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THE MECHANISMS AND EFFECTS OF THE PLANT-ACTIVATION OF CHEMICALS IN THE ENVIRONMENT

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INTRODUCTION

Plants can activate promutagens into stable mutagens and these genotoxic agents may be hazardous to the environment and to the public health. Plant systems have been widely employed in classical and environmental mutagenesis. However, the environmental and human health impact of plants exposed to environmental xenobiotics were not well recognized until the presence of pesticide contaminants in food supplies caused alarm. The capability of plants to bioconcentrate environmental agents and activate promutagens into toxic metabolites is significant when one realizes the immense diversity of xenobiotics to which plants are intentionally and unintentionally exposed. Finally, we all must be attentive to the effects that toxic agents may have on the biosphere and the grave global consequences that would result in a disruption in the carbon cycle.

Plant activation is the process by which a promutagen is metabolically transformed into a mutagen by a plant system. In mammalian systems the majority of enzymes participating in oxidative desulfuration, dealkylation, epoxidation, or ring hydroxylation involve cytochrome P-450-type monooxygenases. It is unknown if microsomal cytochrome P-450 in plants have enzymatic characteristics similar to those of mammalian liver. The optical and magnetic properties of plant cytochrome P-450 are similar to those of hepatic microsomes. Although limited data exist about the inducibility of plant cytochrome P-450, it is unknown if there is an equivalent inducible system to hepatic monooxygenases. Plant peroxidases catalyze the oxidation of a diverse class of xenobiotics. Peroxidases are ubiquitous in plants, however, only limited data are available that demonstrate their participation in the *in vivo* metabolism of foreign compounds.

The purpose of this report is to review our recent findings on the plant activation of aromatic amines, to supplement the last report which was submitted in April 1992 and to list the publications that are the result of the funding of this research by the Air Force Office of Scientific Research. The results for each project in my laboratory are presented below.

RESULTS AND DISCUSSION FOR SPECIFIC PROJECTS

Mutagenic Characterization of the Plant-activated Products of Benzidine and 4-aminobiphenyl

Objectives

- Analyze the capacity of *Nicotiana tabacum* cells (line TX1) to activate benzidine and 4-aminobiphenyl into forms that would induce base pair substitution and frameshift mutations in *Salmonella typhimurium*.

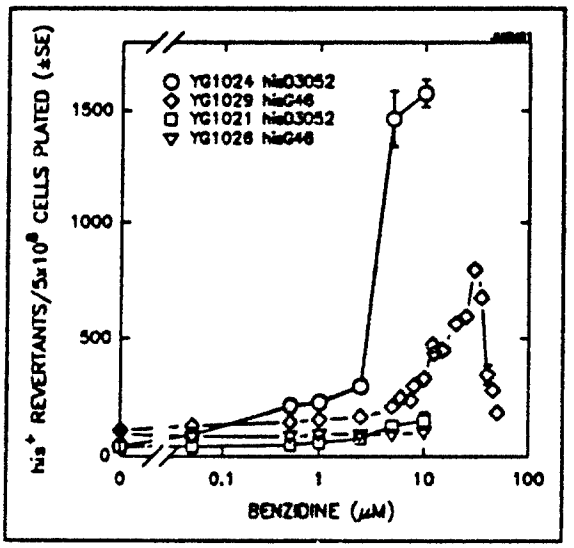

Figure 1 Benzidine was activated by tobacco cells and the products were mutagenic in *S. typhimurium* YG1024 and YG1029.

- Isolate the benzidine and 4-aminobiphenyl plant-activated products using membrane ultrafiltration.
- Determine if the high molecular weight plant activated products can serve as substrates for *Salmonella* acetyl-CoA: *N*-hydroxyarylamine *O*-acetyltransferase and for human CoASAc-dependent arylamine *N*-acetyltransferase.

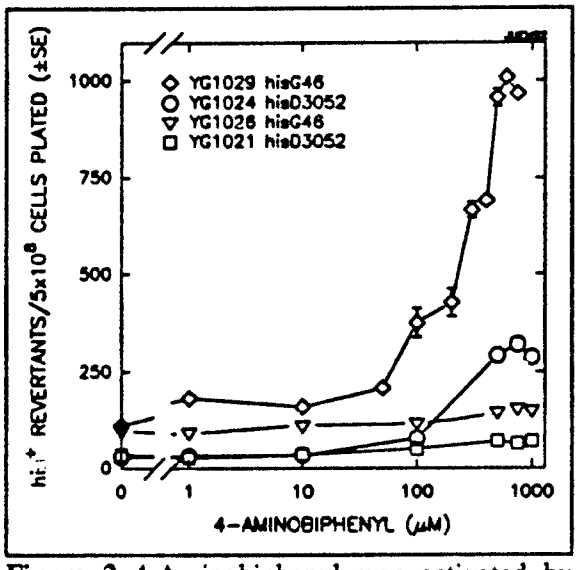

Figure 2 4-Aminobiphenyl was activated by tobacco cells and the products were mutagenic in *S. typhimurium* YG1029 and YG1024.

Plant Cell/microbe Coincubation Analysis of Benzidine and 4-aminobiphenyl

The plant cell/microbe Coincubation assay employs living plant cells in suspension culture as the activating system and a specific microbial strain as the genetic indicator organism. Plant cells from a 7-day culture were harvested, washed, and adjusted to 100 mg fresh weight/ml in MX$^-$ medium. An overnight culture of each specific *S. typhimurium* strain was grown from a single colony isolate. The bacterial suspension was washed in 100 mM phosphate buffer and the titer was adjusted to 1×10^{10} cells/ml. Each reaction tube contained 4.5 ml of the TX1 cell suspension, 5×10^9 bacterial cells and varying concentrations of the aromatic amine chemical. These components were incubated at 28°C for 1 h with shaking (150 rpm). Concurrent negative controls consisted of plant and bacterial cells alone, heat-killed plant cells plus bacteria and the aromatic amine, and both buffer and solvent controls. Triplicate 0.5 ml aliquots (5×10^8 bacteria) were removed from each reaction tube and added to molten top agar supplemented with 550 μM histidine and biotin. The top agar was poured onto Vogel Bonner (VB) minimal medium plates, incubated for 48–72 h at 37°C, and revertant *his*$^+$ colonies were scored.

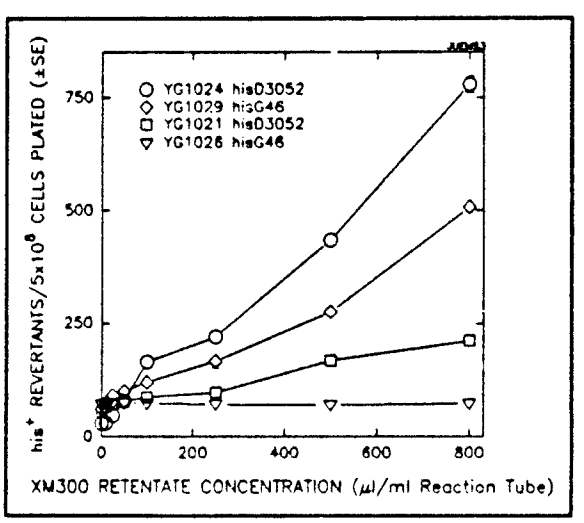

Figure 3 Concentration-response curves of XM300 retentate isolated from TX1 cells treated with 50 μM benzidine for 3 h at 28°C using a preincubation assay.

Figure 1 demonstrates that TX1 cells can activate benzidine into a mutagen that can induce frameshift mutations at *hisD3052* (○) and base pair substitution mutations at *hisG46* (◇) in the *S. typhimurium* strains that

over express *O*-acetyltransferase. Strains YG1021 and YG1026 over express nitrate reductase.

Figure 2 illustrates the data for 4-aminobiphenyl. The sensitivity of the two strains expressing high *O*-acetyltransferase activity are reversed as compared to the data presented for benzidine. Plant-activated 4-aminobiphenyl induced a significantly higher reversion frequency at *hisG46* (YG1029 ◊) as compared to the frameshift tester strain YG1024 (O).

