Deoxyribonuclease I Sensitivity of the T-DNA *ipt* Gene Is Associated with Gene Expression†

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ABSTRACT: We have analyzed the chromatin structure of the T-DNA isopentenyl transferase gene, *ipt*, in four *Nicotiana tabacum* crown gall tumor lines. These four transformed lines contain identical T-DNA inserts and are derivatives of a single clone that did not exhibit any tumorous properties and contained a highly methylated, nonexpressed copy of T-DNA. One of the derivatives also does not exhibit tumorous properties, and the T-DNA of this line is not expressed. The other three lines have reverted to tumorous growth either spontaneously or after treatment with the inhibitor of DNA methylation, 5-azacytidine. Concomitant with this reversion to tumorous growth, expression of the *ipt* gene of these lines has reinitiated. In the lines that express the *ipt* gene, the chromatin structure of this gene exists in a conformation that is more accessible to DNase I than in the line in which this gene is not expressed. The level of *ipt* expression and DNase I sensitivity was independent of the process by which the transformed cell lines reverted to tumorous growth. The relationship of chromatin structure to gene expression and DNA methylation in these lines is discussed.

Agrobacterium tumefaciens causes crown gall tumors on many species of plants. The ability of this soil bacterium to neoplastically transform plant cells is conferred by the Ti (tumor-inducing) plasmid. Tumor formation occurs when a portion of the Ti plasmid, the T-DNA (transferred DNA), is transferred from the bacteria to the plant cell, integrated into the host genome, and expressed (Nester et al., 1984). The T-DNA genes responsible for oncogenesis, *ipt*, *iaaM*, and *iaaH*, encode enzymes that participate in the biosynthesis of zeatin, a cytokinin, and indoleacetic acid, an auxin [for a review, see Weiler and Schroder (1987)]. These growth substances activate cell division and differentiation, resulting in tumor formation and phytohormone-independent growth in vitro.

The regulation of gene expression in eukaryotes is controlled at multiple levels including gene-specific mechanisms involving sequence-specific DNA/protein interactions and general mechanisms involving chromatin conformation [for a review, see Weintraub (1985)]. T-DNA genes contain eukaryotic-type promoter elements which are recognized by the transcriptional machinery of many plants (Bruce & Gurley, 1987; Ellis et al., 1987; An et al., 1986). Furthermore, integrated T-DNA is packaged in a nucleosomal array, resembling the chromatin of "native" plant DNA sequences (Coates et al., 1987; Schafer et al., 1984). Thus, T-DNA genes integrated into the plant genome are likely to be subject to multiple levels of control.

Numerous studies on animal systems suggest that active genes generally exhibit a more open chromatin conformation than inactive genes [for review's, see Eissenberg et al. (1985) and Weintraub (1985)]. Chromatin conformatin is generally defined by the accessibility of the DNA to various endonucleases. DNase I is often used for these studies because this enzyme demonstrates little sequence specificity (Bernardi et al., 1975) and is therefore likely to digest DNA in chromatin solely on the basis of DNA accessibility. Within active animal genes, enhanced general DNase I sensitivity and specific hypersensitive sites have been described. General DNase I sensitivity is characterized by domains of contiguous DNA sequences that are uniformly degraded at a faster rate than neighboring sequences [for a review, see Weintraub (1985)]. DNase I hypersensitivity is characterized by specific sites on the DNA polymer that are more accessible than the surrounding generally sensitive sequences [for a review, see Eissenberg et al. (1985)]. These differences in chromatin structure between active and inactive genes are often associated with chemical modifications of histones and qualitative differences in DNA-associated histone and non-histone proteins [for a review, see Weintraub (1985)]. Furthermore, DNase I resistant chromatin and gene inactivity are often associated with the covalent modification of DNA, cytosine methylation [for a review, see Conklin and Groudine (1984)].

The few DNase I sensitivity studies of DNA in plant chromatin carried out to date suggest that the relationship between gene expression and chromatin conformation resembles that of well-characterized animal systems. Plant genes that are expressed in a tissue-specific manner or in response to environmental factors are more DNase I sensitive when expressed than when not expressed (Paul et al., 1987; Sawyer et al., 1987; Wurtzel et al., 1987; Steinmuller et al., 1986; Vayda & Freeling, 1986; Murray & Kennard, 1984; Spiker et al., 1983). Variations in chromatin conformation have been associated with differences in DNA-associated proteins (Spiker et al., 1983). The expression of certain T-DNA genes and transposon activity in plants appears to be linked to DNA hypomethylation (Bennetzen, 1987; Chomet et al., 1987; Chandler & Walbot, 1986; Peerbolte et al., 1986; Schwartz & Dennis, 1986; Amasino et al., 1984; Hepburn et al., 1983). However, the relationship between chromatin conformation, gene expression, and DNA methylation in these studies has not been addressed. Recent studies have suggested that the level of DNA methylation and the degree of nuclease sensitivity of pea genes encoding ribosomal RNA may be related (Kaufman et al., 1987; Watson et al., 1987).

To further our understanding of the relationship between chromatin conformation, gene expression, and DNA methylation in plant genomes, we have studied four *Nicotiana*

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tabacum crown gall cell lines of identical T-DNA content that vary with respect to *ipt* transcription and DNA methylation. We present evidence that the chromatin of the *ipt* gene is in a more accessible conformation when this gene is expressed and hypomethylated.

