# Base sequence-specific attack of stilbene estrogen metabolite(s) on the mitochondrial DNA: Implications in the induction of instability in the mitochondrial genome in the kidney of Syrian hamsters

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**Abstract.** We have demonstrated previously that diethylstilbestrol is metabolized to diethylstilbestrol reactive metabolites by mitochondrial enzymes *in vitro*. *In vitro*, these reactive intermediates bind to mitochondrial DNA. Here we have investigated the *in vivo* formation of diethylstilbestrol adducts with mitochondrial DNA, the nature of mitochondrial DNA-diethylstilbestrol adducts, and the influence of diethylstilbestrol adduction on *in vitro* replication of a mitochondrial gene. Diethylstilbestrol administration to male hamsters produced several adducts in mitochondrial DNA of both kidney and liver. The total relative adduct levels were 5- to 6-fold higher in mitochondrial DNA than in nuclear DNA. The chromatographic mobility of mitochondrial DNA adducts formed *in vivo* were similar to that of dGMP-DES quinone adducts formed *in vitro*. The identity of mitochondrial DNA adducts formed *in vivo* was further confirmed as dGMP-diethylstilbestrol quinone adducts by rechromatography and cochromatography. Using a DNA polymerase arrest assay we found that the DES quinone attack on a mitochondrial respiratory gene, i.e., the gene for subunit III of cytochrome c oxidase (COIII), was specific for guanine residues that were adjacent to cytosine residues. Long-term treatment with diethylstilbestrol produced tumors in the kidney, and the level of COIII transcripts was 5- to 10-fold higher in tumor samples than age-matched control kidneys. These findings suggest that i) mitochondrial DNA appears more susceptible to formation of diethylstilbestrol adducts than nuclear DNA, ii) the DNA adducts formed by DES were predominantly with guanines, iii) the adducted bases stopped DNA polymerase-mediated *in vitro* replication of the COIII gene, and iv) long-term exposure of hamsters to diethylstilbestrol elevated the expression of COIII mRNA. These results suggest that obstruction of replication of the mitochondrial genes by covalent modifications of the mitochondrial DNA by diethylstilbestrol may produce mitochondrial genomic instability *in vivo* and may provide an explanation for the DES-induced mitochondrial structural abnormality.

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## Introduction

Stilbene estrogen (diethylstilbestrol, DES) is a hormonal chemical that possesses potent estrogenic activity. It is carcinogenic in both animals and humans (1). DES has been shown to damage nuclear DNA by forming adducts (2,3) and single strand breaks and producing chromosomal aberrations (4). The effects of DES on the mitochondrial genome are not clear.

Mitochondria contain their own DNA (mtDNA), which is maternally inherited and have their own transcription and replication machinery. However, most genes encoding mitochondrial proteins are located in the nucleus. The mitochondria are the primary ATP generating organelles in all mammalian cells. The respiratory chain reactions that contribute to ATP production in mitochondria are catalyzed by multiple-polypeptide enzymes the subunits of which are encoded by genes located in the mtDNA as well as nuclear DNA (nDNA). Thirteen essential proteins of oxidative phosphorylation and electron transport, including seven subunits of NADH dehydrogenase, three subunits (I, II, and III) of cytochrome-c oxidase (CO), one subunit of the cytochrome b apoprotein of the coenzyme QH2-cytochrome c reductase, and two subunits (6 and 8) of mtATP synthase are encoded in mtDNA.

The increasing interest in the study of human diseases associated with mtDNA mutations has attracted the attention of investigators to the analysis of possible defects in the mitochondrial genome. A broad spectrum of human diseases associated with defects in mitochondrial function have been identified. In many cases, these deficiencies have been shown to be caused by mutations encoded by the mtDNA. For

example, numerous deleterious point mutations of mtDNA, now amounting to more than 30, have been shown to be associated with various types of human disorders involving deficiencies in the mitochondrial oxidative phosphorylation and electron transport apparatus. There have been many reports of abnormal mitochondria, mitochondrial mutations, and altered energy production in neoplastic cells (5). The levels of mRNA of the mitochondrial genes for NADH dehydrogenase (ND) 2, ND5, ND6, ATPase 6, ATPase 8 and COIII have been shown to be elevated in neoplastic cells, including cervical carcinoma cells, a fibrosarcoma, and an Epstein-Barr virus-transformed lymphomblastoid cell line (6). Mitochondria appear to be more sensitive to the attack of some metals and chemical carcinogens than nuclei. For example, mtDNA is the primary site of attack by reactive metabolites of procarcinogens such as benzo(a)pyrene, aflatoxin $B_1$, 7,12-dimethyl(a)anthracene, and 3-methyl cholanthrene (7).

