar (15a) and that a heavily methylated, silent copy of T-DNA exists in a more DNaseI-resistant chromatin conformation than do unmethylated, expressed T-DNA copies in vivo (24).

Our results do not resolve the question of whether there exists a small number of critical sites in the gene that must be demethylated for gene expression to occur. Since spontaneous revertants arose at a low frequency, we expected that the *ipt* gene in these lines had undergone the minimum amount of demethylation necessary to allow expression and that analysis of the *ipt* gene methylation patterns in these lines might reveal regions in which demethylation is essential for expression. However, as discussed above, demethylation of the *ipt* gene in these lines is as extensive as in lines that were treated with 5-azacytidine. Perhaps extensive demethylation is a prerequisite for gene expression in this system, and the overall density of DNA methylation in the region of the *ipt* gene controls its expression. Alternatively, demethylation of specific sites in the gene may lead to

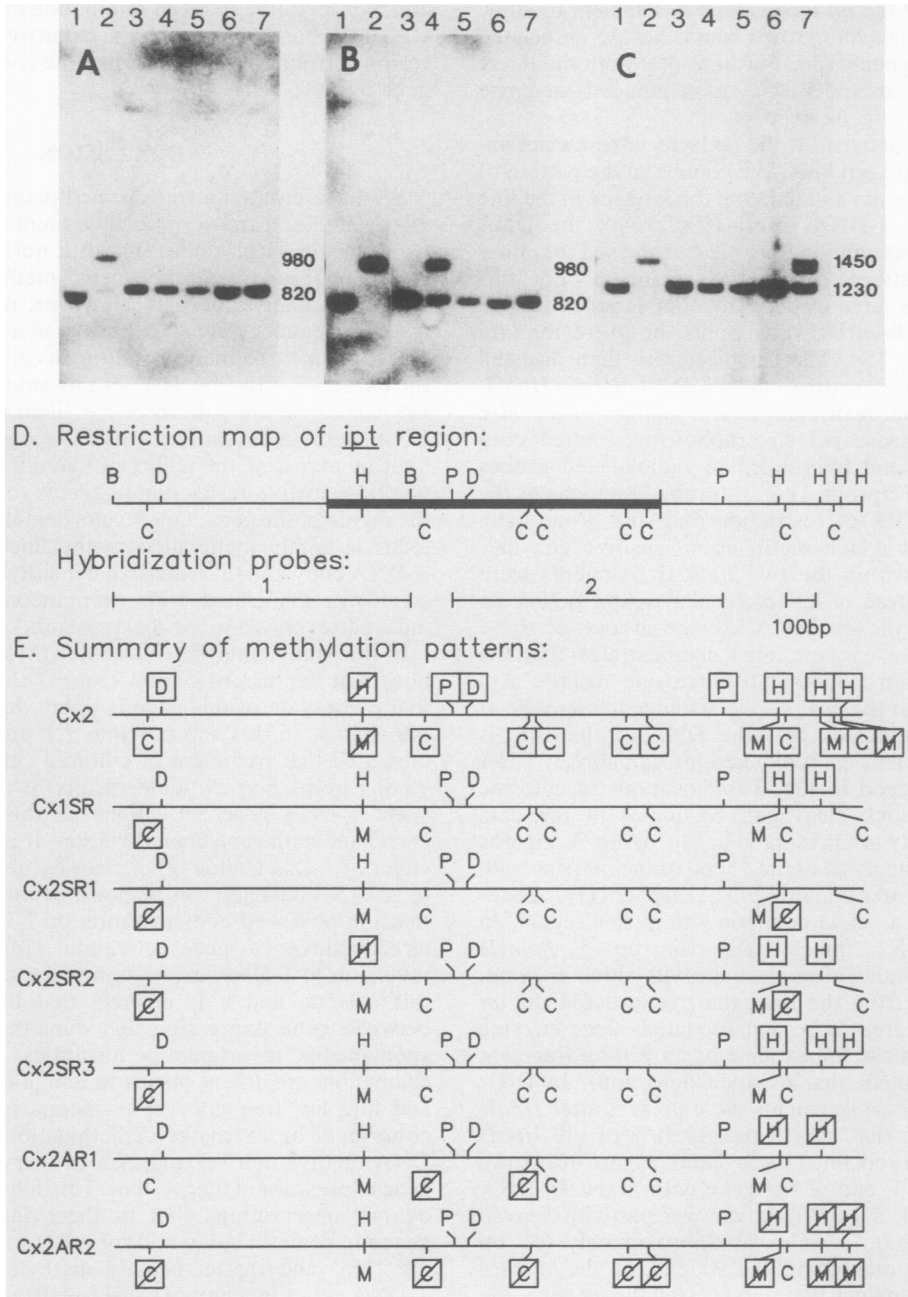

FIG. 3. Analysis of the DNA methylation pattern of the region surrounding the *ipt* gene. (A to C) Southern blots of DNA samples digested with *Bam*HI, followed by *Msp*I (A and C) or *Hpa*II (B). The blots in panels A and B were annealed with probe 1 (panel D), and the blot in panel C was annealed with probe 2. The lanes of each blot contain DNA samples from the following lines: 1, CX1; 2, CX2; 3, CX2SR1; 4, CX2SR2; 5, CX2SR3; 6, CX2AR1; 7, CX2AR2. (D) Restriction map of the *ipt* gene region. (The complete nucleotide sequence of this region has been published [3, 17].) ■, Region found in the mature *ipt* mRNA (17). Restriction site abbreviations: B, *Bam*HI; C, *Hha*I; D, *Dde*I; H, *Hpa*II; M, *Msp*I; P, *Pst*I. *Hpa*II and *Msp*I recognize the same site. The regions of this locus used as hybridization probes are illustrated below the restriction map. (E) Summary of the methylation patterns of the *ipt* region in various cell lines. Boxed restriction sites are fully methylated, boxed sites containing a diagonal line are partially methylated, and unboxed sites are unmethylated. The positions of methylated cytosines that prevent cleavage by the enzymes used are as follows: *Hha*I, G^{m}CGC or GCG^{m}C; *Hpa*II, ^{m}CCGG or C^{m}CGG; *Msp*I, ^{m}CCGG; *Dde*I, ^{m}CTNAG; *Pst*I, ^{m}CTGCAG. Only those *Dde*I sites where N = G and are therefore methylatable are listed.

transcription, and transcription may then cause further demethylation. We cannot distinguish between these possibilities.