Experimental Procedures

Derivation of Transformed Cell Lines. The clonal *Nicotiana tabacum* crown gall tumor line that gave rise to a normal-appearing plant (plant C) containing nonexpressed T-DNA inserts has been described (Amasino et al., 1984). Plant C, which lacked viable pollen, was cross-pollinated with pollen from nontransformed *N. tabacum* variety Xanthi, and a normal seed set was obtained. Seed was germinated on agar-solidified MS (Murashige–Skoog) medium (Murashige & Skoog, 1962) without phytohormones (MS 0/0), and two seedlings, CX1 and CX2, were chosen for further study (see Results). The region of plant CX1 stem that contacted the growth medium developed into a tumorous growth and was maintained as shoot-forming (teratomous), phytohormone-independent clumps of tissue on liquid MSO medium at 27 °C in diffuse constant light at 100 rpm. The CX2 seedling grew as a normal-appearing plant on solidified MS 0/0 medium, and tissues of this plant required phytohormones for callus growth in culture. For RNA and chromatin analysis, line CX2 was maintained as a shoot-forming phenocopy of line CX1 by culture as described previously in liquid MS medium containing 2 mg/L benzyladenine to fulfill the phytohormone requirement of this tissue. Phytohormone-independent revertants of plant CX2 were obtained in two ways. First, numerous leaf pieces of plant CX2 were plated on MS 0/0 medium. At a frequency of approximately 10^{-7}, phytohormone-independent foci were observed at the cut surface of leaf pieces. This frequency was calculated by dividing the number of foci by an estimate of the number of cells at the perimeter of the leaf pieces (40 cells/mm). One of these spontaneous revertants, line CX2 spontaneous revertant 3 (CX2SR3), was used for further study. Second, leaf pieces of plant CX2 were plated on MS 0/0 medium containing 5 μM 5-azacytidine (Sigma, St. Louis, MO) for 5 days and then transferred to medium without 5-azacytidine. Phytohormone-independent tissue arose at a frequency estimated to be greater than 10^{-1} as a confluent ring of tissue developing from the cut surface of the leaf piece. A teratomous segment of this tissue, designated line CX2 5-azacytidine revertant 2 (CX2AR2), was chosen for further study. Both phytohormone-independent revertants were maintained as shoot-forming tumor lines on liquid MSO medium as described previously for CX1.

Nuclei Isolations from Tissue Lines. The procedure for nuclei isolation from cell cultures was adapted from that of Willmitzer and Wagner (1981). Teratomous cell clumps were obtained in mid-log-phase growth, 7 days from subculture. Approximately 40 g fresh weight of tissue was washed with cell digestion buffer (CDB) (CDB = 0.4 M mannitol, 1.3 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM KNO_3, and 6.8 mM $CaCl_2$, pH 5.6, with KOH). Tissue clumps were suspended in 100 mL of CDB and broken down to smaller aggregates by using a Waring blender at low speed. Cells were rinsed and resuspended to a total volume of 60 mL with CDB, and cellulase and pectinase (Worthington Biochemicals, Malvern, PA) were added to 1 and 0.2 mg, respectively, per gram fresh weight of tissue. The samples were then incubated at 28 °C for 2 h. From this step on, all buffers and materials were kept on ice. Cells were rinsed with grinding buffer (GB) [GB = 15 mM NaCl, 0.25 M ultrapure, nuclease-free sucrose, 60 mM KCl, 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.6% v/v Nonidet P-40, 5 mM $MgCl_2$, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM ascorbic acid, and 5 mM β-mercaptoethanol, pH 6.0, with KOH] and resuspended in 50 mL of GB. Cell disruption was achieved by blending with a polytron (Brinkmann generator PT 10 ST) for four, 15-s pulses. The homogenate was centrifuged for 3 min at 636*g*, and pellets were resuspended in 25 mL of GB. This pelleting and resuspension was repeated twice. The final resuspension was filtered through a series of nylon filters with pore sizes of 150, 88, and 37 μm. The filtrate was centrifuged as described above, and the pellets were resuspended in 20 mL of GB without EGTA, Nonidet P-40, and ascorbic acid (GBO). Three additional rounds of centrifugation and resuspension were performed: twice in GBO and once in DNase I buffer (60 mM KCl, 15 mM NaCl, 0.5 mM $CaCl_2$, and 0.5 mM dithiothreitol, pH 7.4 with HCl). In the final resuspension in DNase I buffer, the nuclei were adjusted to a concentration of 10^6/mL as determined by counting on a hemacytometer.

DNase I Digestions. Nuclei preparations were divided into 10 samples. Nine of these samples were removed from ice and preincubated in a 25 °C water bath for 5 min. DNase I (Worthington Biochemicals, Malvern, PA) was added to concentrations of 0, 0.005, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, or 1.00 unit/mL (unit as defined by the manufacturer), and the samples were briefly vortexed and incubated at 25 °C for 3 min. The reactions were stopped by the addition of 1 volume of DNase I stop solution [100 mM ethylenediaminetetraacetate (EDTA), 2% w/v sodium dodecyl sulfate, 200 mM NaCl, 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 8.0), and 50 μg/mL protease K]. Samples were vortexed and incubated at 37 °C for 1 h. The sample of nuclei to which no DNase I was added controlled for endogenous nuclease activity during the 3-min, 25 °C incubation. The following control for endogenous nuclease activity during the preparation of the nuclei, prior to the 25 °C incubation, was also carried out: a sample of nuclei was mixed with an equal volume of DNase I stop solution while still on ice and then incubated at 37 °C for 1 h.

