Nucleotide sequence of the tms genes of the pTiA6NC octopine Ti plasmid: Two gene products involved in plant tumorigenesis

(Agrobacterium tumefaciens)

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ABSTRACT

The nucleotide sequence of the tumor morphology locus, tms, from pTiA6NC has been determined. The sequence analysis indicates that each of two polyadenylated transcripts encoded by this locus contains an open reading frame; the predicted transcript 1 gene product has a molecular size of 83,769 daltons, and the predicted transcript 2 gene product, of 49,588 daltons. The precise start and stop positions of the transcript 2 RNA have been mapped with S1 nuclease. Several insertion mutations have been constructed. One of these localizes the transcript 2 promoter within the 72 base pairs 5' to transcription initiation. Significant homology was observed between the protein encoded by transcript 1 and the adenine binding region of p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens, suggesting that the transcript 1 protein binds adenine either as substrate or cofactor.

Crown gall is a neoplastic disease induced by Agrobacterium tumefaciens affecting most dicotyledonous plants (reviewed in refs. 1 and 2). Both the ability to transform plant cells and host range of the bacteria reside on a large plasmid, the Ti plasmid. The bacteria transfer a specific portion of the Ti plasmid to the plant cell, where this transferred DNA (T-DNA) is stably integrated into plant nuclear DNA. Axenic cultures of crown gall tissue exhibit autonomous growth in the absence of the phytohormones, auxin, and cytokinin. In addition, transformed tissue directs the synthesis of opiates, derivatives of certain amino acids that Ti plasmid-containing strains of A. tumefaciens can use as sole sources of carbon and nitrogen.

Tumors induced by the octopine-type Ti plasmid, pTiA6NC, or the closely related pTiB6S3 synthesize eight polyadenylated transcripts from genes entirely within the T-DNA (3, 4). The DNA sequences of two of these genes, neither essential for tumour formation, have been reported. The product of transcript 3 is responsible for synthesis of octopine by transformed plant tissue (5). The function of the protein encoded by transcript 7 is unknown (6). Five additional transcripts encoded from three distinct genetic loci appear to play a role in tumor morphology (7). Mutations in these loci result in synthesis of shoots (tms), synthesis of roots (tmr), or enlarged tumors (tml). The phenotypes of these different transformed tissues can be correlated with alterations in normal cytokinin/auxin ratio of the tissue (8) and can be corrected by addition of exogenous phytohormones (9). The function of the transcript 2 protein appears to be conversion of indole-3-acetamide to the auxin, indoleacetic acid (J. Schröder, personal communication). It seems likely that the other tumor morphology genes are also involved in phytohormone biosynthesis, although this conclusion is by no means certain. We have determined the nucleo-

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Fig. 1. Restriction endonuclease map of the tms region of pTiA6NC. Numbering of fragments is based on sizes in kb. The positions and directions of transcripts 1 and 2 as well as for the tmr gene product, transcript 4, are indicated by horizontal arrows. The exact positions of initiation and termination of transcription 1 have not been determined. Positions of insertions resulting in a tms morphology (A2100, 328, and 344) or a wild-type morphology (A2101, A2102, and 334) are indicated by vertical arrows.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England BioLabs or Bethesda Research Laboratories and used according to the manufacturers’ specifications. DNA polymerase I Klenow fragment was obtained from New England BioLabs. S1 nuclease was from Bethesda Research Laboratories. [α-32P]dNTPs were purchased from New England Nuclear, and nonradioabeled nucleotides were purchased from P-L Biochemicals. Isopropylthio-β-D-galactoside and 5-bromo-4-chloro-indolyl β-D-galactoside were from Bethesda Research Laboratories.

Culture Conditions. LB broth and agar were prepared as described by Miller (10). Agrobacterium strains were grown on AB minimal medium (11).

Nucleic Acid Sequence Determination. All plasmid and phage replicative form DNAs were prepared by the method

Abbreviations: T-DNA, DNA transferred from the Ti plasmid to a plant cell; orf, open reading frame; bp, base pair(s); kb, kilobase pair(s).