At some of the restriction sites analyzed, partial methylation occurred in DNA samples from certain cell lines, indicating that these cell lines were mixtures of cells differing slightly in *ipt* gene methylation patterns. This heterogeneity was mainly observed at the borders of the demethylated region surrounding the *ipt* gene. We cannot determine whether all of the cells in this mixed population express the *ipt* gene. However, in numerous RNA analyses, we never detected any differences in the steady-state levels of *ipt* mRNA among cell lines that exhibit heterogeneities in *ipt* gene methylation patterns and those that are homogeneously

demethylated at the *ipt* locus, indicating that the majority of cells in these cultures do express the *ipt* gene.

We have observed the phenomenon of spontaneous reversion only in cell culture. In plant CX1, reversion occurs at a high frequency exclusively in regions of contact with the medium. The upper regions of the CX1 shoot maintain a relatively normal phenotype and contain a silent, heavily methylated copy of the *ipt* gene (data not shown). Spontaneous revertants of plant CX2, which occur at a low frequency, have been obtained only by plating large numbers of leaf pieces on medium lacking phytohormones. We believe that we would detect any reversion that occurred in the intact CX1 or CX2 plant because demethylation of T-DNA in cells of such plants results in obvious tumor development (2). However, we have maintained hundreds of vegetatively propagated plants in sterile culture and have never observed any tumorous growth on the parts of the shoot not in contact with the medium. Therefore, this spontaneous reversion phenomenon is a form of somaclonal variation (i.e., a change in phenotype induced by plant cell culture) (16). Changes in the methylation patterns of other T-DNA genes (21) and rRNA genes (22) during cell culture have also been observed. It will be interesting to determine the mechanism(s) by which cell culture induces changes in the program of gene expression and the role of DNA methylation in this cell culture-induced genetic change. The activation of T-DNA genes in the cell lines described in this study provides a useful system for investigating these phenomena.

ACKNOWLEDGMENTS

We thank the members of the laboratory for many helpful discussions, Dring N. Crowell for comments on the manuscript, and Carolyn Kunen and Joann Trodahl for preparation of the manuscript.

This research was supported by grant DCB-8602500 from the National Science Foundation and a Steenbock career development award to R.M.A. R.M.A. is a scholar of the James D. and Dorothy Shaw Scholar Program.