We also examined the DNase I sensitivity of protein-free DNA. For this control, replicate samples of protein-free DNA purified from cell lines CX1 and CX2 (see Direct Nucleic Acid Isolation from Whole Cells) were prepared in DNase I buffer to a concentration of 0.1 mg/mL. Samples were preincubated for 5 min at 25 °C and digested with DNase I at a concentration of 0, 0.05, 0.10, or 0.20 unit/mL for 3 min. The reaction was stopped by the addition of an equal volume of DNase I stop solution.

Micrococcal Nuclease (MNase) Digestions. Nuclei were divided into four samples, brought to 8 mM $CaCl_2$, and preincubated for 5 min at 25 °C. MNase (Worthington Biochemicals, Malvern, PA) was added to a final concentration of 25 units/mL (unit as defined by the manufacturer) and allowed to react for 1, 2, 4, or 8 min at 37 °C. The reaction was terminated by the addition of an equal volume of DNase I stop solution followed by incubation at 37 °C for 1 h.

DNA Isolation from MNase and DNase I Digestions. Nuclease and protease K treated samples were extracted once with phenol/chloroform/isobutyl alcohol (24:24:1) and then extracted with chloroform/isobutyl alcohol (24:1). RNase A (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 15 μg/mL, and the samples were incubated for 30 min at 37 °C. DNA was precipitated with 2 M ammonium acetate and 2 volumes of ethanol (EtOH), and then

DNA was dissolved in TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5). The ammonium acetate precipitation was repeated 3 times. The final pellet was washed 3 times with 70% EtOH and once with 100% EtOH and dried under reduced pressure. DNA was dissolved in TE and quantitated by the 260:280-nm ratio of absorption.

Direct Nucleic Acid Isolation from Whole Cells. For DNA and RNA isolations, tissues were frozen in liquid nitrogen and ground to a fine powder in a precooled metal blender cup. The cup was transferred to a 60 °C water bath and immediately mixed with 1.7 mL/g fresh weight tissue of a 60 °C emulsion of extraction buffer/phenol (1:0.7). Extraction buffer consisted of 100 mM Tris-HCl, 20 mM EDTA, 0.5% w/v sodium dodecyl sulfate, 0.5 M NaCl, and 0.5% β-mercaptoethanol. The tissue–buffer–phenol emulsion was stirred until the temperature reached 50 °C, then transferred to a screw-cap centrifuge tube containing 0.7 mL/g fresh weight tissue of chloroform/isobutyl alcohol (24:1), mixed thoroughly, and then centrifuged for 10 min at 10000*g*. The upper aqueous phase was transferred to another tube containing 0.7 mL/g fresh weight tissue of chloroform/isobutyl alcohol (24:1), mixed, and centrifuged as described above. The aqueous phase was then transferred to a tube containing $^1/_3$ volume of 10 M LiCl and gently mixed, and the RNA was precipitated overnight at 4 °C. The RNA was sedimented by centrifugation for 15 min at 10000*g*, dissolved in CE [CE = 10 mM sodium citrate and 1 mM EDTA, pH 7.0, treated with 0.05% diethyl pyrocarbonate (DEPC)], and reprecipitated with 2 M ammonium acetate and 2 volumes of EtOH. RNA was then resuspended in CE and quantitated spectrophotometrically. To the LiCl supernatant, which contained DNA and small RNAs with a high degree of secondary structure, 2 volumes of EtOH were added to precipitate nucleic acids. The nucleic acid was recovered by centrifugation for 5 min at 2000*g* and dissolved in TE, and 0.95 g/mL CsCl and 350 μg/mL ethidium bromide were added. The nucleic acid was then subjected to equilibrium centrifugation in a Beckman TLA-100.2 fixed-angle rotor at 100000 rpm for 4 h. The band containing genomic DNA was removed, and ethidium bromide was extracted as described by Maniatis et al. (1982) and purified as described previously (Amasino et al., 1984).

Restriction Endonuclease Digestions of DNA and Gel Electrophoresis and Blotting of DNA and RNA. For blot analysis, DNA was digested to completion with *Bam*HI (New England Biolabs, Beverly, MA) or *Bam*HI and *Hae*III (Bethesda Research Laboratories, Gaithersburg, MD) using the manufacturer's recommended buffers with the addition of 1 mM spermidine. Digested DNA samples were then precipitated with 300 mM sodium acetate and 2 volumes of EtOH in the presence of 10 μg of glycogen, sedimented, rinsed once with 70% EtOH and once with 100% EtOH, dried under reduced pressure, and dissolved in TE. Equal amounts of restricted endogenous nuclease control DNA and DNase I digested DNA were fractionated by gel electrophoresis through 1.2% agarose gels in TBE (0.089 M Tris–borate, 0.089 M boric acid, and 2 mM EDTA, pH 8.3). DNA in the gels was acid-depurinated in 0.25 M HCl for 18 min, neutralized in 0.4 M NaOH for 15 min, and transferred onto nylon membranes (Biotrace, Gelman Scientific, Ann Arbor, MI) by capillary blotting in 20 mM NaOH and 1 mM EDTA.

To assess the extent of DNA digestion, nuclease-treated and control DNA samples were electrophoresed through agarose gels in TBE. The nucleosomal repeat length was calculated by averaging the difference in size of the successive nucleosomal fragments. The fragment lengths were determined by comparison to DNA size standards.