Isolation and Sizing of Plant-activated Benzidine and 4-aminobiphenyl Products

Ultracentrifuged supernatant fluids from the control (untreated TX1 cells) and TX1 cells treated with 50 μM benzidine or 600 μM 4-aminobiphenyl were fractionated according to the molecular size of the component molecules. Sterile ultrafiltration membranes were used in an Amicon model 8400 stirred cell using nitrogen gas at 20 – 25 psi at 4°C. The retentate fraction ≥300,000 MW and the filtrate fraction ≤300,000 MW, were stored under darkened conditions at 4°C. The XM300 retentates and filtrates were tested in *S. typhimurium* by varying the concentration of the XM300 product in 100 mM phosphate buffer and preincubating the cells for 1 h at 37°C while shaking.

Figure 3 illustrates that the *O*-acetyltransferase over expressing strains YG1024 and YG1029 were the most sensitive to the plant-activated benzidine XM300 retentate. Their order of sensitivity — YG1024 and YG1029 — was the same as seen in the coincubation assay (Figure 1). The XM300 retentate isolated from 4-aminobiphenyl-treated TX1 cells induced mutation in YG1029 and YG1024 with reversion of *hisG46* being the most sensitive target (Figure 4). This agreed with the strain sensitivities seen in the coincubation assay (Figure 2). In all cases the filtrates from each extraction as well as XM300 retentates isolated from untreated control TX1 cells did not induce mutation in any of the YG tester strains (data not shown). Thus, the plant-activated mutagenic products inducing mutation in the coincubation assays were completely recovered in the ≥300 kDa ultrafiltration membrane fraction. The plant-activated products of benzidine and 4-aminobiphenyl are high molecular weight metabolites, most probably plant-mediated conjugates.

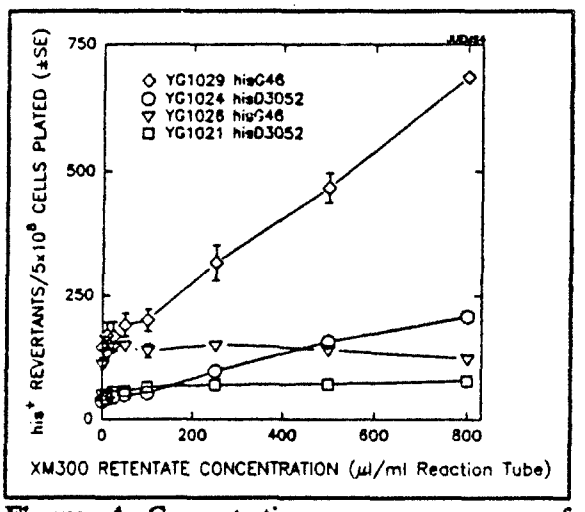

Figure 4 Concentration-response curves of XM300 retentate isolated from TX1 cells treated with 600 μM 4-aminobiphenyl for 3 h at 28°C using a preincubation assay.

Plant-activated Aromatic Amine Products as Substrates for N/O-acetyltransferases

The first step in the mammalian hepatic activation of aromatic amines is *N*-hydroxylation. The *N/O*-acetyltransferases are essential in metabolizing *N*-hydroxyarylamines to their ultimate mutagenic products. In previous work we demonstrated that the plant-activated products of 2-aminofluorene, benzidine, *m*-phenylenediamine, 4-aminobiphenyl, 2,4-diaminotoluene and 2-

naphthylamine served as substrates for bacterial *O*-acetyltransferase and induced mutation in *Salmonella*. In mammals *O*-acetyltransferase and *N*-acetyltransferase activities are catalyzed by the same enzyme, CoASAc-dependent arylamine *N*-acetyltransferase. This dual function is also characteristic of the *S. typhimurium O*-acetyltransferase.

NAT1 and NAT2 are two human genes which produce functionally distinct but similar cytosolic CoASAc:arylamine *N*-acetyltransferase. Grant et al., (*Cancer Res.* 52:3961-64, 1992) generated *S. typhimurium* strains that contain human *NAT1* and *NAT2*, DJ400 (TA1538/1,8-DNP:pNAT1) and DJ460 (TA1538/1,8-DNP:pNAT2), respectively. As illustrated in Figures 5 and 6, plant-activated benzidine and 4-aminobiphenyl XM300 products were substrates for human NAT1 and NAT2. The XM300 benzidine retentate was an excellent substrate for NAT2 and was highly responsive in *S. typhimurium* strain DJ460 (Figure 5). The plant-activated benzidine product only weakly induced reversion at *hisD3052* in strain DJ400 and suggests that it is a poor substrate for human NAT1. Strain YG1024 bacterial *O*-acetyltransferase expressed an intermediate level of response. The concentration-response results for the XM300 4-aminobiphenyl retentate were lower than for the benzidine XM300 retentate. Figure 6 illustrates that YG1024 was the most responsive strain while DJ460 (NAT2) showed a weak response. However, these studies are incomplete in that we have not investigated the role of these acetyltransferases on modulating the reversion at *hisG46* with the XM300 retentates. From the data presented in Figures 2 and 4, the plant-activated 4-aminobiphenyl product preferentially induces mutation at *hisG46* (YG1029). These experiments are planned for the future.

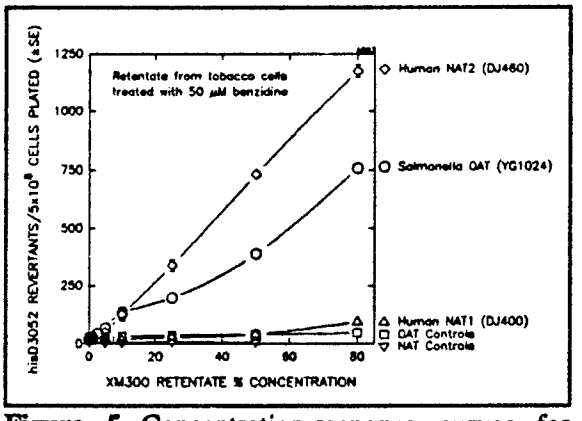

Figure 5 Concentration-response curves for plant-activated benzidine XM300 retentate in tester strains expressing Human or *Salmonella* acetyltransferases.

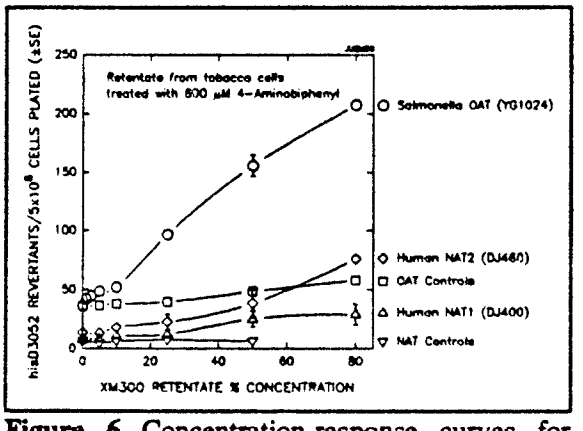

Figure 6 Concentration-response curves for plant-activated 4-aminobiphenyl XM300 retentate in tester strains expressing Human or *Salmonella* acetyltransferases.

Conclusions

- The procarcinogens benzidine and 4-aminobiphenyl are activated by tobacco cells into mutagens and the mutagenic potency of plant-activated benzidine is greater than 4-aminobiphenyl (Figures 1 and 2).

- The plant-activated products of benzidine and 4-aminobiphenyl can be isolated by ultrafiltration in a retentate fraction with a molecular weight of ≥300 kDa (Figures 3 and 4).
- Bacterial or human *N/O*-acetyltransferase is required to further metabolize the high molecular weight plant-activated products of benzidine and 4-aminobiphenyl into their ultimate mutagenic forms (Figures 5 and 6).