LITERATURE CITED

1. **Akiyoshi, D. E., H. Klee, R. M. Amasino, E. W. Nester, and M. P. Gordon.** 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA **81**:5994–5998.
2. **Amasino, R. M., A. L. T. Powell, and M. P. Gordon.** 1984. Changes in T-DNA methylation and expression are associated with phenotypic variation and plant regeneration in a crown gall tumor line. Mol. Gen. Genet. **197**:437–446.
3. **Barker, R. F., K. B. Ilder, D. V. Thompson, and J. D. Kemp.** 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. **2**:335–350.
4. **Barry, G. F., S. G. Rogers, R. T. Fraley, and L. Brand.** 1984. Identification of a cloned cytokinin biosynthetic gene. Proc. Natl. Acad. Sci. USA **81**:4776–4780.
5. **Becker, P. B., S. Ruppert, and G. Schütz.** 1987. Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. Cell **5**:435–443.
6. **Bianchi, M. W., and A. Viotti.** 1988. DNA methylation and tissue-specific transcription of the storage protein genes of maize. Plant Mol. Biol. **11**:203–214.
7. **Busslinger, M., J. Hurst, and R. A. Flavell.** 1983. DNA methylation and the regulation of globin gene expression. Cell **34**: 197–206.
8. **Cedar, H.** 1988. DNA methylation and gene activity. Cell **53**:3–4.
9. **Chandler, V. L., and V. Walbot.** 1986. DNA modification of a maize transposable element correlates with loss of activity. Proc. Natl. Acad. Sci. USA **83**:1767–1771.
10. **Gierl, A., S. Lütticke, and H. Saedler.** 1988. *TnpA* product encoded by the transposable element En-1 of *Zea mays* is a DNA binding protein. EMBO J. **7**:4045–4053.
11. **Gruenbaum, Y., T. Naveh-Mary, H. Cedar, and A. Razin.** 1981. Sequence specificity of methylation in higher plant DNA. Nature (London) **292**:860–862.
12. **Hepburn, A. G., L. E. Clarke, L. Pearson, and J. White.** 1983. The role of cytosine methylation in the control of nopaline synthase gene expression in a plant tumor. J. Mol. Appl. Genet. **2**:315–329.
13. **Jones, P. A.** 1985. Altering gene expression with 5-azacytidine. Cell **40**:485–486.
14. **Keshet, I., J. Lieman-Hurwitz, and H. Cedar.** 1986. DNA methylation affects the formation of active chromatin. Cell **44**:535–543.
15. **Keshet, I., J. Yisraeli, and H. Cedar.** 1985. Effect of regional DNA methylation on gene expression. Proc. Natl. Acad. Sci. USA **82**:2560–2564.

15a. **Klaas, M., M. C. John, D. N. Crowell, and R. M. Amasino.** 1989. Rapid induction of genomic demethylation and T-DNA gene expression in plant cells by 5-azacytosine derivatives. Plant Mol. Biol. **12**:413–423.

16. **Larkin, P. J., and W. R. Scowcroft.** 1983. Somaclonal variation and crop improvement, p. 289–314. *In* T. Kosuge, C. P. Meredith, and A. Hollaender (ed.), Genetic engineering of plants. An agricultural perspective. Plenum Publishing Corp., New York.
17. **Lichtenstein, C., H. Klee, A. Montoya, D. Garfinkel, S. Fuller, C. Flores, E. W. Nester, and M. P. Gordon.** 1984. Nucleotide sequence and transcript mapping of the *tmr* gene of the pTiA6NC octopine Ti plasmid: a bacterial gene involved in plant tumorigenesis. J. Mol. Appl. Genet. **2**:354–362.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**:473–497.
20. **Nester, E. W., M. P. Gordon, R. M. Amasino and M. F. Yanofsky.** 1984. Crown gall: a molecular and physiological analysis. Annu. Rev. Plant Physiol. **35**:387–413.
21. **Peerbolte, R., K. Leenhouts, G. M. S. Hooykaas-van Slogeren, G. J. Wullems, and R. A. Schilperoort.** 1986. Clones from a shooty tobacco crown gall tumor. II. Irregular T-DNA structures and organization, T-DNA methylation and conditional expression of opine genes. Plant Mol. Biol. **7**:285–299.
22. **Quemada, H., E. J. Roth, and K. G. Lark.** 1987. Changes in methylation of tissue cultured soybean cells detected by digestion with the restriction enzymes HpaII and MspI. Plant Cell Rep. **6**:63–66.
23. **Razin, A.** 1984. DNA methylation patterns: formation and biological functions. *In* A. Razin, H. Cedar, and A. D. Riggs (ed.), DNA methylation. Biochemistry and biological significance. Springer-Verlag, New York.
24. **Reid, R. A., M. C. John, and R. M. Amasino.** 1988. Deoxyribonuclease I sensitivity of the T-DNA *ipt* gene is associated with gene expression. Biochemistry **27**:5748–5754.
25. **Schwartz, D., and E. Dennis.** 1986. Transposase activity of the *Ac* controlling element in maize is regulated by its degree of methylation. Mol. Gen. Genet. **205**:476–482.
26. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
27. **Watson, J. C., L. S. Kaufman, and W. R. Thompson.** 1987. Developmental regulation of cytosine methylation in the nuclear ribosomal RNA genes of *Pisum sativum*. J. Mol. Biol. **193**:15–26.
28. **Watt, F., and P. L. Molloy.** 1988. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev. **2**:1136–1143.
29. **Weiler, E. W., and J. Schroder.** 1987. Hormone genes and crown gall disease. Trends Biochem. Sci. **12**:271–275.
30. **Yisraeli, J., D. Frank, A. Razin, and H. Cedar.** 1988. Effect of *in vitro* DNA methylation on β-globin gene expression. Proc. Natl. Acad. Sci. USA **85**:4638–4642.