REGULATION OF T-DNA GENE 7

By

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We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August 1987

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ABSTRACT

The purpose of this study was two-fold. The first objective was to determine if *Saccharomyces cerevisiae* is a useful system for investigating the expression of T-DNA (it takes several months to obtain sufficient bacteria-free transformed plant tissue to investigate T-DNA transcription). A short fragment of T-DNA carrying T-DNA gene 7 was cloned into a yeast plasmid in an attempt to investigate the expression of gene 7 in yeast. The second objective was to determine the significance of a heat shock related sequence identified in the 5' region of T-DNA gene 7.

Primer extension analysis, S1 nuclease mapping, and Northern hybridizations indicate that transcription of T-DNA gene 7 in yeast is different from that of transcription of gene 7 in crown gall tumors. Transcription is different because the distance between the TATA box and the transcription initiation sites must be at least 40 nucleotides in yeast. Therefore, *Saccharomyces cerevisiae* does not appear to be a useful system for investigating the expression of T-DNA.

Crown gall tumors were subjected to a number of stress agents, including heat shock, to determine the significance of the heat shock related sequence identified in gene 7. Primer extension analyses indicate that only cadmium and mercury have a significant effect on the expression of T-DNA gene 7. Although gene 7 responds to cadmium and mercury, the increase in transcription does not appear to be heat shock or metallothionein related, indicating that another mechanism is involved in the enhanced transcription of T-DNA gene 7 in crown gall tumors.
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ACKNOWLEDGEMENTS

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ABBREVIATIONS USED

DNA - deoxyribonucleic acid

cDNA - complementary DNA

T-DNA - transferred DNA

RNA - ribonucleic acid

mRNA - messenger ribonucleic acid

rRNA - ribosomal ribonucleic acid

A or ATP - adenosine triphosphate

C or CTP - cytidine triphosphate

G or GTP - guanosine triphosphate

T or TTP - thymidine triphosphate

HS - heat shock

HSE - heat shock element

HSTF - heat shock transcription factor

Ti - tumor inducing

TRP2 - tryptophan gene 2

TRP3 - tryptophan gene 3

TRIS - tris (hydroxymethyl) aminomethane

EDTA - ethylene diamine tetraacetic acid

SDS - sodium lauryl sulphate

DNP - dinitrophenol

TBE - tris-borate-EDTA

DTT - dithiothreitol

BSA - bovine serum albumin

lambda - lambda virus
INTRODUCTION

The molecular basis for crown gall, a neoplastic transformation of plant cells, involves the transfer and integration of a segment of the Agrobacterium tumefaciens Ti plasmid (T-DNA) into plant nuclear DNA (Chilton et al., 1977; Thomashow et al., 1980; Zambryski et al., 1980). Ti plasmids are classified according to the novel amino acids (opiines) produced in the plant tumors (Sciaky et al., 1978). The T-DNA of nopaline tumors is integrated into plant DNA as a contiguous 22 kbp segment (Leemans et al., 1982). T-DNA from octopine Ti plasmids contain a contiguous 13 kbp segment from the left side of the T-region, TL-DNA (Leemans et al., 1982). Some tissue culture lines contain an 8 kbp fragment from the right side (TR-DNA) of the octopine T-region.

TL-DNA in octopine tumors encodes several polyadenylated transcripts whose synthesis is sensitive to amanitin (Willmitzer et al., 1981). These findings suggest that T-DNA genes are transcribed by the host RNA polymerase II. The 5' flanking regions of these genes have some characteristics in common with other eukaryotic genes. Most T-DNA genes contain a Goldberg-Hogness (TATA) box at a position similar to that of other eukaryotic genes (Barker et al., 1983; de Greve et al., 1983; Joos et al., 1983; Klee et al., 1984; Lichtenstein et al., 1984). A TATA box in the 5' region of T-DNA gene 7 maps 30 bp upstream from the mRNA start site (McPherson, 1984). Studies have indicated that the TATA box is involved in specifying the site of mRNA initiation, but sequences upstream of the TATA box determine both qualitative and quantitative aspects of transcription (Breathnach and Chambon 1981; McKnight et al., 1981; McKnight et al., 1984; Wright et al., 1984). The deletion or mutation of the TATA box results in a decrease in transcription initiation, and the initiation of transcripts at new sites (Osborne et al., 1982; Schulz et al., 1982; Orkin
et al., 1983). CAAT sequences have been also identified in some T-DNA genes, 30 to 50 bases upstream of the TATA box (Barker et al., 1983). However, no CAAT sequence is observed in gene 7.

The 0.7 kb transcript is rather abundant compared with the other mRNAs specified by octopine TL-DNA (Gelvin et al., 1982; Willmitzer et al., 1982). The function of T-DNA gene 7 with regards to crown gall tumorigenesis is unknown, whereas specific roles have been assigned to most of the other TL-DNA encoded genes (Garfinkel et al., 1981; Leemans et al., 1982).

The difficulty with studies of T-DNA is that it takes several months to obtain sufficient bacteria-free transformed plant tissue to investigate its transcription and it would be convenient to have a rapid system in which to investigate expression of T-DNA sequences. *Saccharomyces cerevisiae* is an excellent system for investigating the expression of cloned eukaryotic genes and the functional expression of several foreign genes has been reported (Henikoff et al., 1981; Jacquet et al., 1982). Most of these studies have involved DNA from higher animals or animal viruses (Hitzeman et al., 1981; Mellor et al., 1983; Stepien et al., 1983; Tuite et al., 1982; Valenzuela et al., 1982); however, experiments have shown that products encoded by plant genes can also be synthesized and processed in yeast (Edens et al., 1984; Rothstein et al., 1984). A short fragment of the T-DNA carrying T-DNA gene 7 was cloned into the multicopy yeast plasmid, YEpl3 (a non-expression vector), in an attempt to investigate the expression of gene 7 in yeast.

In addition to the TATA box, a heat shock related sequence was identified in T-DNA gene 7, 40 nucleotides upstream of the TATA box. In most organisms, heat shock genes contain DNA sequences homologous to the heat shock element, as defined by Pelham (1982), in their promoter regions. The heat shock consensus sequence, 5' CTgGAAtnTTTcAg, is active in controlling the heat shock regulated
expression of foreign genes even when synthetic promoter elements are used which only have an 8 of 10 match with the HSE consensus sequence (Pelham and Bienz, 1982). The heat shock related sequence of T-DNA gene 7 (CTTGAAAATTAAGC) has a 7 of 10 match with the consensus sequence.

The heat shock elements of the Drosophila melanogaster hsp 70 and hsp 83 are protected from nucleases in the nuclei of heat shocked cells (Wu, 1984a; Wu, 1984b). The HSEs of hsp 70 are the binding sites for a Drosophila heat shock transcription factor (HSTF) that is specifically required for heat shock protein gene transcription in vitro (Parker and Topol, 1984; Top et al., 1985). Recent evidence also suggests that the number of heat shock elements can be a major determinant of the promoter strength of heat-inducible genes in mammalian cells (Kay et al., 1986).

The heat shock response of plants is similar to the response observed in bacteria, fungi, insects, and mammals (Craig, 1985). Features of the heat shock response that are conserved across a broad spectrum of organisms include: (1) the sequences of the 5' flanking regions of the HS genes which appear to regulate expression of these genes during heat shock, (2) the rapid switch of the cell's transcriptional and translational machinery to the production of HS mRNAs and HS proteins, and (3) the structural features of the HS proteins which may be important for their role in thermal tolerance (Kimpel and Key, 1985). However, the HS response of plants is easily distinguished from other organisms by the complexity and relative abundance of the low molecular weight HS proteins (15 to 18 Kd) (Lin et al., 1984). The significance of this observation is not yet known. It is interesting to note that T-DNA gene 7 encodes a low molecular weight protein (14Kd).

From a physiological point of view, the only biological function assigned to HS proteins to date is their possible role in mediating the expression of
thermal tolerance (Kimpel and Key, 1985). The heat shock response is thought to contribute to homeostasis with the heat shock proteins having a protective role presumed to counteract or prevent deleterious effects induced by heat shock (Ashburner and Bonner, 1979; Schoffl et al., 1984; Velasquez and Lindquist, 1984). In soybean seedlings, an absolute correlation has been found between the synthesis and accumulation of HS proteins and the ability to survive short heat treatments at otherwise lethal temperatures (Kimpel and Key, 1985). Although the exact functions of the plant heat shock proteins remain unidentified, it is known that at least one heat shock protein is transported into the chloroplast during heat shock (Vierling et al., 1986).