Characterization and Biological Responses of the Plant-activated Products of *m*-Phenylenediamine

Objectives

- Determine if the plant-activated products of *m*-phenylenediamine could serve as substrates for human *NAT1* and *NAT2* gene products.
- Determine the toxicity of *m*-phenylenediamine to tobacco cells.
- Measure the effects of *m*-phenylenediamine on genomic DNA of TX1 cells by using flow cytometric analysis.

The Plant Activated M-phenylenediamine Product Is a Substrate for Bacterial and Human N/O-Acetyltransferases

The first step in the mammalian hepatic activation of aromatic amines is *N*-hydroxylation. The *N/O*-acetyltransferases are essential in metabolizing *N*-hydroxyarylamines to their ultimate mutagenic products. *NAT1* and *NAT2* are two human genes which produce functionally distinct but similar cytosolic CoASAc:arylamine *N*-acetyltransferase. Grant et al., (*Cancer Res.* 52:3961-64, 1992) generated *S. typhimurium* strains that contain human *NAT1* and *NAT2*, DJ400 (TA1538/1,8-DNP:pNAT1) and DJ460 (TA1538/1,8-DNP:pNAT2), respectively.

Ultracentrifuged supernatant fluids from the control (untreated TX1 cells) and TX1 cells treated with 500 μM *m*-phenylenediamine were fractionated by molecular size. Sterile ultrafiltration membranes were used in an Amicon model 8400 stirred cell using N_2 at 20 - 25 psi at 4°C. The retentate fraction ≥300 kDa and the filtrate fraction ≤300 kDa, were stored in the dark at 4°C. The XM300 retentates and filtrates were tested in *S. typhimurium* by varying the concentration of the XM300 product in 100 mM phosphate buffer and preincubating the cells for 1 h at 37°C while shaking. In all cases the filtrates from each extraction as well as XM300 retentates isolated from untreated control TX1 cells did not induce mutation in any of the YG or DJ tester strains (data not shown). The plant-activated products of *m*-phenylenediamine are high molecular weight

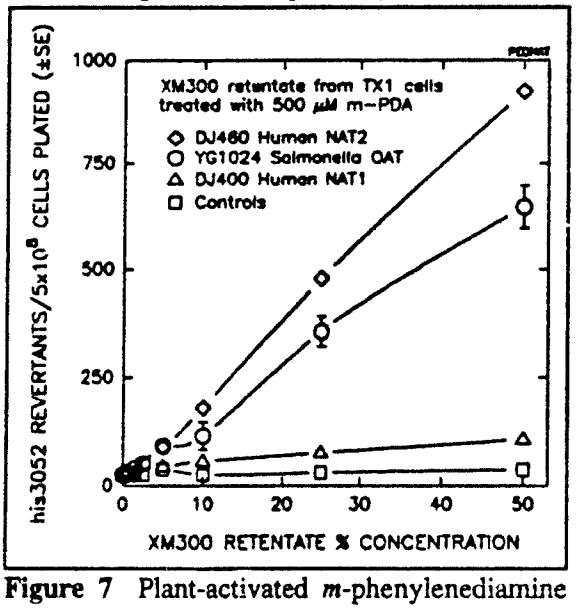

Figure 7 Plant-activated *m*-phenylenediamine product induced mutation in *S. typhimurium* strains DJ460 and YG1024.

metabolites, most probably plant-mediated conjugates.

As illustrated in Figure 7, plant-activated *m*-phenylenediamine XM300 products were substrates for human NAT1 and NAT2. The XM300 *m*-phenylenediamine retentate was an excellent substrate for NAT2 and was highly responsive in *S. typhimurium* strain DJ460. The plant-activated *m*-phenylenediamine product only weakly induced reversion at *hisD3052* in strain DJ400 and suggests that it is a poor substrate for human NAT1. Strain YG1024 bacterial *O*-acetyltransferase expressed an intermediate level of response. It is clear that acetyltransferase is essential for the metabolism of the plant-activated products of *m*-phenylenediamine into their ultimate mutagenic forms. We found that the plant-activated product(s) of *m*-phenylenediamine were associated with a high molecular weight ultrafiltration fraction (≥300 KDa), which suggests that the mechanism of plant activation of this promutagen is far different from that of mammalian system.

Tx1 Cell Viability Assay

A TX1 cell culture was grown at 28°C to mid-log phase and the cells were washed and suspended in MX⁻ medium. MX⁻ medium lacks a plant growth hormone. The fresh weight of the cells was adjusted to 100 mg/ml. Aliquots were prepared from this TX1 cell stock and treated with 0, 10, 25, 50, 100, 250 and 500 μM *m*-phenylenediamine at 28°C for 48 h. After incubation, a small portion of each culture was mixed with one volume of MX⁻ medium and stained with a vital dye (phenosafranine 1 mg/ml). The cells were determined as viable by their ability to exclude the dye from their cytoplasm. As illustrated in Figure 8 *m*-phenylenediamine was not toxic to TX1 cells. This suggests that *m*-phenylenediamine may be metabolized and compartmentalized in the plant cell.

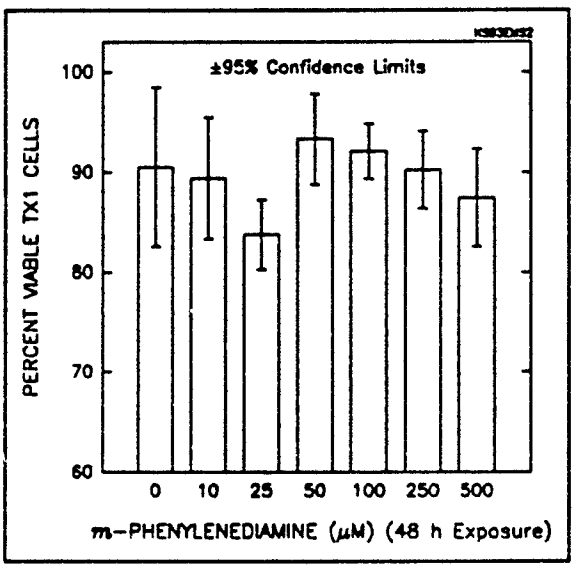

Figure 8 Toxicity of *m*-phenylenediamine to tobacco cells.

Effect of m-Phenylenediamine on Nuclear DNA Content in Tx1 Cells

A TX1 cell culture (100 mg/ml fresh weight) was treated with 500 μM *m*-phenylenediamine and grown for 6 days to early stationary phase. The cells were washed and suspended in MX$^-$ medium. The cells were dried under vacuum and suspended in a nuclear extraction buffer (0.1 M hexylene glycol, 10 mM Tris (pH 8.0), 10 mM $MgCl_2$ and 0.5% Triton X-100). The cells were homogenized for 30 sec at 4500 rpm. The suspension was filtered through two layers of nylon meshes of 250 μm and 57 μm pore sizes, respectively. The filtrate was centrifuged for 15 min at 500 ×g for 15 min. After discarding the supernatant, the nuclei pellet was stained with 500 μl of nuclear staining solution. The fluorochrome used in this study was propidium iodide and its stock solution was prepared as 1 mg/ml. The final concentration of this dye in the nuclear staining solution was 100 μg/ml. After staining, the nuclear suspension was incubated at 37°C for 20 min and stored at 4°C in the dark. The nuclei of untreated TX1 cells were isolated and used as the control. The DNA content of the TX1-cell nuclei was determined using a Coulter EPICS 750 series flow cytometer-cell sorter system. The laser beam for excitation of the fluorescent dye was provided by a 5W argon-ion laser which was adjusted to an excitation wavelength band of 488 nm. A minimum of 5,000 nuclei were analyzed for *m*-phenylenediamine-treated and untreated TX1 cells.