Equal amounts of RNA isolated from the four cell lines were electrophoresed in denaturing formaldehyde gels as described (Maniatis et al., 1982) and blotted as described above for DNA, except that CE was used as the transfer buffer and nucleic acid depurination was omitted.

Production of Probes, Blot Hybridizations, and Autoradiography. Three radiolabeled nucleic acid probes were utilized in this study. The regions of the T-DNA to which the probes correspond are shown in Figure 2. Two antisense RNA probes, *Bam*HI 29 and *Pst*I A, were made by in vitro SP6 RNA polymerase transcription [as described in Amasino (1986)] of T-DNA sequences subcloned into pGEM 2 (Promega Biotec, Madison, WI). One DNA probe, *Hae*III 2022, was produced by using the random-primer oligo labeling technique (Feinberg & Vogelstein, 1983).

Prehybridization was carried out in a sealed tray at 65 °C for 4 h in the following hybridization solution modified from that of Amasino (1986): 0.25 M $NaHPO_4$, 5% w/v sodium dodecyl sulfate, 0.5% w/v nonfat dry milk, 2.5 mM EDTA, 10% w/v poly(ethylene glycol) (M_r 8000), 25 μM aurintricarboxylic acid, and 25 μg/mL carrier yeast RNA, pH 7.2. Probe was added to 10^6 cpm/mL and allowed to hybridize for a minimum of 18 h. Blots were washed in 0.25 M $NaHPO_4$, 2% w/v sodium dodecyl sulfate, and 1 mM EDTA (pH 7.2) 6 times for 30 min each at 65 °C and used to expose Kodak X-Omat AR film at −70 °C with a Dupont Cronex Lightning Plus intensifying screen.

RESULTS

Derivation, Phenotype, T-DNA Expression, and DNA Methylation of Transformed Cell Lines. The derivation of normal-appearing plants containing a T-DNA insert that was transcriptionally inactive from a clonal line of a *Nicotiana tabacum* crown gall tumor has been described (Amasino et al., 1984). One of these plants (plant C) was crossed with wild-type *N. tabacum*, and seed was collected. One hundred seeds, germinated in sterile culture, gave rise to 99 normal-appearing plants of which approximately 50% inherited a silent T-DNA copy as determined by DNA analysis and the ability of 5-azacytidine to reactivate tumorous growth. The remaining seedling, however, exhibited tumorous traits: tissues that contacted the growth medium developed into a tumorous growth (line CX1) that grew as a phytohormone-independent teratoma in vitro. This phenotype suggested that the T-DNA *ipt* gene of line CX1 had been activated spontaneously. Line CX1 and one of the normal-appearing plants that contained a silent T-DNA insert (plant CX2) were chosen for further study. Tissues of line CX2 require phytohormones for growth in vitro.

To obtain revertants of line CX2 that were able to proliferate without phytohormones, leaf pieces of plant CX2 were plated on phytohormone-free media directly or after exposure to the DNA-demethylating agent 5-azacytidine (Jones, 1985). By selecting for cells that reverted to phytohormone-independent growth, we obtained derivatives of plant CX2 in which *ipt* expression had reinitiated. The rate of reversion was approximately 10^{-7} without 5-azacytidine treatment and greater than 10^{-1} after exposure to 5-azacytidine (see Experimental Procedures). One of each class of these revertants was chosen for study: line CX2 spontaneous revertant 3 (CX2SR3) and line CX2 5-azacytidine revertant 2 (CX2AR2).

Two characteristics of these four lines provided a controlled situation for this study. First, the lines were all derivatives of the same clonal line (Amasino et al., 1984) and, at the level

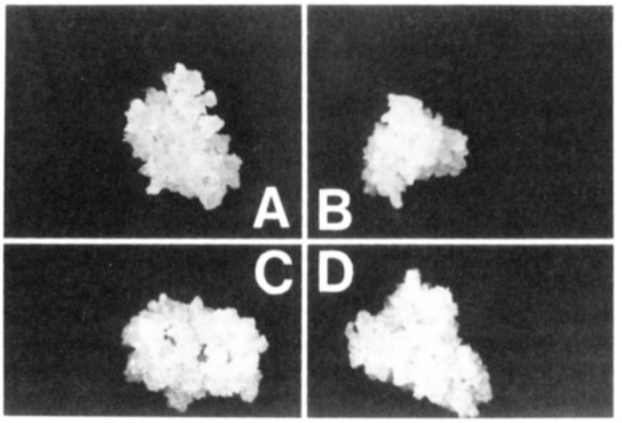

FIGURE 1: Phenotypes of the tumor lines. The tumor lines used in this study were cultured as described under Experimental Procedures either on liquid MS medium without phytohormones, lines CX1 (A), CX2SR3 (C), and CX2AR2 (D), or on liquid MS medium containing 2 mg/L benzyladenine, line CX2 (B). As illustrated, the phenotypes of all of these lines were similar.