While many aspects of the HS response of plants have been investigated, there is little information on the mechanisms of regulation of the response. Results from other organisms including soybeans indicate that during HS, normal cellular mRNAs persist in the cells but are translated very inefficiently if at all (Schoffl and Key, 1982). In soybean seedlings, the level of actin mRNAs remains unchanged during HS, but the mRNA levels of an auxin regulated gene decrease dramatically during HS (Schoffl and Key, 1982). Since the total poly (A) RNA content remains fairly constant and since HS mRNAs represent over 20% of the total poly (A) RNA pool during HS, the concentrations of many normal poly (A) RNAs must decline (Schoffl and Key, 1982). Expression of the heat shock proteins in plants and other organisms is dependent on the rapid and coordinate onset of HS protein gene transcription (Kimpel and Key, 1985).

By definition, heat shock proteins are a new set of proteins rapidly and abundantly produced in response to a heat shock, but in many organisms other stresses, such as ethanol, anoxia, arsenite, or heavy metal ions will also induce the synthesis of heat shock proteins. The Drosophila system responds to a wide range of stress agents (e.g., DNP, arsenite, release from anoxia) by altered puffing patterns (Ellgaard, 1972) and synthesis of HS proteins.
(Ashburner and Bonner, 1979). Arsenite and heavy metals seem to induce a set of proteins similar to HS proteins in a number of systems (Ashburner and Bonner, 1979; Johnston et al., 1980; Levinson et al., 1980). Soybean seedlings respond to stresses such as arsenite and cadmium as well as heat shock, although induction of heat shock proteins under other stress conditions was not detected (Czarnecka et al., 1984). It is not understood why arsenite and cadmium in particular induce the heat shock proteins in soybean while many other stresses, which are effective in many other organisms, do not appear to do so.

The possibility of the conserved heat shock sequence of T-DNA gene 7 having significance in its response to stress related stimuli was investigated.
MATERIALS AND METHODS

REAGENTS

Enzymes

Restriction endonucleases were purchased from New England Biolabs (NEBL) or Bethesda Research Laboratories (BRL). T4 DNA ligase, T4 polynucleotide kinase, RNase T1, and DNA polymerase I (Klenow fragment) were also supplied by BRL. Proteinase K and S1 nuclease were from Boehringer Mannheim. Lysozyme was supplied by Sigma, reverse transcriptase was from Pharmacia, and Glusulase was from Endo laboratories. DNase I and DNA polymerase I were supplied by Amersham (Nick Translation Kit).

Conditions for each enzyme are described later in this section.

Nucleotides

2'-Deoxyribonucleotide triphosphates were supplied by Pharmacia. dNTPs were dissolved in water to a concentration of 10 mM. The pH was adjusted to pH 7.0 by adding a dilute solution (0.05 M) of Tris base. The exact concentration of each stock was determined spectrophotometrically. Small aliquots were frozen at -70°C.

α[32P]-2'-deoxyribonucleotide triphosphates and γ[32P]-adenosine triphosphate were supplied as aqueous solutions containing 10 uCi/ul. Vanadyl ribonucleoside complexes (0.2 M) were purchased from BRL.

Phenol

Ultra Pure Phenol was supplied by BRL. Phenol was equilibrated with either 0.1 M Tris (pH 8.0) or sterile H2O.
Chloroform

Chloroform was supplied by BDH Chemicals.

Formamide

Ultra Pure Formamide was purchased from BRL. Formamide was deionized by mixing 50 ml with 5 g of mixed bed, ion-exchange resin (Bio-Rad AG 501-X8-10) for 2 hours at room temperature. The mixture was filtered twice through Whatman No. 1 filter paper and dispensed into 1 ml aliquots and stored at -20°C.

Formaldehyde

Formaldehyde was supplied by BDH Chemicals. The pH of this solution was 3.8. The solution was deionized with AG 501-X8-D mixed bed resin. The pH was then raised to 6.8.

Agarose

Ultra Pure Agarose was purchased from BRL.

Acrylamide

Acrylamide and bis-acrylamide were supplied by Bio Rad.

Culture Media

Bacto-tryptone, Bacto-yeast extract, Bacto-peptone, and Bacto-yeast nitrogen base without amino acids were supplied by Difco. Amino acids were from Sigma. Ampicillin, tetracycline, and chloramphenicol were purchased from Sigma. Murashige and Skoog plant salt medium (MS media) was obtained from Flow Laboratories.
Others

All other chemicals used were of reagent grade.

MICROBIAL STRAINS

Bacteria

The following strains of E. coli were used as hosts for recombinant DNA molecules.

E. coli RR1, F', hsdS20, ara-l4, po A2, lacY, gal Ks, rspsL20, Xyl-5, mll-l, sup E44, lambda-, was constructed by Bolivar et al. (1977).

E. coli JM 101, (lac, pro), sup E, thi, strA, sbcBl5, end A, hspR4, F'traD36, proAB, lacI, lacZ M15, was constructed by Messing (1981).

Yeast

Strain GM-3C-2 was described by Fay et al. (1981). Its genotype was alpha, leu2-3, 112, trp 1-1, his 4-519, cycl-1, cyp3-1.

TOBACCO TISSUE CULTURE LINES

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<th>Tumor line</th>
<th>Inciting plasmid</th>
<th>N. tobacum cultivar</th>
<th>Octopine biosynthesis</th>
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<td>A6S/2</td>
<td>pTiA6</td>
<td>White Burley</td>
<td>+</td>
<td>(Gelvin et al., 1982)</td>
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<td>E9</td>
<td>pTiB 806</td>
<td>Xanthic nc.</td>
<td>+</td>
<td>(Gelvin et al., 1982)</td>
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<tr>
<td>16-12-C</td>
<td>pTL1</td>
<td>Xanthic nc.</td>
<td>-</td>
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CULTURE MEDIA AND CONDITIONS

E. coli

The following media were used for the growth of E. coli:

LB- 1.0% Bacto-tryptone, 0.5% Bacto-yeast extract,

0.5% NaCl, 0.1% glucose.
M9-50 mM \( \text{Na}_2\text{HPO}_4 \), 25 mM \( \text{KH}_2\text{PO}_4 \).

0.1 mM \( \text{CaCl}_2 \), 10 mM glucose,

0.001% thiamine.

For plates, Bacto-agar was added to the appropriate liquid medium at 20 g/l. \( \text{E. coli} \) was cultured at a temperature of 37°C. Growth was monitored by measuring the optical density at 600 nm. Antibiotics were added to the appropriate concentrations.

Yeast

Media for the culture of yeast have been described by Sherman et al. (1981). The following were used in this study:

YPD - 2% Bacto-peptone, 1% Bacto-yeast extract, 2% glucose, pH 5.8.

YNB - 0.7% Bacto-yeast nitrogen base without amino acids, 2% glucose, pH 5.8. YNB was supplemented with tryptophan (20 mg/l) and histidine (20 mg/l).

For plates, agar was added to a concentration of 2% to the above media.

Regeneration Agar - YNB supplemented with 1 M sorbitol, 2% YPD, and 3% agar.

Yeast cultures were grown at 30°C with mild shaking.

Tissue Cultures

The tobacco crown gall tumors lines A6 and E9 were grown in Murashige-Skook (MS) medium (Murashige et al., 1962) at 25°C in the absence of plant growth hormones. Transformed tobacco cultures (16-12-C) were grown in MS media containing plant hormones, naphthalene acetic acid (2 mg/L) and benzylaminopurine (0.2 mg/L), at 25°C. Stress inducing compounds were added at the appropriate concentrations.
PLASMIDS AND BACTERIOPHAGES

Plasmids and bacteriophages provided by other workers are listed in Table I.

Table I

<table>
<thead>
<tr>
<th>Name</th>
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<td>pBR322</td>
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<td>R. Kay (U.B.C.)</td>
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<td>J. McPherson (U.B.C.)</td>
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<tr>
<td>YEp13</td>
<td>Broach et al. (1979)</td>
<td>J. Ngsee (U.B.C.)</td>
</tr>
<tr>
<td>HpaII 0.8</td>
<td></td>
<td>J. McPherson (U.B.C.)</td>
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TRANSFORMATION OF E. COLI

E. coli was transformed by a modification of the procedure of Mandel and Higa (1970). One bacterial colony was picked and inoculated in 5 ml of LB-glucose media. The bacterial cells were grown overnight at 37°C with mild shaking. The overnight culture (0.5 ml) was inoculated into 50 ml of LB-glucose media and incubated at 37°C with shaking to a density of approximately 5 x 10^7 cells/ml (A_{600} = 0.25). The culture was chilled on ice for 10 minutes and then centrifuged at 4000 g for 5 minutes at 4°C. The supernatant was discarded and the remaining cells were resuspended in 0.25 ml of 100 mM CaCl_2 and 10 mM Tris-Cl (pH 8.0) and incubated at 0°C for 1 hour. The cell suspension was divided into 0.1 ml aliquots and 16 ul of sterile glycerol was added to the mixture. The cell suspensions were stored at -70°C and removed from the freezer as needed.