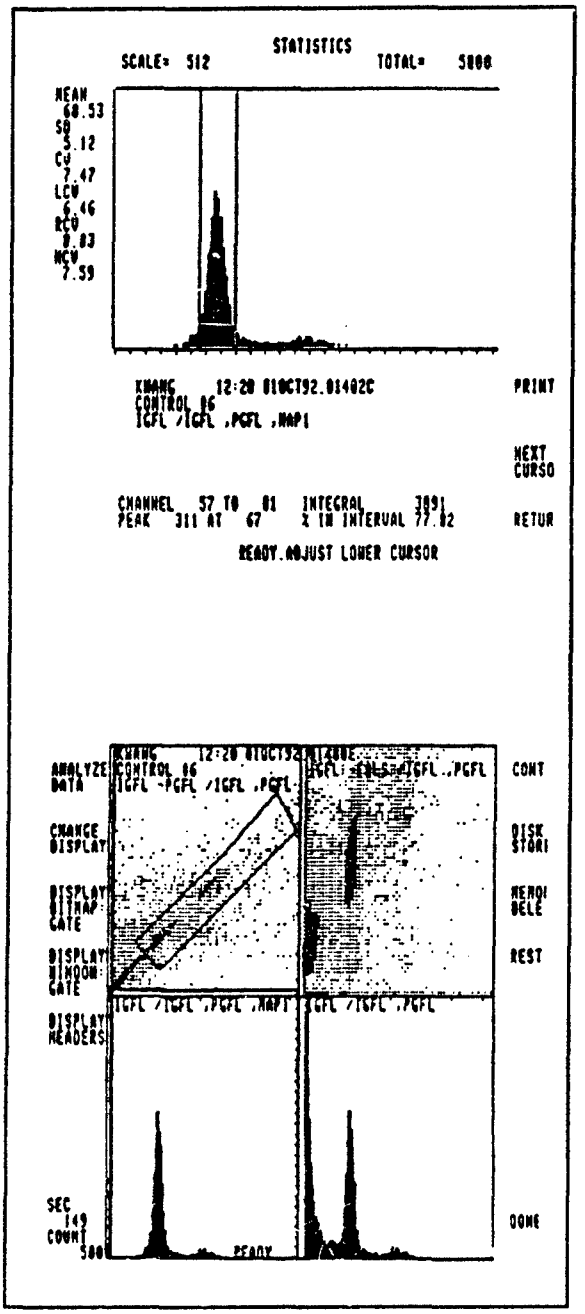

Figure 9 Distribution of DNA content in nuclei isolated from control TX1 cells.

As illustrated in Figures 9 and 10 the DNA content in nuclei isolated from *m*-phenylenediamine-treated and untreated TX1 cells was not significantly different. Both nuclear DNA profiles obtained from treated and untreated TX1 cells show similar patterns in their 2N and 4N state of DNA and indicate that the plant-activated product(s) of *m*-phenylenediamine did not induce major alterations in the genomic DNA content.

Conclusions

- The isolated, XM300 plant-activated *m*-phenylenediamine product must be further metabolized by bacterial OAT or human NAT2 acetyltransferases to express its mutagenic properties. Thus the plant-activated *m*-phenylenediamine product is a proximal mutagen.
- The promutagen *m*-phenylenediamine is not toxic to cultured tobacco cells at concentrations in which it is activated by the plant cells. We hypothesize that the plant cell is able to compartmentalize the activated products and nutralize its toxicity..
- *m*-Phenylenediamine does not induce alterations in the nuclear DNA of tobacco cells. This suggests that the plant-activated product is non-toxic or that the cells compartmentalize the plant-activated product.

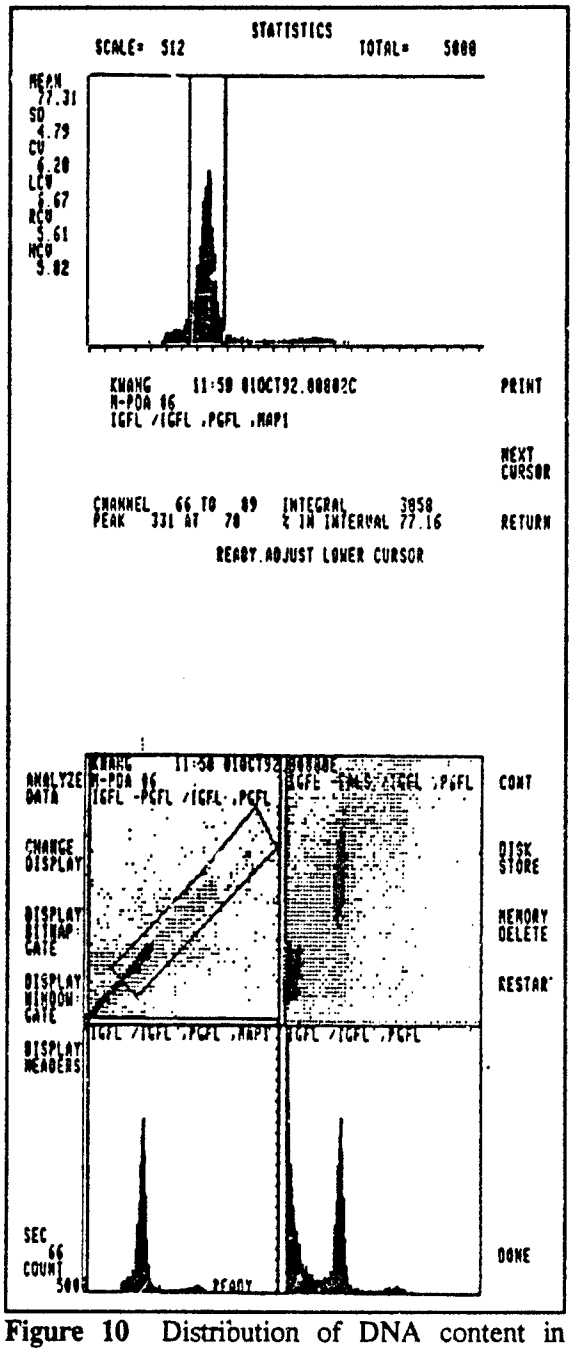

Figure 10 Distribution of DNA content in nuclei isolated from treated TX1 cells.

A Comparison of the Mutational Spectra of Yg1024 Revertants Induced by the Plant-activated Products(s) of Monocyclic and Bicyclic Aromatic Amines

We are conducting mutation spectra analysis on the plant-activated 2-Aminofluorene-induced *his*$^+$ revertants and the XM300 retentate-induced *his*$^+$ revertants of YG1024. Other studies demonstrate that a high frequency of reversion occurs by a CG/GC deletion which is located in an alternating CG octamer of the *hisD3052* allele (D878-885). A colony-probe hybridization procedure was developed to detect this -2 deletion in the 2-aminofluorene-induced revertants (Cebula and Koch, 1990). An improved procedure with non-isotopic probing of PCR products dot blotted on nylon membranes was used to detect this deletion in the XM300 retentate-induced revertants.

The objectives of this research were to construct and compare the mutation spectra of the YG1024 revertants induced by both plant-activated 2-aminofluorene and *m*-Phenylenediamine metabolite(s). These data will be compared with spectra generated from spontaneous revertants of YG1024.

Revertant collection

Because of the difference in mutagenic potency between 2-aminofluorene and XM300 retentate, YG1024 was exposed to different concentrations of both mutagens to determine the equivalent biological effect. YG1024 was exposed to 0-2.5 μM 2-aminofluorene and 0-90% XM300 retentate (v/v). 0.25 μM 2-aminofluorene resulted in a 12-15 fold increase in mutagenic activity over the spontaneous reversion frequency. 33% (v/v) XM300 retentate elicited the same fold increase over background. These were the concentrations selected for collecting the plant-activated 2-aminofluorene-induced revertants and the XM300 retentate-induced revertants.

Colony-Probe Hybridization Assay

The plant-activated 2-aminofluorene-induced revertants were isolated by picking individual colonies from minimal medium plates and inoculating them in minimal (VB + biotin) liquid medium. These were grown for 1-2 days at 37° C. Brain heart infusion (BHI) agar plates, a very rich growth medium, were divided into grids of 50 sections and numbered. One drop of each revertant suspension was transferred to its corresponding section. The BHI plates were made in duplicate and incubated overnight at 37° C. The plates were sealed with parafilm and stored at 4° C.