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of Clewell and Helsinki (12). The strategies used in the sequence determination are described elsewhere (13). Briefly, 100- to 300-base-pair (bp) fragments of T-DNA were generated by restriction endonuclease digestion or by sonication. These fragments were cloned into M13 mp7, mp8, or mp9 (14) and screened by plaque hybridization (15); their sequences were determined by the dideoxy methods described by Sanger et al. (16, 17). The results are shown in Fig. 1.

Isolation of RNA From Crowded Gall Tissue. The octopus crown gall line A62 (18) was grown as a suspension in hormone-free MS medium (19) and harvested in late logarithmic phase. Total RNA was prepared as described by Taylor and Powell (20) and polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography (21).

S1 Nuclease Mapping of Transcript 2. Unique single-strand-labeled probes spanning the 5' and 3' termini of transcript 2 RNA were prepared as described (13) by in vitro-primed synthesis of M13 cloned DNAs. Hybridizations and S1 nuclease digestions were carried out as described (13).

Isolation and Characterization of Mutations. Tn5 insertion mutations previously isolated in this laboratory (7), which are located in and around the tms locus, were accurately mapped (within 10 bp) by restriction endonuclease digestions. Additional mutations in and adjacent to transcript 2 were made in the following way: a clone of BamHI fragment 8 [the 7.6-kilobase-pair (kb) fragment in Fig. 1], in the Bgl II site of pRK290 (7), was partially digested with EcoRI. A gene encoding kanamycin resistance isolated from pUC4K

Fig. 2. Nucleotide sequence of transcript 1. The orf is shown in uppercase letters, and the amino acid sequence is shown below the nucleotide sequence.
containing EcoRI sticky ends was ligated into each of the EcoRI sites in the Bam 8 clone. After selection on kanamycin-containing medium, clones having the kanamycin-resistance gene inserted into either the left or right end of the 1.2-kb EcoRI fragment were identified. These mutations were introduced into pTiA6NC by the marker-rescue technique as described by Garfinkel et al. (7). Mutant strains were tested for virulence on Kalanchee diurantiana and Nicotiana tabacum as described (23).

RESULTS

DNA Sequence Determination. Mutational analyses using a variety of techniques have indicated that the tms locus in pTiA6NC covers approximately 5 kb (7, 24, 25). RNA blotting of polyadenylated transcripts has shown that the locus actually encodes two RNAs; transcript 1 (2.7 kb) and transcript 2 (1.75 kb) (Fig. 1). The sequence of the DNA of the region covering these two transcripts has been determined and is shown in Figs. 2 and 3. There are two orfs extending in opposite orientations out from the 0.42-kb HindIII fragment and separated by 250 bp. The first orf (tms1) encodes a protein with a subunit molecular weight of 83,769 and is consistent with the position and direction of transcription of transcript 1. Transcript 2 contains a second orf (tms2) encoding a protein with a subunit molecular weight of 49,588.

Amino Acid Sequence Homology. To obtain some insight into the functions of the tms gene products, a search for homology to known proteins was carried out using the protein sequence database of the Atlas of Protein Sequence and Structure (27). Tms1 displayed significant homology to p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens. Specifically, residues 239–263 of tms1 showed a high degree of identity to residues 5–29 of the hydroxylase (Fig. 4). Residues 5–31 of the hydroxylase have been determined by crystallographic analysis to form a pocket that binds the adenine moiety of FAD (28). It thus seems likely that tms1 also binds adenine in some form either as substrate or cofac-

![Image](Fig. 3. Nucleotide sequence of transcript 2. The transcribed nucleotides, as determined by S1 nuclease mapping, are indicated by upper-case letters. Numbers are given with respect to the start of transcription. Possible regulatory sequences are underlined with a single line. A sequence capable of forming an 8-bp hairpin structure in the mRNA is indicated by a heavy line. Asterisks indicate the two alternative transcription termination sites.)
No significant homology was found for the transcript 2 gene product.