Plasmid DNA (40-100 ng in of 10-100 ul of H_2O) was added to 0.1 ml of the thawed cell suspension on ice. The mixture was incubated on ice for 15 minutes and then incubated at 37°C for 5 minutes. LB-glucose media (1 ml) was added and
the mixture was incubated for 2 hours at 37°C without shaking. Aliquots (50 ul to 200 ul) were spread on selective media. The plates were incubated at 37°C overnight.

LARGE SCALE ISOLATION OF PLASMID DNA

This procedure was described by Maniatis et al. (1982) and is a modification of the method of Birnboim and Doly (1979). The bacterial pellet from a 500 ml culture was resuspended in 10 ml of a solution containing 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 5 mg/ml lysozyme and was incubated for 5 minutes at 4°C. A solution consisting of 0.2 N NaOH and 1% SDS (20 ml) was added to the cell suspension and the contents of the tube mixed by gentle inversion. The suspension was then incubated on ice for 10 minutes. An ice-cold solution of 3 M potassium acetate and 2 M HOAc (15 ml) was added and the contents were mixed. The mixture was incubated on ice for 10 minutes and then centrifuged at 20,000 rpm for 20 minutes at 4°C. The supernatant was removed and transferred to 30 ml Corex tubes. Two volumes of 95% ethanol were added to each tube and incubated at -20°C for one hour. The DNA was recovered by centrifugation at 10,000 g for 20 minutes at room temperature. The DNA pellet was washed with 70% ethanol and air dried. The pellet was dissolved in TE (pH 7.6) and purified by centrifugation in cesium chloride-ethidium bromide density gradients.

PURIFICATION OF PLASMID DNA BY CESIUM CHLORIDE GRADIENT CENTRIFUGATION

Plasmid DNA was purified according to Maniatis et al. (1982). For every milliliter of plasmid DNA isolated, exactly 1 g of cesium chloride was added. Ethidium bromide (0.8 ml of 10 mg/ml in H₂O) was added for every 10 ml of cesium chloride solution. The cesium chloride solution was transferred to a
tube suitable for centrifugation in a Beckman type 50 rotor. The remainder of the tube was filled with paraffin oil and the tubes were sealed using the Beckman tube sealer. The tubes were spun at 50,000 rpm for 40 hours at 20°C. The tubes were mounted on a stand and illuminated with an ultraviolet lamp. Two bands were normally visible, and the lower band containing plasmid DNA was collected with an 18 gauge hypodermic needle. Ethidium bromide was removed from the solution by repeated extractions with n-butanol equilibrated with 20XSSC. The solution was diluted with two or three volumes of H₂O and 2 volumes of ethanol were added to precipitate the DNA.

The mixture was incubated at -20°C overnight and spun down at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. The pellet was resuspended in 10 mM Tris-Cl (pH 7.6) and 1 mM EDTA (pH 8.0).

SMALL SCALE ISOLATION OF PLASMID DNA

This procedure, described by Maniatis et al. (1982), is a modification of the method of Birnboim and Doly (1979). LB-glucose media (5 ml) containing either ampicillin (50 ug/ml) or tetracycline (15 ug/ml) was inoculated with a single bacterial colony and grown overnight at 37°C with mild shaking. A 1.5 ml portion of the culture was poured into a microfuge tube. The tube was centrifuged for one minute and the supernatant was poured off. The remaining pellet was resuspended in 100 ul a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH 8.0), and lysozyme 4 mg/ml. The mixture was incubated at room temperature for 5 minutes.

A solution containing 0.2 N NaOH and 1% SDS (200 ul) was added and mixed without vortexing. The solution was stored on ice for 5 minutes. A 150 ul solution of potassium acetate was added and the mixture was stored on ice for
5 minutes. The mixture was centrifuged in a microfuge for 5 minutes at 4°C and the supernatant was transferred to a new tube. An equal volume of phenol/chloroform was added and mixed by vortexing. The tube was centrifuged at room temperature for one minute and the supernatant was transferred to a new tube. Two volumes of ethanol were added and the solution was incubated at -20°C for one hour. The tube was then centrifuged for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air dried and redissolved in 10 mM Tris-Cl (pH 7.6) and 1 mM EDTA (pH 8.0).

**RESTRICTION ENDONUCLEASE DIGESTION**

Restriction endonucleases used in this work were Hind III, Bgl II, Eco RI, Bam HI, PvuII, and Eco RV. A 10X buffer solution consisting of 500 mM Tris-Cl (pH 8.0) 100 mM MgCl$_2$, 500 mM NaCl was used for each enzyme reaction. Reactions typically contained between 0.1 and 1 ug of DNA in a total volume of 50 ul. The DNA to be digested was dissolved in 10 mM Tris-Cl (pH 7.6) and 1 mM EDTA (pH 8.0). The 10X buffer concentrate was added (one-tenth of the final volume of the mixture) and the final volume was brought up to 50 ul with sterile H$_2$O. About 1 to 5 units of the appropriate restriction endonuclease were added per ug of DNA. The mixture was then incubated at 37°C for approximately 2 hours. The extent of the digestion was checked by agarose gel electrophoresis.

If the DNA was to be used in a subsequent reaction (a ligase reaction or another restriction digestion), the DNA was purified by adding an equal volume of phenol. The aqueous layer was removed and placed in another microfuge tube. An equal volume of chloroform was added and the aqueous layer was transferred to another microfuge tube. Sodium acetate was added to 0.3 M and
the DNA was precipitated by adding 2 volumes of ethanol. The mixture was incubated at -20°C for 1-12 hours. The precipitate was collected by centrifugation for 10 minutes at 4°C. The ethanol was removed and approximately 1 ml of 70% ethanol was added to the pellet. The mixture was then vortexed and centrifuged for 5 minutes. The ethanol was removed and the pellet was air dried for one hour. The pellet was then dissolved in water or Tris-Cl.

LIGATIONS

Ligation reactions were done in a total volume of 20 ul using 10 to 50 ng of vector DNA and a three to ten-fold molar excess of insert fragment. Fragments were ligated in a buffer containing 66 mM Tris-Cl, 10 mM MgCl₂, 10 mM DTT, and 1.0 mM ATP. T4 DNA ligase enzyme was added (0.1 unit for overhanding-end ligations and 1 unit for blunt end ligations) and the mixture incubated at 4°C overnight. The ligation mixtures were used without further treatment to transform E. coli.

AGAROSE GEL ELECTROPHORESIS OF DNA

Agarose gels (0.7% in 1 TBE) were prepared for electrophoretic fractionation of DNA fragments. Electrophoresis was carried out horizontally at a voltage gradient of 2-5 V/cm.

TRANSFORMATION OF YEAST

The procedure used for yeast transformation was similar to those described by Hinnen et al. (1978), Beggs (1978), Sherman et al. (1981), and Orr-Weaver et al. (1983). A single colony of the yeast strain to be transformed was used to inoculate 5 ml of YEPD, and the culture was incubated at
30°C overnight with mild shaking. One ml of the overnight culture was inoculated into 80 ml of YEPD. The yeast were then grown with mild shaking for approximately 8 hours (O.D.₆₆₀ = 0.33 - 0.35). The cells were harvested by centrifugation at 3,000 rpm for 2 minutes at room temperature. The pellets were resuspended in 2.5 ml of 1 M sorbitol per tube and centrifuged as before. The cells were then resuspended in 2.5 ml of 1 M sorbitol per tube and 10 mg of DTT and 100 ul of glusulase were added to each tube. The suspension was incubated at 30°C with mild shaking, and 5-10 ul samples were removed periodically, diluted with 50 ul H₂O and examined under a phase-contrast microscope. Spheroplasts are non-refractile and appear dark under the phase-contrast microscope, while normal cells are refractile. More glusulase was added if the spheroplasts had not been formed after 1.5 hours.