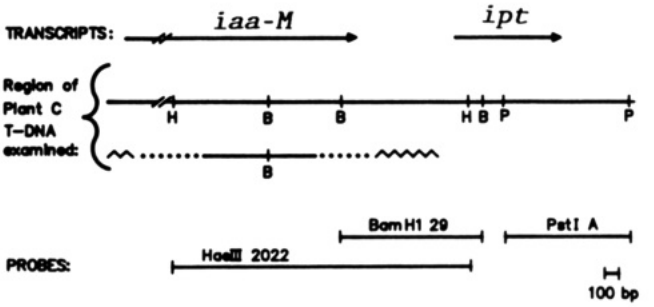

FIGURE 2: Restriction map of the relevant regions of the T-DNA of plant C, T-DNA transcripts, and DNA sequences used as hybridization probes. The cell lines used in this study contain two copies of T-DNA: one full-length copy that contains the complete sequence of the *iaaM* and *ipt* genes (upper T-DNA map) and one truncated copy that does not contain the complete coding sequence of any T-DNA *iaaM* gene (lower T-DNA map). Only the region of the full-length T-DNA relevant to this study is shown. The complete map of this T-DNA is described in Amasino et al. (1984). The truncated T-DNA copy is diagrammed as follows: T-DNA sequences are indicated by the straight line, plant sequences are indicated by a zig-zag line, and the level of uncertainty as to the location of T-DNA to plant DNA junctions is represented by the dotted line. The following probes were used in this study: *Pst*I A probe to detect *ipt* mRNA and *Bam*HI 29 and *Hae*III 2022 probes to detect DNase I and restriction endonuclease digested DNA fragments. H = *Hae*III, B = *Bam*HI, and P = *Pst*I.

of extensive Southern blot analysis, contained T-DNA inserts of identical length integrated into the same genomic site (unpublished experiments and Figure 5A–D, panels II and III, as described below). Thus, differences in the level of expression and chromatin structure of the *ipt* gene are not likely due to differences in T-DNA primary structure. Second, the lines were maintained in culture as phenotypically identical teratomas (Figure 1). The teratomous phenotype of the phytohormone-independent lines CX1, CX2AR2, and CX2SR3 resulted from endogenous production of cytokinin. Therefore, we chose to maintain the hormone-dependent line CX2 as a teratoma by adding cytokinin to the media. Consequently, nuclei were prepared from tissues with similar morphologies at similar stages of culture growth to allow for consistency in the nuclei preparations and minimize the potential effects of phenotypic variation on gene expression.

To verify that expression of the *ipt* gene was responsible for the phytohormone autonomy of lines CX1, CX2AR2, and CX2SR3, and that lack of expression resulted in the phytohormone requirement of line CX2, mRNA blot analyses were performed on these lines (Figures 2 and 3). Total RNA from these lines was size-fractionated using gel electrophoresis, transferred onto a membrane, and hybridized to a radiolabeled probe corresponding to the *ipt* gene. The hybrid signal intensities of RNA samples from the phytohormone-independent lines confirm that the phytohormone autonomy of these lines results from high steady-state levels of *ipt* mRNA (Figure 3, lanes CX1, CX2AR2, and CX2SR3). Furthermore, the level of *ipt* expression is independent of which reversion process (i.e., spontaneously or after 5-azacytidine treatment) gave rise to expression. No *ipt* mRNA, however, was detected in RNA from the phytohormone-requiring line CX2 (Figure 3, lane CX2). Additional mRNA analyses, similar to those above, showed that all T-DNA *iaaM* sequences in all four lines were transcriptionally inactive (data not shown).

DNase I Sensitivity of the ipt Gene. We first analyzed the relative DNase I sensitivity of the *ipt* region of the T-DNA in nuclei prepared from lines CX1 and CX2. Microscopic examination showed that these preparations consisted primarily of intact nuclei. The nucleosome conformation of the chromatin within these nuclei appeared undisturbed since we were able to reproducibly generate identical nucleosomal ladders from these nuclei preparations by micrococcal nuclease digestions. A representative ladder of nucleosomal fragments is shown in Figure 4. The size of the nucleosomal repeat length in these *N. tabacum* cell lines was found to be approximately 190 base pairs (bp) and is similar to that observed in other studies of plant chromatin (Coates et al., 1987; Murray & Kennard, 1984; Spiker et al., 1984). In addition, there was undetectable endogenous nuclease activity within these nuclei preparations since DNA prepared from these nuclei was of the same high molecular weight as DNA directly isolated under conditions that prevent nuclease activity (data not shown), and incubation of the nuclei preparations under conditions favorable for nuclease activity did not cause a decrease in DNA size or a loss in the hybridization signal corresponding to the *ipt* gene (Figure 5A–D, panels I and II, lanes C and 0).

Nuclei were incubated with DNase I at concentrations ranging from 0 to 1.0 unit/mL for 3 min at 25 °C. DNA was isolated from the DNase I treated nuclei, size-fractionated by agarose gel electrophoresis, and visualized by ethidium bromide staining. The extent of DNase I degradation at specific DNase I concentrations was identical for DNA isolated from nuclei of line CX1 and line CX2 (Figure 5A,B, panel I). Most notably, a major shift in DNA fragment size range occurs between 0.2 and 0.5 DNase I unit/mL. These reproducibly identical degradation patterns of DNase I treated samples from different tissues provide a reference with which to determine differences in the DNase I sensitivity of specific genes in the different lines. Since the chromatin isolated from both lines exhibits the same overall DNase I sensitivity, any differences in the DNase I sensitivity of a specific gene are not due to differences in the integrity of total chromatin isolated from the different tissues.