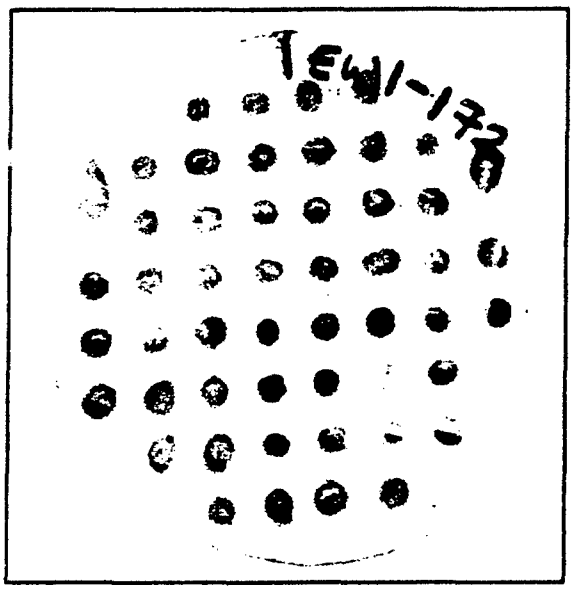

Figure 11 Colony lifts on filter paper from a BHI plate with *S. typhimurium* revertants.

A disk of Nº541 Whatman filter paper was placed on the revertant colonies of each BHI plate. The colonies adhered to the filter disk by applying pressure to the surface of the agar and peeling back the filter. The disks were placed in a denaturing solution for 5 min, irradiated in the microwave to lyse the cells, transferred to a neutralizing solution for 5 min, and dried overnight (Figure 11).

Each induced *his*$^+$ revertant was analyzed by a modified version of the Cebula colony-probe hybridization assay (Cebula and Koch, 1990). Filters containing the lysed *hisD3052* revertants were put in petri plates with 10 ml of hybridization solution. TC13, an unlabelled competitive probe, was added to linearize the DNA at a secondary hairpin loop that contains the common -2 deletion (Figure 12).

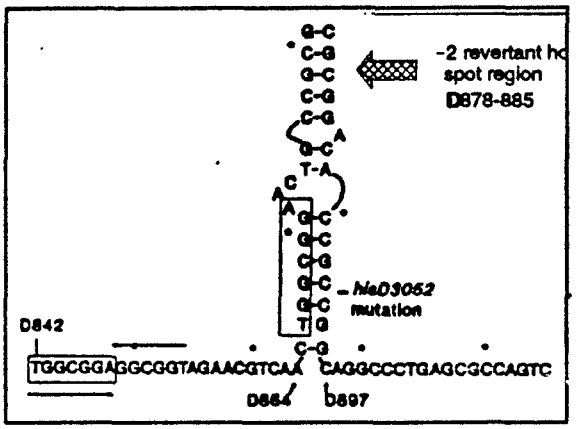

Figure 12 *HisD3052* region from Cebula, T.A., and W.H. Koch (1990) Mutation and the Environ., part D, 367-377.

After 20 min at 60°C, a ^{32}P-labelled probe, TC5, was added and incubated for 2 h at 60°C. TC5 is a probe that contains the -CG deletion at D878-885. The filters were then washed in 3 × SSC for 30 min at 60°C. They were washed a second time, dried, and exposed to Kodak XAR-5 X-ray film with intensifier screens overnight at -70°C. This procedure was repeated on the same filters a second time to obtain enhanced signals. The number of revertants with the -CG deletion were recorded. A majority of the TA98 *his*$^+$ revertants hybridized with the probe and thus contained the -2 deletion. As illustrated in Figure 13, three colonies did not hybridize and are presumed to be mutations at some other location in the *hisD3052* region.

The revertant colonies which were negative or unclear in the colony-probe hybridization procedure were grown overnight in minimal media. The cultures were washed and suspended in 200 μl TE buffer, pH 7.4. The suspensions were gently boiled for 10 min, centrifuged for 2 min to remove cell debris, and the resulting supernatant was stored at -20°C for DNA sequencing.

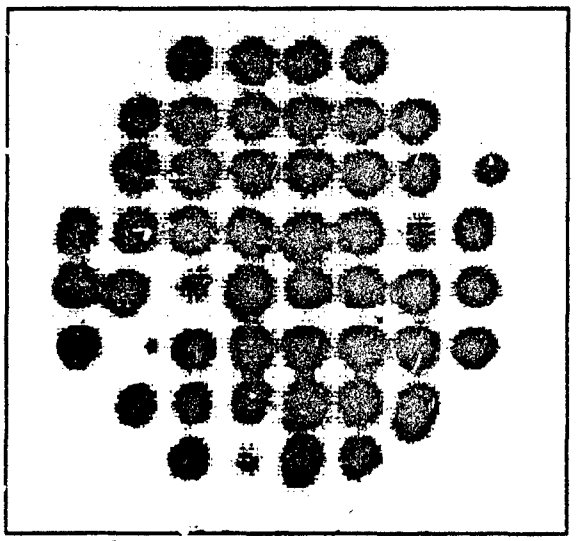

Figure 13 Autoradiograph of TC13/TC5 probed TA98 revertants induced by plant-activated 2-AF.

Non-isotopic probing

The XM300 retentate-induced revertants were isolated by picking individual colonies from minimal medium plates and streaking them onto VB-biotin quartet plates. These plates were incubated for 48 h and stored at 4°C. Samples from these plates were grown for 1-2 days in VB + biotin liquid media at 37°C. Genomic DNA was extracted using a shortened version of the bacterial DNA minipreparation procedure described in *Current Protocols in Molecular Biology* (Ausubel et al., 1992). A 1:10 dilution of the DNA served as a template to amplify a 635 base pair DNA

fragment containing the *hisD3052* region. Amplification was conducted on a DNA thermocycler programmed for 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 30 sec (extension). The PCR products were electrophoresed on 2% agarose gels. These were stained and checked for DNA bands (Figure 14).

The DNA was transferred to nylon membranes using a Bio-Rad dot blotting apparatus in which a dilution of the DNA was heated to 95°C for 10 min and pulled onto a charged nylon membrane under vacuum. O.4 N NaOH was pulled through the membrane. The membrane was removed from the apparatus, washed in 2× SSC, and crosslinked by UV. Each membrane was analyzed with the Amersham nonradioactive ECL3'-oligolabelling and detection system by using the specific probes for known mutantion events. Membranes were hybridized for 30 min at 60°C with TC-13. Fluorescein-dUTP-labelled TC5 was added and incubated for 1 h at 50°C. The membranes were washed according to the procedure outlined by the Amersham kit RPN2131 with the final wash conditions at 0.75 × SSC, 0.1% SDS at 62°C. The membrane was incubated for 30 min in block solution, rinsed, and incubated for 30 min with anti-fluorescein horseradish peroxidase conjugate. Following extensive washing, the reduction of the bound peroxidase was coupled to the oxidation of luminol resulting in light emission (428nm). The membranes were exposed for 5 min to Kodak XAR-5 X-ray film.

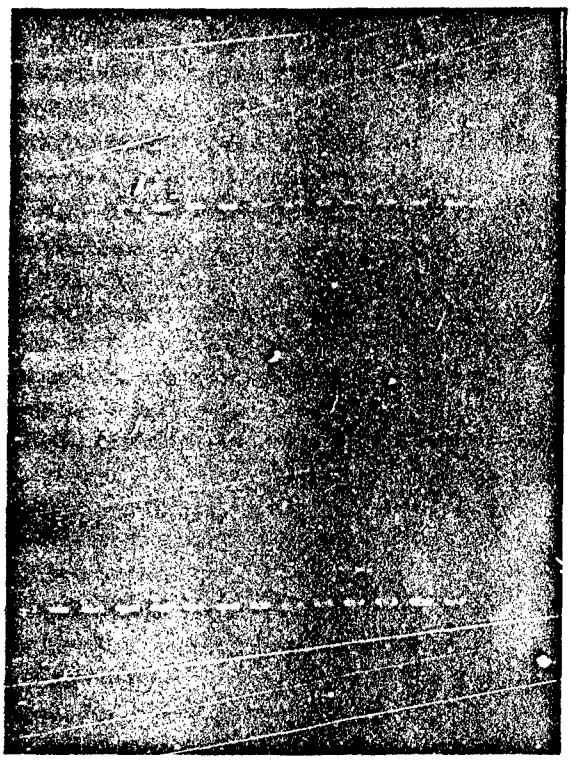

Figure 14 Gel electrophoresis illustrating the PCR products of the *hisD3052* revertants.