When the cells had been converted to spheroplasts, they were harvested by centrifugation at 2,000 rpm for 5 minutes at room temperature. The pellet was washed by resuspending the cells in 2.5 ml of 1M sorbitol per tube and centrifuged as before. The cells were resuspended in 2.5 ml of STC (1 M sorbitol, 10 mM Tris-Cl, 10 mM CaCl₂) per tube and centrifuged at 2,000 rpm for 5 minutes. The cells were resuspended in 0.2 ml of STC per tube.

Approximately 50 ul of plasmid (1 ug) was added to 50 ul of competent yeast cells. The mixture was incubated on ice for 15 minutes, and 0.5 ml of PEG-TC (20% polyethylene glycol, 10 mM Tris, 10 mM CaCl₂, pH 8.0) was added to each tube and incubated on ice for 20 minutes. The entire solution containing the DNA was added to a sterile centrifuge tube, and 0.3 ml of YEPD and 15 ml of regeneration agar (50-55°C) was added to each tube. The solution was then immediately poured on a YNB plate containing the appropriate amino acids. Transformants gave rise to colonies embedded within the regeneration agar which were easily visible after 2-3 days of incubation at 30°C.
YEAST PLASMID PURIFICATION

Plasmids were isolated from yeast as described by Lorincz (1984). A medium sized yeast colony (approximately 3 mm in diameter) was picked and placed in a microfuge tube containing 200 ul of 100 mM CaCl₂, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.1% SDS. Glass beads (0.45 mm diameter) were added to just below the level of the liquid and the contents mixed vigorously on a vortex mixer for 1 minute. The suspension was extracted with an equal volume of Tris-buffered phenol and then extracted with an equal volume of chloroform. Sodium acetate was added to 0.3 M, and 2 volumes of cold ethanol were added. After 30 minutes at -60°C, nucleic acid was precipitated by centrifugation (10 minutes). The air dried pellet was resuspended and the entire DNA solution was used to transform E. coli (0.2 ml suspension).

ISOLATION OF YEAST RNA

Yeast RNA was isolated as described by McNeil and Smith (1986) with a few modifications. A single colony of yeast was used to inoculate 5 ml of YNB plus the appropriate amino acids. The culture was grown with mild shaking for 1-2 days and 1 ml of the culture was inoculated into 100 ml of fresh YNB media. The culture was then incubated at 30°C with mild shaking until its A₅₃₀ was 0.8. Cycloheximide was added to a concentration of 0.1 mg/ml and the culture was incubated at 30°C for 5 minutes with mild shaking. The culture was then poured into two 520 ml centrifuge bottles, each half-full of crushed ice. The chilled cells were transferred to centrifuge tubes and centrifuged at 4000 rpm for 1 minutes at 40°C. The cells were resuspended into about 10 ml of ice cold H₂O and cycloheximide (0.1 mg/ml) per tube. The mixture was transferred to a weighed 30 ml Corex tube and the cells were pelleted at 4000 rpm for 3 minutes at 4°C. The cells were then immediately frozen in a dry ice/ethanol bath.
Acid-washed glass beads were added to the frozen cell pellets (3g beads/g wet weight cells) followed by 3 ml/g of ice-cold RNA extraction buffer (0.15 M NaCl, 0.1 M Tris-HCl) and 50 ul/g of Vanadyl ribonucleoside complexes (VRC: 0.2 M). The cells were broken by vortexing hard for six 15-second intervals, each followed by 45 seconds of cooling on ice. The mixture was centrifuged at 9,000 rpm for 5 minutes at 40°C. The supernatent was transferred to a sterile 30 ml Corex tube and placed on ice. Extraction buffer and VRC were added to the pellet as before and vortexed. The cells were centrifuged as before and the supernatant was removed and combined with the previous supernatant. SDS and proteinase K were added to concentrations of 0.5% and 0.5 mg/ml, respectively. The mixture was incubated in a 37° water bath for 60 minutes.

The proteinase K-digested solution was extracted with 1 volume of phenol/chloroform/amyl alcohol (24/24/1). The aqueous supernatant was transferred to a clean tube and nucleic acid was precipitated by adding sodium acetate to 0.3 M, 2.5 volumes of ethanol, and chilling at -20°C overnight. The precipitate was collected by centrifugation (9,000 rpm, 20 minutes, 40°C) rinsed in cold ethanol, dried and dissolved in 1 ml of 20 mM EDTA. An equal volume of 4M LiCl was added and the mixture was incubated at 4°C for 16 hours. The RNA precipitate was collected by centrifugation (9,000 rpm, 40 minutes, 4°C) and dissolved in sterile H₂O. A final ethanol precipitation from 0.3M sodium acetate, followed by rinsing with ethanol served to desalt the RNA. The precipitate was air dried, dissolved in H₂O and stored at -20°C. Up to 5 mg of RNA was obtained from 1 g (wet weight) of cells.

**SELECTION OF POLY A+ RNA**

Polyadenylated RNA molecules were isolated by chromatography using oligo dT cellulose by a modification of the procedure of Aviv and Leder (1972).
Oligo dT cellulose was equilibrated with RNA loading buffer (0.5 M NaCl, 10 mM Tris, 4 mM EDTA) and the mixture was applied to a small sterile column with a built-in-filter. RNA (100 ug aliquots) was loaded in 3 ml of loading buffer. The run-through was collected and reapplied to the column. The RNA which ran through the column was non-polyadenylated. The column was washed with 5 ml of loading buffer and 2 ml of 0.5 M KCl. The polyadenylated RNA was eluted by applying 200 ul of sterile H2O 5 times and collecting the fractions. The fractions were then pooled, precipitated in ethanol, and dissolved in sterile H2O. RNA was stored at -20°C.

End-labeling of oligonucleotides

The oligonucleotide (10 pmoles) was 5'-ended labeled in a reaction (50 ul) containing [gamma-32P]ATP (120 Ci of 3000 Ci/mmole), polynucleotide kinase (10 units), 50 mM Tris-HCl, pH 7.7, 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM spermidine, for 45 minutes at 37°C. The reaction was terminated by addition of 2 ul of 0.5 M EDTA and heating to 67°C (10 minutes).

The labeled oligonucleotide was separated from unincorporated (gamma-32P) ATP by chromatography through a column of Sephadex G-25. Approximately 8 grams of Sephadex G-25 and 100 ml of H2O (this was enough for 4 columns) were autoclaved for 15 minutes. The mixture was cooled down to room temperature, the water was poured off, and 40 ml of column buffer [10 mM Tris (pH 8.0), 5 mM EDTA] was added. The columns were poured slowly and continuously. Approximately 200 ul of column buffer was added to the oligonucleotide solution and applied to the top of the column. Column buffer was added as needed and fractions were collected in microfuge tubes. The leading peak of radioactivity which contained the labeled oligonucleotide was pooled and stored at -20°C.
**PRIMER EXTENSION ANALYSIS**

The oligonucleotide was desalted and hybridized [approximately 1 ng ($10^5$ cpm) per reaction] with samples of RNA. Annealing was performed at 65°C in 250 mM KCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Following hybridization to specific RNA, the oligonucleotide primer was extended using Avian myeloma virus reverse transcriptase using the conditions described (McKnight et al., 1981). The reaction products were denatured and the lengths of the extended products estimated by denaturing acrylamide gel electrophoresis.

**DENATURING UREA-ACRYLAMIDE GEL**

Denaturing acrylamide gels (6% and 8%) containing 7 M urea were prepared in 1 X TBE. Samples were denatured in formamide (100°C, 4 minutes) prior to loading and electrophoresis (30-40 V/cm).

**SI NUCLEASE MAPPING**

DNA fragments were $^{32}$P-labelled at the 5' end using [gamma-$^{32}$P] ATP and T4 polynucleotide kinase. Stand separation was accomplished as described (Maxam and Gilbert, 1980), using acrylamide concentrations varying between 3% and 7% according to the fragment molecular weight.

Single strands were eluted from the gel in 50 mM Tris pH 8, 20 mM EDTA, 0.5 M NaCl overnight at room temperature. The single stranded DNA probes were hybridized to RNA and subsequently digested with SI nuclease as described (De Greve et al., 1982).

**GEL ELECTROPHORESIS OF RNA AND NORTHERN HYBRIDIZATION**

RNA was analyzed by electrophoresis in formaldehyde-agarose gels after denaturation with formaldehyde and formamide (Lehrach et al., 1977; Maniatis
et al., 1982). As much as 30 ug of RNA was denatured in a volume of 50 ul containing 2.2 M formaldehyde, 50% formamide, and 1/2 MOPS buffer (1X MOPS buffer contained 40 mM Na MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.9) by heating at 55°C for 15 minutes. Sample loading buffer (2 ul) was added and the RNA samples were loaded on a 1.1% agarose gel. The gel was made up in 1X MOPS buffer containing 2.2 M formaldehyde and the gel was run in 1X MOPS at 0.5 - 1 V/cm for 6-12 hours. After electrophoresis, the gel was soaked in sterile H₂O for 10 minutes and then soaked in 20X SSC (1X SSC is 0.15 M NaCl, 0.15 M Na citrate) for 30 minutes prior to transferring the RNA to nitrocellulose.