Estrogens undergo redox cycling by mitochondrial enzymes (8,9). During the redox cycling of DES, semiquinones and quinones are produced. *In vitro*, these reactive intermediates can bind to crucial cellular macromolecules of organelles, including mtDNA (9). Whether DES is able to bind to mtDNA *in vivo* is not clear. Therefore, we determined the *in vivo* capability of stilbene estrogen to covalently bind to mtDNA and the site and base specificity of DES attack on mtDNA. We then examined the possible biological significance of adduct formation by studying the effects of DES adducts on the *in vitro* replication of a mitochondrial gene, COIII. Cytochrome c-oxidase or complex IV of the respiratory chain, is a multiple polypeptide enzyme composed of thirteen subunits. The three largest subunits (COI, COII, COIII) are encoded by mtDNA and confer the catalytic and proton pumping activities to the enzyme. The ten smaller subunits are encoded by nDNA and are thought to provide tissue specificity by adjusting the enzymatic activity to the metabolic demands of the different tissues. We selected COIII gene as a template for *in vitro* replication study, because of i) positive correlations of abnormal mitochondria and changes in mitochondrial genes associated with energy production in the neoplastic cells, particularly with COIII, ii) insertion of the mtDNA sequence encoding COIII into the 5' of exons 2 and 3 of the c-*myc* and transcription of this chimeric gene, and iii) the dual genetic make up of CO genes, i.e., encoded by both the mitochondrial and the nuclear genomes. An understanding of the molecular genetic mechanisms by which defects are produced in the mitochondrial genome by DES will provide insights into the etiology of possible defects in mitochondria and a possible role in estrogen-induced cancer.

## Materials and methods

*Materials.* The mitochondrial COIII gene was purchased from ATCC, Rockville, MD. Sequenase II kit was purchased from United States Biochemical Corporation, Cleveland, OH. RNAzol was purchased from Tel Test Inc., Friendswood, TX. Random priming DNA labeling system and RNA low molecular weight standards were purchased from Gibco BRL Life Technologies, Gaithesburg, MD. Radio-labeled $^{32}$P-dCTP (specific activity 10 mCi/ml) was purchased from Amersham Life Sciences, Arlington Heights, IL. Carrier-free [$^{32}$P]-phosphate (specific activity 285 Ci/mg at 100% isotopic enrichment or 9120 Ci/mmol) in water was purchased from ICN Radiochemical, Irvine, CA. Materials and chemicals needed for $^{32}$P-postlabeling assays were obtained from the sources described previously (2,10). DES Q was prepared as described by Roy and Liehr (2). Agarose, acrylamide, chloroform, formamide, formaldehyde, ethidium bromide and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

*Preparation of mitochondrial and nuclear DNA.* Five male hamsters were treated with 50 mg/kg of DES i.p. After 4 h, the animals were sacrificed. The livers and kidneys were removed and immediately homogenized in a buffer containing sucrose 25 mM; EDTA 1 mM and Tris 10 mM pH 7.5. The mitochondria and nuclei were isolated by differential centrifugation. DNA was isolated from purified mitoplasts and nuclei (8,9). The mitochondria were treated with 1.6% digitonin and centrifuged to collect pure mitoplasts. The mitoplasts and nuclei were resuspended in Tris pH 8.0; 10 mM EDTA; 1%SDS and gently vortexed to release the nucleic acids. The homogenate was incubated with RNAse A (10 μg/ml) and RNAse $T_1$ (20 U/ml) at 37°C for 30 min then incubated with protease K (500 μg/ml) for 1 h at 37°C. The solution was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and three times with chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated with 2 volumes ice cold ethanol at -80°C for 15 min.

*Chemical reactions between DES quinone and dGMP or COIII gene.* A bluescript plasmid containing the COIII gene was grown in ampicillin resistant *E. coli* and harvested. Plasmid DNA containing the COIII gene was extracted as described previously (11). One mg of plasmid DNA containing the COIII gene or dGMP (dissolved in 1.6 ml water) was reacted with one mg DES quinone (dissolved in 0.6 ml) for 4 h at 25°C. Reaction mixtures were extracted four times with chloroform and six times with water-saturated ethyl acetate. The aqueous phase was precipitated with ice-cold 100% ethanol. The DNA pellet was reconstituted in 10 mM citrate buffer, pH 6.0, and concentration of DNA or dGMP was estimated at 260 nm. The samples were stored at -70°C unless used immediately for adduct analysis.