A majority of the XM300-induced revertants hybridized with the probe and therefore contain the -2 deletion (Figure 15). Five colonies did not hybridize. Spontaneous revertant DC-89 was used as a negative control in these studies because it is a true -2 hotspot revertant that contains a base pair substitution within the probing region. Thus, these hybridization conditions were able to distinguish mutants that differ by only a single base pair. These remaining non-hotspot mutants were sequenced using dsDNA cycle sequencing with ^{33}P.

This -CG deletion accounted for approximately 50% of spontaneous TA98 revertants (DeMarini et al., 1991). In this study, a control spectra of 225 YG1024 spontaneous revertants was generated. This deletion accounted for 70.7% of spontaneous YG1024 frameshift mutations.

98% of the 2-aminofluorene-induced revertants and 97% of the XM300 retentate-induced revertants had the common -2 hotspot deletion of the *hisD3052* allele. There is a significant increase in the specific -2 deletion between the spontaneous and induced spectra.

The sequences of *his*$^+$ revertants with non-hotspot mutations for both the spontaneous and induced spectra are represented in Table 1. We propose that all or most of the non-hotspot mutants are actually spontaneous mutations. Spontaneous mutants can not be selected against. The mutants were collected at a mutagen concentration yielding a 15 fold increase in revertants over the spontaneous rate. Therefore, 1 out of 15 induced mutants collected are likely to be spontaneous. This accounts for the 2-3% of non-hotspot mutants in the induced spectra.

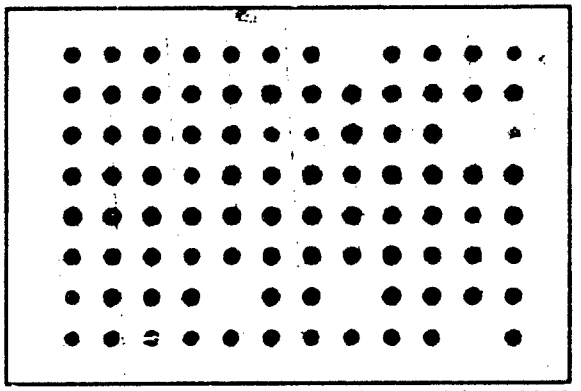

Figure 15 Southern blot analysis of *hisD3052* revertants (-2 events at D878-885) induced by the XM300 retentate fraction from plant-activated *m*-PDA.

Some of the spontaneous non-hotspot revertants have predictable mutational mechanisms. Two revertants, DC-80 and DC-54, are duplications which can be explained by the following events: polymerase fall off, template misalignment, and repolymerization. Two deletions, DC-45 and DC-52, could result from formation of a hairpin loop during DNA replication while the region is single stranded. Finally, two additions, DC-46 and DC-68, could result from a 1-base slippage within a 2 base pair repeat during replication.

Table 1. Summary of the Non-hotspot Revertant Sequences

```
                850       860       870       880       890       900       910
                 *         *         *         *         *         *         *
CONTROL  TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC

Spontaneous Revertant Sequences:

DC-13    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTG    GCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-4 & +11,877)
                                      -CCGC,+GCGGAACTGAC

DC-21    TGGCGGAGGCGGTAGAACGTCAACT           CGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-11,867)
                               -GGCGGAACTGC

DC-33    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGC    AGGCCCTGAGCGCCAGTC (-4 & +8,894)
                                                  -CGGC,+GGGGAGGC

DC-38    TGGCGGAGGCGGTAGAACGTCA        AACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-8,863/864)
                         -ACTGGCGG or -CTGGCGGA

DC-41    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGAC CC CCGGCAGGCCCTGAGCGCCAGTC (-1,889;-1,892)
                                                  -A -G

DC-44    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGC        ACCGCCGGCAGGCCCTGAGCGCCAGTC (-8,881)
                                        -GCGCGGAC
```

DC-45 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGC AGGCCCTGAGCGCCAGTC (-17,879/881)
-GCGCGGACACCGCCGGC or -GCGCGCGGACACCCGCCG

DC-46 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,870)
+G

DC-48 TGGCGGAGGCGGTAGAACGTCAACT CGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,867)
-GG

DC-49 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGC GCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-3 & +1,878)
-CGC, +A

DC-52 TGGCGGAGGCGGTAGAACGTCAACTGG GACACCGCCGGCAGGCCCTGAGCGCCAGTC (-17,868/869)
-CGGAACTGCCGCGCGCG or -GCGGAACTGCCGCGCGC

DC-54 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+4,889)
+GGAC

DC-55 TGGCGGAGGCGGT ACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-3 & +1,885)
-AGA,+T

DC-60 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGC ACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,885)
-GG

DC-68 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,898)
+G

DC-73 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGC ACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,885)
-GG

DC-76 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTAAGCGCCAGTC (+1,901 & b.s.,905)
+C G

DC-80 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+13,887)
+ACACCGCCGGCAG

DC-86 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCC T AGCGCCAGTC (-1,901;-1,905)
-C-G

DC-88 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,857)
+A

DC-89 TGGCGGAGGCGGTACAACGTCAACTGGCGGAACTGA CGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,878 & b.s.,877)
(C)-CG

DC-91 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,879)
+G

DC-94 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGAC GCCCTGAGCGCCAGTC (-11,889)
-ACCGCCGGCAG

DC-100 TGGCGGAGGCGGTAGAA ACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-14,859)
-CGTCAACTGGCGGA

Sequences of induced revertants using partially purified plant-activated 4-phenylenediamine products

D93 TGGCGGAGGCGGTAGAACGTCAACT CGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-11,867)
-GGCGGAACTGC

D209 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCG GGCAGGCCCTGAGCGCCAGTC (-2,894)
-CC

D253 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,884)
+C

D263 TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTG GCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,877)
-CC

```
D482    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGC   CGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-3 & +1,881)
                                        -GCG,+T

D574    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGCGGACACCGCCGGCAGGCCCTTAGCGCCAGTC (+1,901 & b.p.,905)
                                                          +C  (G)

D576    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGC  ACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,885)
                                              -GG

D805    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCC CGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-1 & +2,879)
                                        +CC,-G
```

Sequences of induced revertants using plant-activated 2-aminofluorene

```
EW71    TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGC  ACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,885)
                                              -GG

EW190   TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (+1,898)
                                                          +G

EW228   TGGCGGAGGCGGTAGAACGTCAACTGGCGG              GACACCGCCGGCAGGCCCTGAGCGCCAGTC (-14,873)
                                  -AACTGCCGCGCGCG

EW1269  TGGCGGAGGCGGTAGAACGTCAACTGGCGGAACTGG  CGCGCGGACACCGCCGGCAGGCCCTGAGCGCCAGTC (-2,878 & b.p.877)
                                    (C)-CG
```

Conclusions

Concentrations of plant-activated 2-aminofluorene and *m*-phenylenediamine metabolite which elicited equivalent biological effect (identical mutant fold increases over spontaneous levels) resulted in a majority of -CG deletions at D878-885 in the *hisD3052* region. The plant-activated 2-aminofluorene and *m*-phenylenediamine-induced spectra are fundamentally different from the spontaneous spectrum. This significant difference between the spontaneous and induced spectra indicates that the plant-activated *m*-phenylenediamine and 2-aminofluorene product(s) act preferentially on the hotspot region of DNA and perhaps promote or stabilize a secondary structure which leads to the hotspot mutation. Both 2-aminofluorene and *m*-phenylenediamine, in the presence of TX1 cells, are metabolized into activated products that induce the same significant increase above background levels of the -2 deletion. This suggests that the mutagenic metabolites of both a monocyclic and bicyclic aromatic amine interact with the DNA in the *hisD3052* allele in the same manner. Further resolution of non-hotspot revertants through DNA sequencing reveals that exposure to both 2-aminofluorene and *m*-phenylenediamine results in no large deletions or insertions in YG1024.