The procedures of Thomas (1980) were used to transfer RNA from agarose gels to nitrocellulose and hybridize the RNA to radioactively labelled probes. The Northern hybridization apparatus was cleaned out with sterile H₂O and the chamber was filled with 20X SSC. Two pieces of filter paper soaked in 20X SSC were laid across the box so that the papers just touched the bottom of each chamber. The gel was then laid on the filter paper, and cut X-ray film was placed around the gel so that no liquid diffused out of the gel. A piece of nitrocellulose, which had been soaked in sterile H₂O for one minute and then soaked in 20X SSC for one minute, was placed on top of the gel. The agarose not covered by nitrocellulose was removed by a razor blade. Two more pieces of Whatman 3 MM paper were placed on top of the nitrocellulose, followed by a 6 cm stack of paper towels. A book was placed on top of the assemblage and transfer of RNA to the nitrocellulose was allowed to proceed for about 16 hours. The nitrocellulose was then baked at 80°C for 2 hours.

Nitrocellulose filters with bound RNA were prehybridized for 4-16 hours at 42°C in sealed plastic bags containing 10 ml of a mixture of 50% formamide, 5X SSC, 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA) 50 mM NaH₂PO₄ and Na₂HPO₄, pH 7.0, and denatured, sheared salmon sperm
DNA (250 ug/ml). The prehybridization mixture was removed and replaced with a hybridization mixture (10 ml) which contained the radioactively-labelled probe. The hybridization mixture was composed of four parts of prehybridization mixture and one part of 50% dextran sulfate. The probe was boiled for 5 minutes and chilled on ice before adding it to the hybridization mixture. Hybridization was allowed to proceed for about 18 hours at 42°C. The filter was washed in 2 changes of 0.1% SDS, 2X SSC at room temperature for 5 minutes each time. The filter was then washed in two changes of 0.1X SSC, 0.1% SDS at 55°C for 20 minutes each. The filter was subjected to autoradiography.

**NICK TRANSLATION**

Nick translation of DNA was done using the Amersham Nick Translation Kit. Typically 500 ng of DNA was radioactively labeled to a specific activity of $4 \times 10^7 - 10 \times 10^7$ cpm/ug DNA in a 50 ul reaction containing 10 ul of nucleotide buffer (dGTP, dCTP, dTTP, 50 uCi of [alpha-$^{32}$P] ATP, and 5 ul of an enzyme solution containing DNA polymerase I and DNase I. The reaction was incubated at 15°C for 2 hours. Unincorporated nucleotides were removed by running the solution through an Ultrogel column.

**ISOLATION OF PLANT TUMOR RNA**

RNA was extracted from total callus tissue by a modification of the procedure described by McPherson et al. (1980). Callus tissue (50 g fresh weight) was frozen in liquid nitrogen and ground in a blender. The finely divided tissue (frozen powder) was extracted by homogenization (2 minutes) in buffer (100 ml) containing 1 M Tris, 10% SDS, 10% Triton, and 0.5 mg/ml Heparin. The cell debris were precipitated following centrifugation at 4°C (10,000 g for 5 minutes).
The supernatant was filtered through cheesecloth and extracted with phenol: chloroform (1:0.5). The aqueous layer was re-extracted with phenol: chloroform and then with chloroform. Nucleic acids were recovered by ethanol precipitation (2.5 volumes) at -20°C overnight. The pellet was reconstituted in 0.1 M NaOAc to which an equal volume of LiCl (5 M) was added. RNA was recovered by precipitating with LiCl (2.5 M) at -20°C overnight. The resulting RNA was precipitated from ethanol (2.5 volumes).

**STRESS INDUCTION OF PLANT TUMORS**

Crown gall tumors and transformed tobacco cultures (16-12-C) were subjected to various stress treatments. Cadmium, mercury, zinc, and arsenite at concentrations of $10^{-4}$ M, $10^{-5}$ M, $10^{-6}$ M, and $10^{-7}$ M were added to MS media (plant hormones were added to MS media for growth of 16-12-C) for different periods of time and RNA was isolated. The plant tumors were heat shocked at 40°C for different times and RNA was isolated.
RESULTS

A. T-DNA Gene 7 in Yeast

Plasmid Constructions

Construction of plasmids for T-DNA gene 7 expression in yeast were done as shown in Figures 1 and 2. Plasmid Y-BG.E9 consists of the yeast plasmid, YEpl3 (Broach et al., 1979), and a 2,637 bp T-DNA segment containing gene 7 (Fig. 1). Y-BGE.E9 consists of YEpl3 and a 1,547 bp T-DNA segment containing gene 7 (Fig. 2). Restriction site analysis was done on these plasmids as described in Figure 3.

Primer Extension Analysis

The primer extension method was used to identify the number and position of T-DNA gene 7 RNA 5' ends. The source of T-DNA gene 7 RNA was the S. cerevisiae strain GM-3C-2 (Orr-Weaver et al., 1981) transformed with Y-BG.E9 and Y-BGE.E9. An oligonucleotide primer (oligo-7) complementary to T-DNA gene 7 RNA (Fig. 5) was added to yeast RNA and used to prime reverse transcriptase. The cDNA products were electrophoresed through a DNA sequencing gel (Fig. 6). The results obtained for transcript 7 from octopine tumor line E9 (Fig. 6, lane 5) show that the major cDNA product is 45 bases long which indicates that the major transcription initiation site is the adenosine residue located 15 nucleotides upstream of the ATG start codon of the open reading frame (Fig. 4). This corresponds to transcript initiation at a distance of 29 bases from the center of the TATA sequence. The results obtained when no RNA was added to the primer extension reaction (Fig. 6, lane 4) indicate that there was an unexpected 60 nucleotide stretch. The 60 nucleotide stretch appears in all the primer extensions. It is thought that this 60 nucleotide
Figure 1. Construction of Y-BG.E9

Construction of yeast plasmid for T-DNA gene 7 expression in yeast. Y-BG.E9 was constructed as follows: LE.E9 which is pBR325 plus the EcoRI left junction fragment of T-DNA/plant DNA sequences of the E9 tumor line was digested with BglII. The 2,637 bp T-DNA fragment containing gene 7 was ligated to YEpl3 which had been digested with Bam HI (Bam HI and BglII generate cohesie ends). The ligation reaction was then treated with Bam HI to digest all ligation products except for the recombinant plasmid Y-BG.E9 which consists of YEpl3 plus the 2,637 bp T-DNA fragment containing gene 7. E=EcoRl, H=HindIII, Bg=BglII, B=Bam HI; the dashed lines indicate T-DNA sequences.
Figure 2. Construction of Y-BGE.E9

Construction of yeast plasmid for T-DNA gene 7 expression in yeast. Y-BGE.E9 was constructed by digesting LE.E9 with Eco RV and BglII. The 1,547 bp T-DNA fragment containing gene 7 was ligated to YEpl3 which had been digested with Bam H1 and PvuII (Bam H1 and BglII have cohesive ends, and PvuII and Eco RV have cohesive ends). The ligation reaction was then treated with Bam H1 to digest all the ligation products except for the recombinant plasmid Y-BGE.E9 which is YEpl3 plus the 1,547 bp T-DNA fragment containing gene 7. EV=Eco RV, E=EcoRI, H=HindIII, Bg=BglII, B=Bam H1; the dashed lines indicate T-DNA sequences.
Figure 3. Restriction site analysis of plasmids Y-BG.E9, Y-BGE.E9, YEpl3, and LE.E9. DNA was digested with restriction enzymes indicated below and the products were analyzed by electrophoresis through a 0.7% agarose gel in TBE. The gels were stained with ethidium bromide and photographed in UV light. (a) lambda (HindIII); (b) LE.E9 (BglII); (c) YEpl3 (HindIII); (d) YEpl3 (BglII); (e) Y-BGE.E9 (HindIII); (f) Y-BGE.E9 (BglII); (g) Y-BG.E9 (Hind III); (h) Y-BG.E9 (BglII); (i) lambda (HindIII).
Figure 4. DNA sequence of T-DNA gene 7. The predicted amino acid sequence of a protein, Mr 14,400 was obtained by analysis of the open reading frame (Staden, 1980). The putative recognition sequence ("TATA box") and the heat shock related sequence (the heat shock consensus sequence is CT-GAA-TTC-AG-) are underlined, the transcription initiation site is indicated ("1"), as are characteristic polyadenylation signals. The nucleotides are numbered from the HindIII site (Base 2,119 in Figures 1 and 2). * marks the beginning of putative transcription initiation sites of gene 7 in yeast as determined by S1 nuclease mapping.
Figure 5. Oligonucleotide corresponding to the 5' region of transcript 7. The DNA sequence represents the 5' flanking region of transcript 7 showing the location and partial sequence of the 30-mer 3'-GTACTAAATCGTCTATGAGGGAACCGGAG-5'. 