*DNA adducts analysis by $^{32}$P-postlabeling.* Analysis of covalent modification in DNA was carried out by the $^{32}$P-postlabeling technique (10). DNA (10 μg) was digested with micrococcal nuclease, spleen phosphodiesterase, and nuclease P1 as described previously (10). The resolution of $^{32}$P-postlabeled adduct spots was carried out with unmodified nucleotides on polyetheleneimine-cellulose TLC plates using the following solvents: D1, 2.3 M sodium phosphate, pH 5.7; D3, 4.2 M lithium formate and 6.8 M urea, pH 3.3; D4, 0.8 M sodium phosphate, 0.5 M Tris and 8.5 M urea, pH 8.2; and D5, 1.7 M phosphate, pH 6.0. The $^{32}$P-labeled adduct spots were detected by autoradiography using Kodak X-Omat film. The spots containing $^{32}$P-postlabeled adducts were excised from TLC plates, and levels of radioactivity were determined by Cerenkov counting. The amount of adducts was calculated according to the procedures described previously (10).


Figure 1. The two dimensional thin layer chromatography of mtDNA and nDNA adducts isolated from hamster kidney. Panel A represents the two dimensional chromatograph of the dGMP-DES quinone adducts. Panel B represents nDNA adducts isolated from hamsters treated with 150 mg/kg DES. Panel C represents mtDNA adducts isolated from hamsters dosed with 150 mg/kg DES.

*Rechromatography and cochromatography.* After the *in vitro* and *in vivo* adducts were chromatographed one adduct was chosen to be further characterized. This adduct, arbitrarily given the no. 7, was found in both the *in vitro* reactions containing DES quinone and dGMP and in mtDNA of treated hamsters. The adducts were cut out and extracted individually with 1 ml of isopropanol/6 N ammonia (1:1 vol). The extract was dried to 100 µl. Five µl was counted. Equal amounts of radioactivity for each adduct were combined (500 CPM dGMP-DES quinone and 500 CPM *in vivo* no. 7). Equal counts (1000 CPM) of each adduct was chromatographed along with 1000 CPM of the mixed adducts. Seven different chromatographic systems were used. System one consisted of PEI-cellulose as the stationary phase and 4.2 M lithium formate and 6.8 M urea, pH 3.3 as the mobile phase. System two consisted of PEI-cellulose as the stationary phase and 0.4 M ammonium formate pH 6.2 as the Mobil phase. System three consists of PEI-cellulose as the stationary phase and 2-propanol/0.4 M ammonium formate pH 6.2 (1:1v/v) as the Mobil phase. System four consists of C18 as the stationary phase and 2-propanol/2-butanol/0.4 M ammonium formate pH 6.2/conc. ammonia (4:1; 14.3:2.4 v/v) as the Mobil phase. System five consists of C18 as the stationary phase and 2-propanol/0.2 M ammonium formate pH 6.2/(1.5:3 v/v) as the Mobil phase. System six consists of C18 as the stationary phase and 2-propanol/0.2 M ammonium formate pH 6.2/(2.:3 v/v) as the mobile phase. System seven consists of silica gel as the stationary phase and 2-propanol/4 N ammonia (11:9 v/v) as the Mobil phase. Each of the systems was allowed to chromatograph until the solvent reached three fourths of the length of the solid phase. The sheets were dried and exposed to X-ray film for 48 h.

*Base specific modifications assayed by DNA polymerase arrest assay.* The non-reacted and reacted COIII genes were used as DNA templates to study the influence of DES adduction on *in vitro* synthesis using DNA polymerase arrest assay in which prematurely terminated replication products were separated on a DNA sequencing gel (12,13). The reacted and non-reacted COIII genes were polymerized with the sequenase enzyme (T7 polymerase) without any dideoxynucleotide, polymerase inhibitor. The unreacted COIII gene was also sequenced using dideoxynucleotide as an inhibitor of polymerase elongation reaction. The stop sites in the reacted COIII gene was compared to the stop sites in the complete sequence to determine where DES quinone binds to DNA.