To analyze the DNase I sensitivity of the *ipt* gene in nuclei prepared from lines CX1 and CX2, DNA from DNase I treated nuclei was digested with the restriction endonuclease *Bam*HI, size-fractionated, transferred onto membranes, and hybridized to a probe corresponding to the 5′ region of the *ipt* gene. The concentration of DNase I and the extent of total DNA digestion necessary to cause a loss of hybridization signal are greater in CX2 than in CX1 (Figure 5A,B, panel II). This demonstrates that the 5′ region of the *ipt* gene is more DNase I sensitive in the expressing line, CX1, than in the nonex-

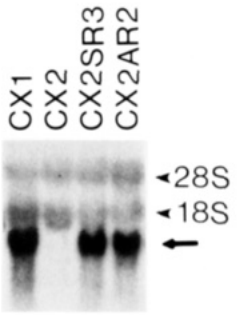

FIGURE 3: Analysis of steady-state levels of *ipt* transcripts. Ten micrograms of total RNA from transformed cell lines was fractionated on a denaturing agarose gel, blotted to a nylon membrane, hybridized to an antisene ^{32}P-labeled RNA probe complementary to the *Pst*I A fragment of the *ipt* gene, and exposed to film as described under Experimental Procedures. The source of the RNA is indicated above each lane of the autoradiogram. Exposure was for 16 h at −70 °C with an intensifying screen. The hybridization band corresponding to the *ipt* mRNA is marked with an arrow. Cross-hybridization of this probe with the 28S and 18S rRNA is indicated.

pressing line, CX2. No DNase I hypersensitive sites were detected in either line.

To further demonstrate that the differences in DNase I sensitivity of the *ipt* gene in lines CX1 and CX2 are associated with differences in gene activity, we analyzed, as an internal control, the DNase I sensitivity of a T-DNA segment that is not expressed in either line. Specifically, this internal control is a truncated T-DNA that contains part of the coding sequence of the *iaaM* gene and is not contiguous with the other T-DNA copy (which contains the *ipt* gene) present in these lines [Figure 2 and Amasino et al. (1984)]. The lack of expression of these incomplete *iaaM* sequences in lines CX1 and CX2 was confirmed by Northern blot analyses that included RNA from a tumor line that expresses the *iaaM* gene to ensure that this low-abundance transcript was detectable (data not shown). The DNase I treated DNA from lines CX1 and CX2 was digested to completion with restriction endonucleases *Bam*HI and *Hae*III, and blots were prepared. These DNA samples were hybridized to a probe complementary to the 3′ end of the nontranscribed *iaaM* sequences and the 5′ end of the *ipt* gene. The resulting autoradiograms are shown in Figure 5A,B, panel III. The probe hybridizes to five DNA fragments in restricted tumor DNA (Figure 2). Two small fragments that correspond to the *iaaM* sequences present in the full-length T-DNA copy were not well resolved. The other three fragments for which an autoradiographic signal was detected are the *Bam*HI–*Hae*III fragment of the complete T-DNA copy (which contains the 5′ end of the *ipt* gene) and fragments of T-DNA and flanking plant DNA on either side of the *Bam*HI site of the truncated T-DNA copy (Figure 2). The hybridization signal corresponding to the 5′ region of the *ipt* gene disappears more rapidly with increasing DNase I concentration in DNA from line CX1 than from line CX2 and confirms our earlier observation that the 5′ region of the *ipt* gene is more DNase I sensitive in line CX1 than in line CX2 chromatin. The profiles of DNase I concentration-dependent disappearance of the hybridization signals corresponding to *iaaM* sequences of the truncated T-DNA are equal in chromatin prepared from lines CX1 and CX2. Thus, control T-DNA sequences that are not expressed in either CX1 or CX2 have identical chromatin conformations under our assay conditions. Moreover, these profiles are similar to the profile of disappearance of the hybridization signal corresponding to the 5′ region of the *ipt* gene in line CX2, a line that does not express the *ipt* gene (Figure 5A,B, panel III). These differences in the DNase I sensitivity of the *ipt* gene in the cell line that expresses this gene, CX1, versus the nonexpressing line, CX2, were reproduced with independent nuclei preparations from both lines.

To show that the difference in DNase I sensitivity was caused by differences in chromatin structure and not directly by covalent modifications of the DNA, such as cytosine methylation, DNase I digestions were carried out on high molecular weight, protein-free DNA isolated from lines CX1 and CX2. Using the same blotting and hybridization conditions, we observed no differences in DNase I sensitivity between lines CX1 and CX2 in the 5′ region of the *ipt* gene when protein-free DNA was analyzed (data not shown).

Since we had demonstrated that differences in the chromatin structure of the *ipt* gene in lines CX1 and CX2 were associated with differences in expression of this gene, it was of interest to extend these studies to revertants of line CX2 that had reinitiated expression of the *ipt* gene. In particular, we sought to determine if alterations in the chromatin structure of the *ipt* gene accompanied the reversion process. Thus, we examined the DNase I sensitivities of this gene in nuclei prepared from a 5-azacytidine-induced and a spontaneous revertant of plant CX2 (CX2AR2 and CX2SR3, respectively). The profile of degradation of total DNA from DNase I treated nuclei of line CX2AR2 was identical with that described for lines CX1 and CX2, exhibiting a major shift in DNA size range between 0.2 and 0.5 DNase I unit/mL (Figure 5A–C, panel I). In this experiment, the extent of DNase I degradation in DNA from line CX2SR3 nuclei is slightly less than that observed in chromatin from the other lines [i.e., a major shift in DNA size range occurs between 0.5 and 1.0 DNase I unit/mL (Figure 5A–D, panel I)]. The extent of DNase I sensitivity of the *ipt* gene in chromatin from lines CX2SR3 and CX2AR2 was assessed by blot hybridization as described above. The profile of disappearance of the hybridization signal corresponding to the 5′ end of the *ipt* gene in line CX2AR2 was identical with that of line CX1 (Figure 5A,C, panel II). Therefore, the *ipt* gene in chromatin of the 5-azacytidine revertant, line CX2AR2, and line CX1 have equal DNase I sensitivities and are more DNase I sensitive than the same sequence in line CX2. The hybridization signal corresponding to the 5′ end of the *ipt* gene in line CX2SR3 diminishes at DNase I concentrations intermediate to those required for signal diminution in DNA from the lines that express the *ipt* gene (CX1 and CX2AR2) and in DNA from the line that does not express the *ipt* gene (CX2) (Figure 5A–D, panel II). These hybridization data and the observation that DNA of line CX2SR3 chromatin is less degraded at specific DNase I concentrations when compared to lines CX1, CX2, and CX2AR2 chromatin demonstrate that the DNase I sensitivity of the *ipt* gene in chromatin of line CX2SR3 is clearly greater than the *ipt* gene in chromatin of line CX2 and comparable to the sensitivity of that same gene in chromatin of lines CX1 and CX2AR2. No hypersensitive sites were detected in line CX2AR2 or line CX2SR3.