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AAG CTT GAA AAT TAA GCC CCC CCC CGA AAT CAT CGC CAC AGG TCG TCC CAG CCC GCC ATC
10    20    30    40    50    60

Met Asn Phe Ala

TAT ATA TAG CGC CAA TAT AGT TTG TCT TAC ACA AAC ACA CCT CAC ATC ATG AAT TTC GCA
70    80    90    100   110   120

3'G TAC TTA------5 oligo
Figure 6. Reverse transcriptase extension products from RNA primed with an oligonucleotide complementary to the 5' coding region of the T-DNA transcript 7 (Figure 5). RNA samples were from yeast (GM-3C-2) transformed with Y-BG.E9 and YEpl3. The $^{32}$P-labelled extension products were sized on a denaturing urea-acrylamide gel.

The results represent expression from the T-DNA clones: lane 1, Y-BG.E9 (2 ug poly A$^+$ selected RNA); lane 2, Y-BG.E9 (100 ug total RNA); Control samples: lane 3, YEpl3(100 ug total RNA); lane 4, no RNA; lane 5, E9 plant tumor (40 ug total RNA); lane 6, $^{32}$P-labelled HpaI fragments of pBR322. The migration of the 30 base $^{32}$P oligonucleotide (oligo-7) is indicated.
stretch was the double stranded form of oligo-7. The absence of any other cDNA products besides the 60 nucleotide stretch in the control lanes containing no RNA and RNA from yeast transformed with YEpl3 (Fig. 6, lanes 3 and 4, respectively) demonstrates that the oligonucleotide primer is not specific for yeast RNA.

The results of the poly A+ selected RNA and the total RNA samples from yeast transformed with Y-BG.E9 (Fig. 6, lanes 1 and 2, respectively) show that there were a large number of T-DNA gene 7 RNAs with different 5' ends. The results from poly A+ selected RNA and total RNA were identical. It should be noted, though, that the primer extension method was limited because this method could not detect transcription products with initiation sites located one base upstream of the ATG translation initiation codon and downstream from that base.

S1 Nuclease Mapping

The S1 nuclease protection method was used to map the 5' ends of T-DNA gene 7 RNAs in yeast. S1 nuclease resistant hybrids were formed between the HindIII/PvuII T-DNA gene 7 fragment shown in Figure 4 and 2 ug each of RNA preparations. This method could detect transcription initiation sites downstream from the ATG translation initiation codon. The results are shown in Figure 7.

The controls (no RNA, lane A; RNA from GM-3C-2, lane B; RNA from GM-3C-2 transformed with YEpl3, lane C) demonstrate that the DNA probe is not specific for yeast RNA. S1 nuclease mapping of RNA isolated from E9 plant tumors (lane F) shows that an RNA fragment of 239 bases was protected. The distance between the normal transcription initiation site and the PvuII restriction site is 239
Figure 7 - SI nuclease mapping of yeast T-DNA gene 7 RNA 5' termini. SI nuclease resistant hybrids were formed between the HindIII/PvuII T-DNA gene 7 fragment shown in Figure 4 and 2 ug each of RNA preparations from yeast (GM-3C-2) transformed with YEpl3, Y-BG.E9, and Y-BGE.E9 (lanes C, D, and E, respectively). Lane A, no RNA; lane B, yeast RNA with no T-DNA; lane F, RNA from E9 plant tumor RNA (2 ug); lane G, 32P-labelled HpaII fragments of pBR322. Protected DNA was electrophoresed on 8% acrylamide/urea gels.
bases, indicating that the S1 nuclease procedure correctly identifies the transcription initiation site of T-DNA gene 7 in E9 plant tumors.

S1 nuclease mapping of RNAs isolated from yeast transformed with Y-BG.E9 and Y-BGE.E9 is shown in lanes D and E, respectively. RNAs of 216 bases and shorter were protected by the DNA probe with some longer minor RNAs being protected as well. These longer minor RNAs correspond to initiation sites identified previously by primer extension analysis, and they are expressed at a much lower level than the shorter protected RNAs (216 bases or less). If it is assumed that one end of the RNA corresponds to the PvuII site of the DNA probe, then the other end of the 216 base RNA would correspond to transcription initiation at a position 20 nucleotides downstream from the normal initiation site in plant tumors or 49 nucleotides downstream from the TATA box (Figure 4). The shorter protected RNAs correspond to initiation sites farther downstream.

Northern Hybridizations

Northern hybridizations were done to determine if full length transcripts were being transcribed from T-DNA gene 7 in yeast. The results are shown in Figure 8. E9 plant tumor poly A+ selected RNA was hybridized to the T-DNA gene 7 probe (lane 4). The gene 7 probe extends from the HindIII site to the SmaI site (1,178 bases) of T-DNA gene 7 as shown in Figure 4. The probe hybridized to RNA approximately 700 bases long, confirming previous results obtained by other (Gelvin et al., 1982; Willmitzer et al., 1982; McPherson, 1984).

Hybridizations between the gene 7 probe and RNA isolated from GM-3C-2 transformed with Y-BG.E9 show a transcript of 710 bases for both total RNA and poly A+ selected RNA, lanes 1 and 2, respectively. This indicates that a full length transcript is transcribed from T-DNA gene 7 in yeast. S1 nuclease
Figure 8 - An autoradiograph of an RNA gel blot hybridized to a T-DNA gene 7 probe (HindIII - SmaI T-DNA fragment, 1,178 bp, see Figure 4). RNA from yeast transformed with Y-BG.E9 was examined. Lane 1, Y-BG.E9 total RNA (30 ug); lane 2, Y-BG.E9 poly A* selected RNA (5 ug); lane 3, 32P-labelled HpaII fragments of pBR322; lane 4, E9 plant tumor poly A* selected RNA (5 ug).
mapping identified the initiation sites of gene 7, but termination sites of gene 7 in yeast are difficult to determine. Transcript sizes from plants and yeast are difficult to compare since the degree of polyadenylation is different (50 or fewer A residues in yeast compared with 100-200 in plants - Sagher et al., 1974).

B. Heat Shock – T-DNA Gene 7

Heat Shock

The effect of a rapid temperature shock on the expression of T-DNA gene 7 in crown gall tumors was investigated. Crown gall tumors were heat shocked at 40°C for 1 hour, 2 hours, 6 hours, and 24 hours. Transcripts were identified and quantified by primer extension analysis as before and the cDNA products were electrophoresed through a DNA sequencing gel (Fig. 9, lanes b-f). The results obtained for transcript 7 from tumor line E9 before heat shock (Fig. 9, lane b) are identical to the results obtained previously.

After 1 hour of heat shock at 40°C, gene 7 RNA levels decrease and continue to decrease until they are no longer detectable after 25 hours of heat shock. These results indicate that heat shock of crown gall tumors does not increase transcription of T-DNA gene 7. In fact, heat shock of tumors decreases transcription of gene 7, as would be expected for a non-heat shock gene.