Transcript Characterization. The 5' end of transcript 2 was precisely mapped by S1 nuclease protection of radiolabeled DNA prepared by in vitro fill-in of an M13 clone spanning this region. A 404-bp Pst I/EcoRI fragment (Fig. 1) was purified following primed radiolabeling of a single-stranded M13 template containing an insertion of the 1.1-kb EcoRI fragment. When the probe complementary to transcript 2 was hybridized to polyadenylated RNA prepared from plant tumors, a fragment of 338 bp was observed after S1 nuclease digestion (Fig. 5A). This fragment determines the 5' end of transcript 2 to be 16 bp 5' of the ATG translation initiation codon. When a probe of opposite orientation was used, no S1 nuclease-protected fragments were observed.

The 3' end of transcript 2 was determined in the same manner except that the labeled probe for protection was a Bgl II/HindIII fragment. In this protected fragments present in approximately equal amounts were observed at 313 bp and 323 bp (Fig. 5B). Thus, this RNA appears to be heterogeneous, ending 118 and 128 nucleotides beyond the termination codon (positions 1530 and 1540, respectively).

Mutational Analysis of the tms Region. Several Tn5 insertion mutations were previously mapped to the tms region (7). Since the DNA sequences of both the tms region and Tn5 (29) have been determined, it was possible to map the sites of insertion by restriction endonuclease digestion. Three mutations, 328, 334, and 344, were mapped in this way. Both 328 and 344 were localized within the orf of transcript 1 (positions 337 and 1934, respectively) and are tms mutants. Mutation 334 mapped 85 bp downstream from the tmsI orf (position 2531) and is wild type.

Because no transposon insertions in the 0.42-kb HindIII fragment were available and this restriction fragment likely contains the promoters for both tms transcripts, we made additional mutations by inserting an EcoRI fragment encoding kanamycin resistance into each of the two EcoRI sites located in the 7.6-kb BamHI fragment (Fig. 1). The leftward insertion, A2100, is within the orf of transcript 2 (position 982) and results in a tms phenotype. The rightward insertion is located 72 bp 5' to the start of transcript 2 (position -72).

Insertion of the kanamycin-resistance gene in either orientation (A2101 and A2102) results in a wild-type phenotype. These insertions thus appear to separate the two tms genes as well as localize the entire promoter for transcript 2 within the 72 bp between the EcoRI site and the start of transcription.

Analysis of Nontranslated Regions. We have observed a conserved 9-nucleotide sequence, T-T-T-C-A-A-G-A, located 100–200 nucleotides upstream from the start of transcription in a number of T-DNA genes including transcripts 3 (octopine synthase), 4, and 7 from pTiA6NC as well as nopaline synthase from the nopaline Ti plasmid, pTiF37 (13). Although the exact position of the start of transcription for transcript 1 is not known, a sequence closely resembling the consensus, T-G-C-A-T-T-A-G-A, appears 85 nucleotides upstream from the start of translation. In contrast, transcript 2 does not have anything resembling this conserved sequence in the proper orientation in the 5' nontranslated region. The upstream region of transcript 2 does contain a sequence homologous to the "TATA" box observed in most eukaryotic genes (30) as well as a sequence resembling the "CCAAT" box, usually located 5' to the TATA box (31).

Both of the tms transcripts are polyadenylated in plant cells. A search of the sequences 3' to the orfs of these two transcripts reveals sequences resembling the canonical polyadenylation sequence, A-A-T-A-A-A. Sequences with a five out of six base match appear in several places 3' to the orf in both transcripts. Two of these, T-A-T-A-A-A and A-A-T-A-A-T, occur immediately preceding the sites of termination in transcript 2. There is also a sequence located midway between translational and transcriptional termination (position 1459) that is a perfect 8-bp palindrome separated by one nucleotide. Such hairpin structures have been implicated in prokaryotic transcription termination but do not appear to be involved in eukaryotic termination (reviewed in ref. 32).

DISCUSSION

A. tumefaciens containing mutations in the tms locus induce tumors that have a proliferation of shoots on many host plants. These tumors have greatly reduced levels of auxins compared with tumors induced by the wild-type strain (8). At least some of these mutants contain elevated cytokinin levels as well. Addition of exogenous auxins to tumor tissue results in a return to the wild-type, unorganized phenotype.
T-DNA promoters for which sequences have been determined show decreased expression of this gene. However, the consensus sequence upstream of the transcript 2 is similar to the Pribnow box, found prior to the start of transcription in E. coli (35).

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