In conclusion, we have shown that the chromatin structure of the *ipt* gene in lines that express this locus (CX1, CX2AR2, and CX2SR3) is more accessible to DNase I than in a line (CX2) in which this gene is inactive. As discussed below, these differences in chromatin conformation and expression are associated with differences in the level of cytosine methylation of the *ipt* gene.

Discussion

We have analyzed the DNase I sensitivity of the 5′ region of the T-DNA *ipt* gene in nuclei of *Agrobacterium*-transformed tobacco cell lines. We have demonstrated that in a

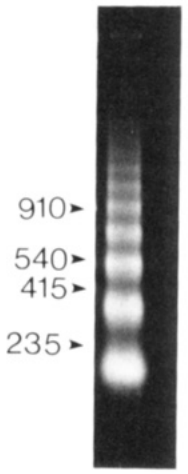

FIGURE 4: Micrococcal nuclease digestion of DNA from line CX1 nuclei. One microgram of DNA isolated from micrococcal nuclease treated nuclei of line CX1 was fractionated through a 2.0% agarose gel and stained with ethidium bromide to illustrate the nucleosomal digestion pattern of line CX1 chromatin. Numbers on the left correspond to DNA size markers in base pairs.

line in which the *ipt* gene is inactive (CX2), this gene is in a chromatin conformation that is relatively DNase I resistant. However, in lines in which this inactive locus reverted to reexpression either spontaneously (CX1, CX2SR3) or after 5-azacytidine treatment (CX2AR2), reexpression is accompanied by an increase in the DNase I sensitivity of the *ipt* gene. Thus, in these examples, transcriptionally active T-DNA genes exhibit a more open chromatin conformation than T-DNA genes that are inactive. Our measure of gene activity is the steady-state level of *ipt* mRNA. We have shown that the three lines that exhibit tumorous properties (CX1, CX2AR2, and CX2SR3) contain high levels of *ipt* mRNA, while the nontumorous line (CX2) does not contain detectable *ipt* transcripts. Our interpretation of these data is that the *ipt* gene is not transcriptionally active in line CX2, whereas in lines CX1, CX2AR2, and CX2SR3 the *ipt* gene is actively transcribed. It is unlikely that differences in *ipt* mRNA turnover are responsible for the observed differences in the steady-state levels of *ipt* mRNA because among phenotypically identical tissues with the same T-DNA insert an identical mRNA would be expected to demonstrate similar rates of turnover.

No DNase I hypersensitive sites were found within the 5′ region of the *ipt* gene and either do not exist within the chromatin of this region or are not detectable by our experimental procedures. DNase I hypersensitive sites have been observed in the chromatin of several plant genes (Paul et al., 1987; Steinmuller et al., 1986; Vayda & Freeling, 1986), although these sites are not always detected in transcribed plant genes (Sawyer et al., 1987; Steinmuller et al., 1986; Murray & Kennard, 1984). Such sites are generally thought to reflect the binding of sequence-specific DNA binding transcription factors [e.g., see Bodner and Karin (1987), Emerson et al. (1985), and Zaret and Yamamoto (1984)]. Whether factors are present in plant cells that are necessary for transcription of and confer DNase I hypersensitivity to T-DNA genes awaits further study.

The differences in the expression and DNase I sensitivity of the *ipt* genes in these lines are associated with variations in the patterns of DNA methylation. We have previously demonstrated that in transformed cell lines containing T-DNA inserts identical with the T-DNA of the lines described herein, actively transcribed *ipt* genes were hypomethylated when compared to inactive *ipt* genes (Amasino et al., 1984). Moreover, we have recently utilized a number of methyla-