Heavy Metal Treatment

Metals which are known to be toxic to plants at high levels were tested for their influence on the accumulation of T-DNA gene 7 RNAs in crown gall tumors and transformed tobacco cultures. As seen in Figure 9 (lanes g-n), there wasn't a significant increase in transcription of gene 7 until after 24 hours of treatment with cadmium (10^{-4}M) at which time there was a two-fold
Figure 9 - The effect of a temperature shock and the effect of treatment of cadmium chloride on the expression of T-DNA gene 7 in crown gall tumors. Cultures were heat shocked at 40°C for 1 hour, 2 hours, 6 hours, and 1 day and RNA was isolated as described in methods. Cultures were also grown on media containing cadmium chloride (10^{-4}M) for 1 hour, 2 hours, 6 hours, 1 day, 2 days and 20 days and RNA was isolated. Reverse transcriptase extension products from crown gall tumor RNA (20 ug) primed with oligonucleotide T7 complementary to the 5' coding region of T-DNA transcript 7 are shown in the figure. Lane a, 32P-labelled Hpa II fragments of pBR322; lane b, crown gall tumor line E9 with no treatment; lane c, E9 at 40°C for 1 hour; lane d, 40°C for 2 hours; lane e, 40°C for 6 hours; lane f, 40°C for 1 day; lane g, crown gall tumor line A6 with no treatment; lane h, A6 treated with 10^{-4}M CdCl_2 for 1 hour; lane i, 10^{-4}M CdCl_2 for 2 hours; lane j, 10^{-4}M CdCl_2 for 6 hours; lane k, 10^{-4}M CdCl_2 for 1 day; lane l, gamma32P-oligonucleotide T7; lane m, 10^{-4}M CdCl_2 for 2 days; lane n, 10^{-4}M CdCl_2 for 10 days; lane o, 32P-labelled Hpa II fragments of pBR322.
increase in transcription in crown gall tumors. After 10 days of treatment with cadmium, gene 7 RNAs accumulated to 3 times the normal level. Different concentrations of cadmium ranging from $10^{-4}$M to $10^{-7}$M were also tested to determine their effects on gene 7 transcription (Fig. 10, lanes c-g). The results indicate that concentrations of $10^{-4}$M to $10^{-7}$M cadmium all increase gene 7 transcription three-fold after 10 days of treatment.

When crown gall tumors were treated with concentrations of mercury ranging from $10^{-4}$M to $10^{-8}$M (Fig. 10, lanes h-l), there was a three-fold increase in gene 7 transcription for all concentrations. When tumors were treated with various concentrations of zinc (Fig. 11), there was no effect on gene 7 transcription.

The effect of metals on gene 7 transcription in the transformed tobacco culture 16-12-C was also investigated (Fig. 10, lanes m-o). The 16-12-C tobacco culture was transformed with plasmid DNA containing a Cauliflower mosaic virus enhancer element upstream of T-DNA gene 7. The transcript level of T-DNA gene 7 in the 16-12-C culture is five times the normal level in crown gall tumors. When the 16-12-C cultures were treated with either mercury or cadmium, transcription of gene 7 increased 100-fold. The reason for this dramatic increase in transcription is unknown at this time.

**Arsenite Treatment**

Crown gall tumors were subjected to various concentrations of sodium arsenite and gene 7 RNAs were quantified. As shown in Figure 12, arsenite appears to have no effect on transcription of T-DNA gene 7.
Figure 10 - The effect of heavy metals on the expression of T-DNA gene 7 in crown gall tumors and transformed tobacco cultures. Cultures were grown on media containing cadmium chloride (0, 10^{-7} M to 10^{-4} M) and mercuric chloride (0, 10^{-8} M to 10^{-4} M) for 10 days after which time RNA was isolated as described in methods. Reverse transcriptase extension products from crown gall tumor RNA (10 ug) primed with oligonucleotide T7 complementary to the 5' coding region of T-DNA transcript 7 are shown in the figure. The 32P-labelled extension products were sized on denaturing urea-acrylamide gels. Lane a, gamma-32P-oligonucleotide T7; lane b, 32P-labelled molecular weight markers (Hpa II fragments of pBR322); lane c, crown gall tumor line E9 treated with 10^{-4} M CdCl_2; lane d, with 10^{-5} M CdCl_2; lane e, with 10^{-6} M CdCl_2; lane f, with 10^{-7} M CdCl_2; lane g, E9 with no treatment; lane h, E9 treated with 10^{-4} M HgCl_2; lane i, with 10^{-5} M HgCl_2; lane j, with 10^{-6} M HgCl_2; lane k, with 10^{-7} M HgCl_2; lane l, 10^{-8} M HgCl_2; lane m, transformed tobacco culture line 16-12-C treated with 10^{-4} M HgCl_2; lane n, 16-12-C treated with 10^{-4} M CdCl_2; lane o, 16-12-C with no treatment; lane p, 32P-labelled Hpa II fragments of pBR322.
Figure 11 - The effect of treatment of zinc chloride on the expression of T-DNA gene 7 in crown gall tumors. Cultures were grown on media containing zinc chloride (0, $10^{-7}$M to $10^{-4}$M) for 10 days after which time RNA was isolated as described in methods. Reverse transcriptase extension products from crown gall tumor RNA (20 ug) primed with oligonucleotide T7 complementary to the 5' coding region of T-DNA transcript 7 are shown in the figure. Lane 1, $^{32}$P-labelled Hpa II fragments of pBR322; lane 2, crown gall tumor line A6 with no treatment; lane 3, A6 treated with $10^{-4}$M ZnCl$_2$; lane 4, with $10^{-5}$M ZnCl$_2$; lane 5, with $10^{-6}$M ZnCl$_2$; lane 6, with $10^{-7}$M ZnCl$_2$; lane 7, A6 with no treatment; lane 8, E9 with no treatment.
Figure 12 - The effect of treatment of sodium arsenite on the expression of T-DNA gene 7 in crown gall tumors. Cultures were grown on media containing sodium arsenite (0, $10^{-7}$M to $10^{-4}$M) for 10 days after which time RNA was isolated as described in methods. Reverse transcriptase extension products from crown gall tumor RNA (20 ug) primed with oligonucleotide T7 complementary to the 5' coding region of T-DNA transcript 7 are shown in the figure. Lane 1, crown gall tumor line A6 with no treatment; lane 2, A6 treated with $10^{-4}$M arsenite; lane 3, with $10^{-5}$M arsenite; lane 4, with $10^{-6}$M arsenite; lane 5, with $10^{-7}$M arsenite.
DISCUSSION

A. T-DNA Gene 7 in Yeast

Primer extension analysis, S1 nuclease mapping, and Northern hybridizations indicate that transcription of T-DNA gene 7 in yeast is different from that of transcription of gene 7 in crown gall tumors. S1 nuclease mapping and primer extension analysis of gene 7 in yeast show that there are multiple transcription initiation sites. The major transcription initiation sites of gene 7 in yeast begin 20 nucleotides downstream from the normal initiation site in crown gall tumors, or 49 nucleotides downstream from the TATA box.

In yeast the structure of the promoter is similar to that of higher eukaryotes. TATA sequences have been observed in all yeast genes studied (Sentenac and Hall, 1982). However, in contrast to higher eukaryotic systems, the distance between a TATA sequence and the corresponding set of transcription initiation sites is variable, ranging from 40 to 150 bp (Sentenac and Hall, 1982). In most eukaryotic cells the TATA box has a separation from the transcription start point that varies from 25 to 30 bp (Breathnach and Chambon, 1981).

It has been shown that for the yeast iso-1-cytochrome c gene there is a loose spatial relationship between the TATA sequences and the mRNA start sites (McNeil and Smith, 1986). This distance relationship varies from 100 to 60 base-pairs (+ 15 base-pairs). There also exists four and possibly five TATA sequences located within the 5' non-coding region of the iso-1-cytochrome c gene (McNeil and Smith, 1986). Each TATA sequence is thought to be required for a specific subset of mRNA starts. McNeil and Smith suggest that the spacing between T-A-T-A and starts is not a single fixed distance and it is very likely that intervening sequences determine the spacing, either directly
or through a series of specific spacing factors. In contrast, Chen and Struhl suggest that the TATA box in yeast does not select initiation sites by measuring a fixed distance from it (Chen and Struhl, 1986). Instead, an initiator element close to the initiation site itself contains the signal for determining where transcription begins. Photoprinting studies indicate, however, that the transcription-dependent alteration in light sensitivity at the hexanucleotide 5' ATATAA3' marks a functional TATA sequence in yeast (Selleck and Majors, 1987). When the hexanucleotide is deleted from the yeast GAL 1 gene, expression decreases 30-fold under inducing conditions (West et al., 1984). These studies indicate the functional importance of the TATA box in yeast. The TATA box appears to be directly involved in the selection of transcription initiation sites in yeast, although the exact function of the TATA box is unknown.