Plant-activated Products of Aromatic Amines Induce Mutation in As52 CHO Cells
AS52 Cells

Carcinogens and mutagens can affect gene function by inducing point mutations, intrachromosomal deletions, mitotic recombination, gene conversion or aneuploidy. All of these diverse mutational events have been implicated in the process of carcinogenesis as well as in the etiology of a variety of human genetic diseases. The characterization of these mutational processes in mammalian cells requires a well defined system with a mutable locus that is easily recovered for detailed molecular analyses. We utilize a Chinese hamster ovary (CHO) cell line (AS52) which carries a single functional copy of the bacterial *gpt* gene stably integrated into the CHO genome. Mutations at the *gpt* locus can be recovered as 6-thioguanine-resistant colonies and point mutations, deletions and complex rearrangements can be defined in molecular terms. Point mutational spectra are generated using the polymerase chain reaction (PCR) and DNA sequence analysis. These data provide insight into the mechanisms by which a mutagen/carcinogen exerts its genotoxic effects, especially when used in combination with defined DNA adduct chemistry.

More complicated is the molecular definition of deletions and complex rearrangements. AS52 cells are particularly well suited for the study of this class of mutations apparently because the genomic site of integration of the *gpt* locus allows the recovery of rearrangements induced by many clastogens and radio-mimetic agents that are not apparently recovered in many other in vitro mammalian mutagenesis assay systems. We have developed methods for the rapid identification of genomic rearrangements in AS52 cells using "multiplex PCR". Thus, AS52 cells are a sensitive indicator of mutagenic activity by agents which have been classified as nonmutagens in other mammalian mutagenesis assays.

Objectives

- Evaluate the mutagenicity of benzidine and *m*-phenylenediamine in AS52 cells.
- Determine if the plant-activated products of these aromatic amines directly induce mutation in AS52 cells.
- Generate a line of AS52 cells that expresses *O*-acetyltransferase and determine its sensitivity to these aromatic amines.

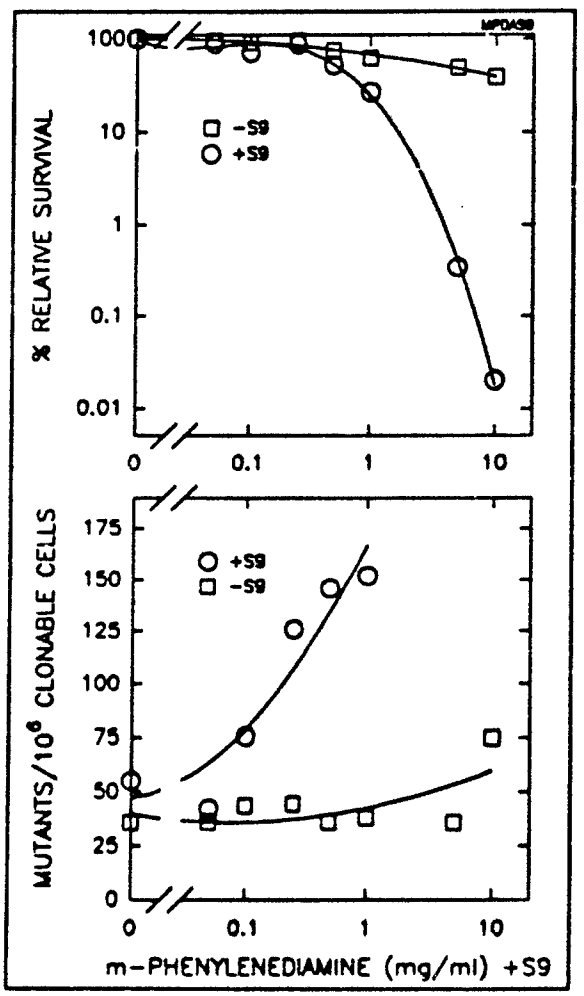

Figure 16 Mutation induction in AS52 cells with S9-activated *m*-phenylenediamine.

Results

AS52 cells were grown in MPA medium and 1×10^6 cells were added to each plate containing 1/4 XAT medium. The cells were grown overnight and the medium changed to F12 without serum. The test chemical was added and incubated for 5 h after which the cells were washed and grown in fresh medium. The percent relative survival was determined after 24 h and the cells were placed in selective medium after 6 days growth. The plates were scored for 6-thioguanine-resistant colonies after an additional 7-8 days of growth.

Benzidine — The human procarcinogen, benzidine, was assayed with AS52 cells with and without Aroclor 1254-induced ♂ rat hepatic S9. The benzidine concentration range was from 0.1 μg/ml to 800 μg/ml. AS52 cells were not mutated by benzidine.

m-Phenylenediamine — The monocyclic aromatic amine *m*-phenylenediamine was assayed for mutagenicity in AS52 cells (Figure 16). Without S9 activation, in a concentration range from 0.05 - 10 mg/ml only the highest concentration induced an increase in *gpt* mutants/10^6 clonable cells. With S9 activation, a significant increase in cell toxicity was seen at concentrations above 1 mg/ml. A concentration-dependent increase in *gpt* mutants was observed at concentrations above 0.05 mg/ml.

Analysis of Plant-Activated Products — In previous work, tobacco cells in suspension (TX1) cells have activated benzidine and *m*-phenylenediamine into mutagens as detected in *Salmonella typhimurium*. Ultracentrifuged supernatant fluids from the control (untreated TX1 cells) and TX1 cells treated with 50 μM benzidine or 500 μM *m*-phenylenediamine were fractionated by molecular size. Sterile ultrafiltration membranes were used in an Amicon model 8400 stirred cell using N_2 at 20 - 25 psi at 4°C. The retentate fraction ≥300,000 MW and the filtrate fraction ≤300,000 MW, were stored in the dark at 4°C. The XM300 retentates and filtrates were tested in *S. typhimurium* by varying the concentration of the XM300 product in 100 mM phosphate buffer and preincubating the cells for 1 h at 37°C while shaking.

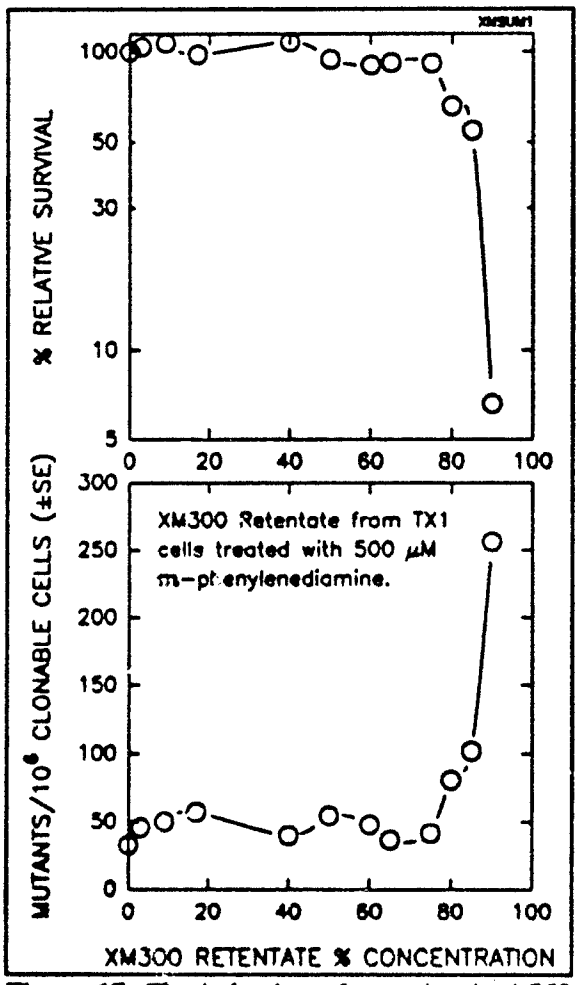

Figure 17 The induction of mutation in AS52 cells by plant-activated *m*-phenylenediamine.