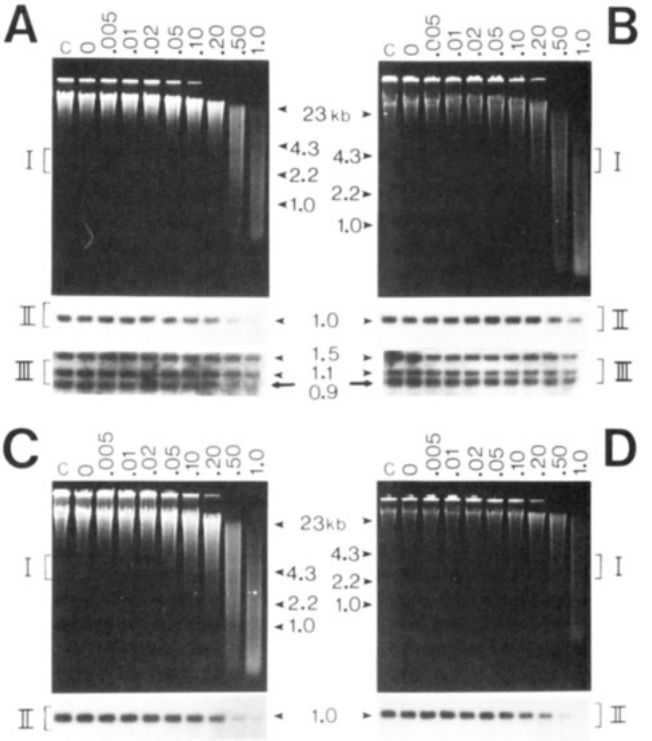

FIGURE 5: Analysis of the DNase I sensitivity of total chromatin, *ipt*, and internal control sequences from DNase I treated nuclei. The data for line CX1 are presented in section A; line CX2, section B; line CX2AR2, section C; and line CX2SR3, section D. Panel I of each section shows the extent of DNA digestion as a function of DNase I concentration. One microgram of DNA from DNase I digested nuclei was fractionated through 0.7% agarose and stained with ethidium bromide. DNase I concentrations are listed above each lane in units per milliliter. Lanes 0 and C correspond to DNA used as controls for endogenous nuclease activity. The DNA in lane C was prepared from nuclei that had been kept on ice; DNA in lane 0 was from nuclei incubated at 25 °C in DNase I digestion buffer without added DNase I. Numbers in the center correspond to DNA size markers in kilobase pairs. In panel II of each section, 3.5 μg of *Bam*HI-restricted DNA isolated from DNase I treated nuclei was fractionated through 1.2% agarose, blotted onto membranes, hybridized to an antisense *Bam*HI 29 RNA probe, and exposed to film. Lanes correspond to those as in (I). The hybridization signal corresponds to the 5′ *ipt* gene coding sequences and upstream, noncoding sequences in the *Bam*HI 29 fragment of the T-DNA (see Figure 2). In panel III of each section, 3.5 μg of *Bam*HI- and *Hae*III-restricted DNA isolated from DNase I treated nuclei was fractionated through 1.2% agarose, blotted onto membranes, hybridized to a *Hae*III 2022 DNA probe, and exposed to film. Lanes correspond to those in (I). The hybridization signal corresponding to the *Bam*HI–*Hae*III fragment of the 5′ region of the *ipt* gene (see Figure 2) is the lowest band in the panel and is indicated by the arrow. The two upper bands indicated by the arrowheads correspond to *iaaM* sequences on either side of the *Bam*HI site within the truncated T-DNA.

tion-sensitive restriction endonucleases to define the states of *ipt* gene methylation in the lines used in this study (unpublished results). We have found that line CX2, which does not express the *ipt* gene, is methylated at all of the methylation-sensitive restriction endonuclease sites analyzed within the coding and near-upstream region of the *ipt* gene. Conversely, in the lines that express the *ipt* gene, cytosine methylation is entirely absent from these same sites. Furthermore, the high frequency of reactivation of silent T-DNA expression we observe in plant CX2 after treatment with the DNA methyltransferase inhibitor 5-azacytidine indicates that DNA hypermethylation is directly involved in suppression of *ipt* gene expression. Thus, DNA methylation may be a primary determinant of *ipt* expression and chromatin structure in these lines. Other studies have also suggested that DNA hyper-

methylation is linked to transcriptional inactivity of genes inserted into the plant genome [e.g., see Peerbolte et al. (1986), Schwartz and Dennis (1986), Amasino et al. (1984), and Hepburn et al. (1983)] although these studies did not address the relationship between DNA methylation and chromatin structure. It was recently reported that the DNase I sensitivity of T-DNA genes in transformed lines did not decrease with increasing levels of DNA methylation (Coates et al., 1987). However, in those lines studied, the complexity and copy number of T-DNA did not permit a comparison of the extent of methylation and transcriptional activity of individual T-DNA genes, and the authors suggested that all of the T-DNA genes studied were transcriptionally competent. Studies of the rRNA genes in pea have demonstrated that hypomethylation and enhanced general DNase I sensitivity of certain gene copies occur during different developmental stages and it is likely that both of these properties are exhibited by the same gene copies (Kaufman et al., 1987; Watson et al., 1987). In the lines studied herein, we demonstrate that for a single T-DNA gene, *ipt*, expression and DNase I sensitivity are associated with DNA hypomethylation.

Studies in animal systems have linked DNA methylation to decreased sensitivity of chromatin to DNase I and transcriptional inactivation of numerous genes [e.g., see Durrin et al. (1984), Mather and Perry (1983), and Groudine et al. (1981)]. Our results link DNA methylation and transcriptional inactivity to a DNase I insensitive chromatin conformation for a T-DNA gene in plants. The recent studies of Keshet et al. (1986) suggest that DNA methylation in animals can direct DNA sequences into a DNase I resistant conformation. Since plant genomes may contain much higher levels of DNA methylation than animal genomes (Gruenbaum, 1981), it will be of interest to understand possible similarities and differences in the mechanisms by which DNA methylation and gene regulation are related in plant and animal genomes.

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Registry No. DNase, 9003-98-9; isopentenyltransferase, 37277-78-4.

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