A TATA box in the 5' region of T-DNA gene 7 maps 30 bp upstream from the normal mRNA start site in crown gall tumors. S1 nuclease mapping shows that the mRNA start sites of gene 7 in yeast begin 49 nucleotides downstream from the TATA box. This confirms previous results which indicate that a TATA box must be at least 40 nucleotides upstream of the transcription initiation site for transcription in yeast (Chen and Struhl, 1986). These facts suggest that when foreign DNA is to be expressed in yeast, the distance between the initiation site and the TATA box in the foreign gene is critical. If the TATA box is less than 40 bp upstream from the ATG translation initiation codon, then it is highly unlikely that a complete, functional protein will be translated. The major transcription initiation sites of T-DNA gene 7 in yeast begin after the ATG translation initiation codon which means that the translation reaction is prevented.
The possible reason for the abnormal expression of foreign genes in yeast in previous studies may have been due to the fact that the distance requirement of the TATA box in yeast is different than in other eukaryotes. When the rabbit B-globin gene was cloned in yeast, the B-globin transcripts from yeast were about 20-40 nucleotides shorter at the 5' end than normal globin mRNA (Beggs et al., 1980). It was suggested that the abnormal 5' termini of the B-globin transcripts represented either transcription initiation sites recognized by a yeast RNA polymerase or degradation of longer transcripts originating at the promoter site or even further upstream. The TATA box in the B-globin gene maps 28 nucleotides upstream of the transcription initiation site. The major initiation sites of the B-globin gene in yeast are 46 and 65 nucleotides downstream from the TATA box. Fortunately, the ATG initiation codon was still downstream from the transcription initiation sites, so normal translation was still a possibility. In this case, however, the gene was not spliced properly and it was concluded that the yeast system was inadequate for the study of transcription and processing of the B-globin gene.

In most eukaryotes, transcription initiation is confined to a short region. In yeast, however, there can be many sites at which transcripts are initiated (Faye et al., 1981; Hsu and Schimmel, 1984; Johnston and Davis, 1984; Zalkin et al., 1984). S1 nuclease mapping of the mRNA 5' termini for the TRP2 and TRP3 yeast genes showed that there were multiple clusters of 5' ends for both TRP2 and TRP3 mRNA (Zalkin et al., 1984). It is not surprising then that there are multiple transcription initiation sites when gene 7 is cloned in yeast.

In a recent paper (Hirsch and Beggs, 1984), it was reported that T-DNA had been cloned in yeast. Northern hybridizations were done to identify T-DNA transcripts with various probes, but no RNA similar in size to the 0.7 kb mRNA
was identified. As for other T-DNA transcripts, the pattern of transcription was complex. RNAs identified by Northern hybridizations appeared to be characteristically larger than expected RNA lengths. Results in this investigation indicate that a full length transcript is transcribed from gene 7 in yeast, although transcription initiation and termination are different than in crown gall tumors. The gene 7 transcript was most likely detected in this investigation because a specific probe for gene 7 was used.

In conclusion, transcription of T-DNA gene 7 in yeast is different than in crown gall tumors. Specifically, transcription initiation is different because the distance between the TATA box and the transcription initiation sites must be at least 40 nucleotides in yeast. Although yeast is an excellent system for investigating the expression of some cloned eukaryotic genes, it does not appear to be a useful system for investigating the expression of T-DNA.

B. Heat Shock - T-DNA Gene 7

By definition, HS proteins are a new set of proteins rapidly and abundantly produced in response to heat shock. The appearance of heat shock proteins is dependent on transcriptional activation of the heat shock genes. This induction is immediate and increases the concentration of hsp mRNA from undetectable (less than one molecule per cell) to a thousand molecules per cell within an hour of heat shock (Schlesinger et al., 1982). Eukaryotic HSP genes have one or more copies of a 14-base-pair (bp) sequence, referred to as a heat shock promoter element (HSE), upstream from the transcription initiation site (Bienz, 1985; Craig, 1985; Pelham, 1985). A heat shock sequence similar to the HSE was identified in the promoter region of T-DNA gene 7.

Although untreated crown gall tissue contains significant levels of gene 7 RNAs, there is at least one case where a plant heat shock gene is expressed at
significant levels prior to heat shock (Czarnecka et al., 1984). When cloned cDNAs to heat shock (hs)-induced mRNAs were used to assess whether various stresses induced the accumulation of poly(A) RNAs in soybean seedlings, one set of poly (A) RNAs which were present at variable levels in control (non-stressed tissue) tissue were increased some 5- to 10-fold by heat shock and other stress agents such as cadmium and arsenite (Czarnecka et al., 1984).

A summary of the influence of a number of stress agents, including heat shock, on T-DNA gene 7 RNA levels based on primer extension analysis is presented in Table 2. The data presented here demonstrate that only cadmium and mercury have a significant effect on the expression of T-DNA gene 7. Cadmium and mercury increase the expression of gene 7 3-fold after 10 days of treatment. Arsenite, zinc, and heat shock do not induce the accumulation of gene 7 RNAs to levels above that of normal levels in crown gall tumors. In fact, when crown gall tumors were subjected to a temperature of 40°C, gene 7 RNA levels decreased until after one day when they were no longer detectable. In soybean seedlings the concentrations of many normal poly(A) RNAs declines after 2 hours of heat shock (Kimpel and Key, 1985). Furthermore, when transformed callus tissue is exposed to high temperatures, nopaline synthase (a non-heat shock gene) transcripts decrease to a very low level immediately after heat shock (Spina et al., 1985). These facts suggest that T-DNA gene 7 is not a heat shock gene.

Due to the fact that T-DNA gene 7 responded only to cadmium and mercury, the possibility of gene 7 being a metallothionein gene was investigated. Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich proteins of about 6,000 to 7,000 molecular weight which are widely distributed in nature (Kagi et al., 1984). They are expressed in many different tissues and cell types when these are exposed to heavy metals such as cadmium, zinc,
<table>
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<th>Treatment</th>
<th>No Treatment (25°C)</th>
<th>1 hour</th>
<th>2h</th>
<th>6h</th>
<th>1 day</th>
<th>2d</th>
<th>10d</th>
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<td>Heat shock 40°C(E9)</td>
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<td>.5X</td>
<td>.25X</td>
<td>0X</td>
<td>--</td>
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<tr>
<td>CdCl₂ 10⁻⁴M(A6)</td>
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<td>1X</td>
<td>1X</td>
<td>1X</td>
<td>2X</td>
<td>2X</td>
<td>3X</td>
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<tr>
<td>CdCl₂ 10⁻⁴M(E9)</td>
<td>1X</td>
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<tr>
<td>HgCl₂ 10⁻⁴M(E9)</td>
<td>1X</td>
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<tr>
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<td>1X</td>
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<td>1X</td>
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<tr>
<td>Arsenite 10⁻⁴M(A6)</td>
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<tr>
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</table>

**Table 2**

Summary of the stress induced accumulation of T-DNA gene 7 RNAs in crown gall tumors and transformed tobacco cultures. A6 = crown gall tumor line A6; E9 = crown tumor line E9; 16-12-C = transformed tobacco culture. X = estimated relative level from primer extension analyses as determined by a densitometer; -- = not analyzed.
copper and mercury (Kagi and Nordberg, 1979). The induction of these proteins is regulated mainly at the level of transcription (Durnam and Palmiter, 1981; Karin et al., 1984; Mayo et al., 1982). Although the gene 7 protein is a low molecular weight protein (14Kd), this protein is not cysteine rich (less than 2% of the amino acid content are cysteines). In addition, a computer analysis was done to determine if there were any sequences similar to the consensus sequence responsible for metal regulation (5' - TGGCGCGCTTC-3') (Carter et al., 1984). No sequence similar to the metal consensus sequence was identified in the 5' region of gene 7. These facts suggest that T-DNA gene 7 is not a metallothionein gene.

In an attempt to identify DNA sequences of functional importance in the 5' region of T-DNA gene 7, the transformed tobacco culture line, 16-12-C, was treated with mercury and cadmium. The 5' flanking region of T-DNA gene 7 is deleted upstream of the HindIII site (Fig. 4) in this tobacco culture line. It should be noted that there is also a Cauliflower mosaic virus enhancer element upstream of T-DNA gene 7 in this culture line. The normal transcript level of T-DNA gene 7 in the 16-12-C culture is five times the normal level of that in crown gall tumors. When the 16-12-C cultures were treated with either mercury or cadmium, transcription of gene 7 increased 100-fold. The reason for this dramatic increase is unknown at this time, but prima facie it appears that the metals are directly influencing the Cauliflower mosaic virus enhancer element to increase transcription of gene 7.

In conclusion, it does not appear that T-DNA gene 7 is a heat shock gene. Although gene 7 responds to cadmium and mercury, the increase in transcription does not appear to be heat shock or metallothionein related. As for the importance of DNA sequences in the 5' region of gene 7, the deletion of the 5' region of gene 7 is complicated by the presence of a Cauliflower mosaic
enhancer element. These facts suggest that another mechanism is involved in the enhanced transcription of gene 7 in response to cadmium and mercury treatment of crown gall tumors.
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