The plant-activated benzidine product was a potent frameshift mutagen in strains DJ460 and YG1024. YG1024 over expresses bacterial *O*-acetyltransferase (OAT) and

DJ460 expresses human *N*-acetyltransferase (NAT2). Similar results were obtained with the plant-activated retentate from *m*-phenylenediamine-treated tobacco cells (Figure 7). The product was further metabolized into a mutagen in strains expressing bacterial OAT or human NAT2. In all cases the filtrates from each extraction as well as XM300 retentates isolated from untreated control TX1 cells did not induce mutation in any of the YG or DJ tester strains (data not shown). The plant-activated products of benzidine and *m*-phenylenediamine are high molecular weight metabolites, most probably plant-mediated conjugates.

The XM300 retentate from tobacco cells treated with 500 μM *m*-phenylenediamine was assayed on AS52 cells (Figure 17). HPLC analysis demonstrated that the XM300 retentate did not contain any of the original unmetabolized *m*-phenylenediamine. It appears that the plant-activated product was mutagenic to AS52 cells at high concentrations. The XM300 retentate at concentrations of 80% or higher was toxic to AS52 cells. From these preliminary data it appears that plant-activated *m*-phenylenediamine was moderately mutagenic to AS52 cells.

Involvement of N/O-Acetyltransferases in the Mutagenicity of Aromatic Amines — The first step in the mammalian hepatic activation of aromatic amines is *N*-hydroxylation. The *N/O*-acetyltransferases are essential in metabolizing *N*-hydroxyarylamines to their ultimate mutagenic products. In mammals *O*-acetyltransferase and *N*-acetyltransferase activities are catalyzed by the same enzyme, CoASAc-dependent arylamine *N*-acetyltransferase (EC 2.3.1.5.).

NAT1 and *NAT2* are two human genes which produce functionally distinct but similar cytosolic CoASAc:arylamine *N*-acetyltransferase. From the data I presented in pages 5-6 of this report, it is clear that acetyltransferase is essential for the metabolism of the plant-activated products of benzidine and *m*-phenylenediamine into their ultimate mutagenic forms.

An analysis of the *N*-acetyltransferase activities of AS52 and YG1024 indicates that the CHO cells have very low NAT activity (Figure 18). We believe that these data explain the poor mutagenic response of AS52 to aromatic amine promutagens.

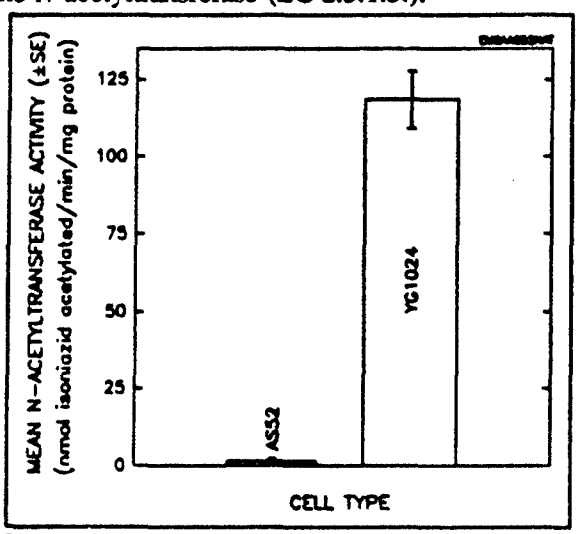

Figure 18 Comparison of the rates of *N*-acetyltransferase in AS52 and YG1024 cells.

Generation of AS52 Cells with Enhanced Acetyltransferase Activity — The *Salmonella O*-acetyltransferase gene was recovered from a *EcoRV* - *BamH*I restriction digest of pYG213. pYG213 was a gift from Dr. T. Nohmi. This *EcoRV* - *BamH*I restriction fragment was inserted in the multiple cloning site of pCDNAII. The resulting construct, pCDNAII/OAT (Figure 19) was cloned and digested with *BamH*I and *Xho*I. The resulting 1.35 kb fragment carrying the *OAT* gene was then inserted in correct orientation in the multiple cloning site of the mammalian expression vector, pCEP4. The resulting construct, pCEP4/OAT, has the *OAT* structural gene under CMV promoter regulation (Figure 20). pCEP4/OAT has an Ori P origin of replication and confers hygromycin resistance. pCEP4/OAT was introduced into AS52 cells by electroporation or lipofection. The cells were grown in medium with 400 μg/ml hygromycin. Over 80 hygromycin-resistant AS52 clones have been isolated. These clones are currently being evaluated for enhanced *O*-acetyltransferase expression. When suitable clones are identified, they will be used in mutation experiments with mammalian- and plant-activated aromatic amines. Our hypothesis predicts that AS52 cells with higher OAT activity will have an increased sensitivity to mutation induction by *N*-hydroxylated aromatic amines.

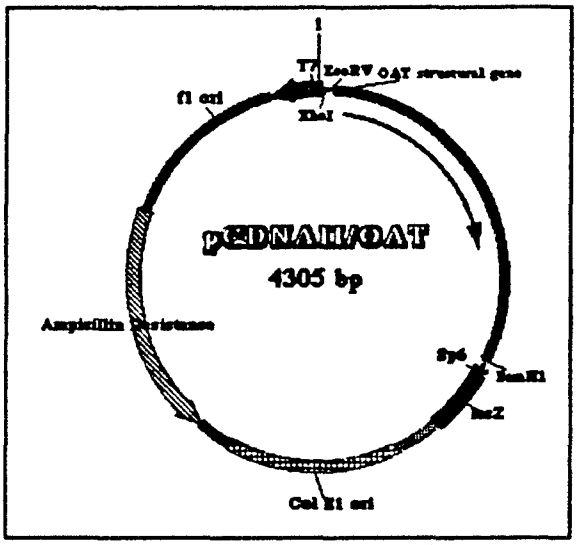

Figure 19 pCDNAII/OAT was used to generate the *Xho*I - *BamH*1 1.35 kb fragment which was used in the construction of pCEP4/OAT.

Conclusions

- Mammalian S9-activated *m*-phenylene diamine, but not benzidine, was mutagenic in AS52 cells. The plant-activated products of *m*-phenylenediamine were moderately mutagenic to AS52 cells.
- *N*-hydroxylated aromatic amines are substrates for *N/O*-acetyltransferases which initiate their ultimate mutagenic forms. AS52 cells have very low acetyltransferase activity. We generated pCEP4/OAT-transfected AS52 cells that may express OAT. We hypothesize that these cells will be sensitive to mammalian and plant-activated aromatic amine promutagens.

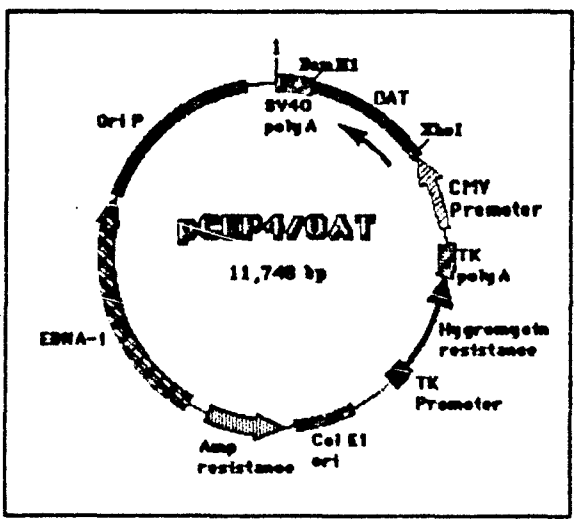

Figure 20 pCEP4/OAT used to transform AS52 cells.

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