*Analysis of COIII expression in DES-induced tumor tissues.* Syrian hamsters were treated with DES pellet (22 mg DES plus 3.0 mg cholesterol) (2). Controls were given 25 mg of cholesterol. The hamsters were sacrificed at three intervals; 8, 15, and 180 days. The kidneys were removed, quick frozen in liquid nitrogen, and stored at -70°C until needed. RNA was isolated from 100 mg of tissues using RNAzol. Forty µg of RNA from each treatment group was run on a 1.5% agarose gel along with low molecular weight RNA standards. The RNA was then transferred to nitrocellulose membrane. The membrane was hybridized with radio-labeled probes for the COIII. The membrane was washed and exposed to autoradiographic film. The relative intensity of the COIII bands were analyzed by densitometry.

## Results

Examination of adducts in untreated and DES treated male hamster kidney and liver mitochondrial DNA by $^{32}$P-postlabeling revealed that DES treatment to Syrian hamsters produced several adducts in both kidney and liver mitochondrial DNA. No adducts were detected in DNA isolated from control animals (Figs. 1 and 2).

In the kidneys of hamsters treated with DES we detected three distinct adducts in the nDNA and two distinct adducts in the mtDNA. The chromatographic mobility of these adducts were different (Fig. 1). However, the mobilities of both sets of adducts were similar to the mobility of the chemically synthesized DES quinone-dGMP (Fig. 1). Although there were more adducts in the nDNA than mitochondrial DNA, the levels of adducts were higher in the mtDNA compared to nDNA. The total adduct level was $3.85 \times 10^{-5}$ and $1.98 \times 10^{-4}$ in nDNA and mDNA, respectively (Table I). The ratio of mtDNA adducts to nDNA adducts was greater than 5-fold in the kidney.

DES also formed DNA adducts in the mtDNA and nDNA of the liver. Five adducts were detected in the nDNA whereas

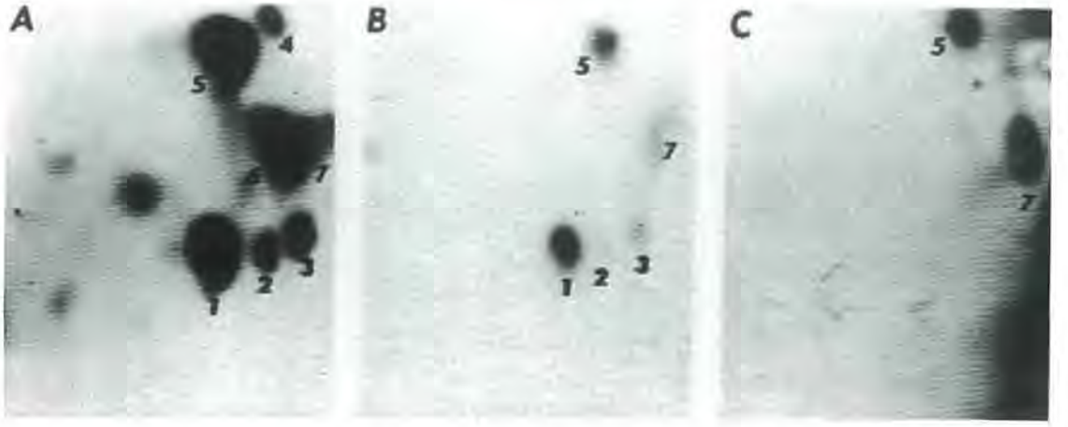


Figure 2. The two dimensional thin layer chromatography of mtDNA and nDNA adducts isolated from hamster liver. Panel A is the chromatographic profile of the DES quinone-dGMP adducts. Panel B represents the chromatographic mobility of nDNA adducts isolated from hamsters dosed with 150 mg/kg DES. Panel C represents the chromatographic mobility of mtDNA adducts isolated from hamsters dosed with 150 mg/kg DES.

Table I. Relative adduct levels (RALs) of mtDNA and nDNA adducts in kidney from DES-treated hamsters.[a]

| Adduct no. | nDNA | mtDNA |
|---|---|---|
| 1 | $2.15 \times 10^{-5}$ | - |
| 2 | $1.38 \times 10^{-5}$ | - |
| 3 | $3.13 \times 10^{-6}$ | - |
| 5 | - | $1.29 \times 10^{-4}$ |
| 7 | - | $6.86 \times 10^{-5}$ |
| Total adducts | $3.85 \times 10^{-5}$ | $1.98 \times 10^{-4}$ |
| mtDNA/nDNA | 5.14 | |

[a]This table demonstrates the RALs for each of the adducts in Fig. 1. The spots in Fig. 1 were counted and the RALs were calculated (10).

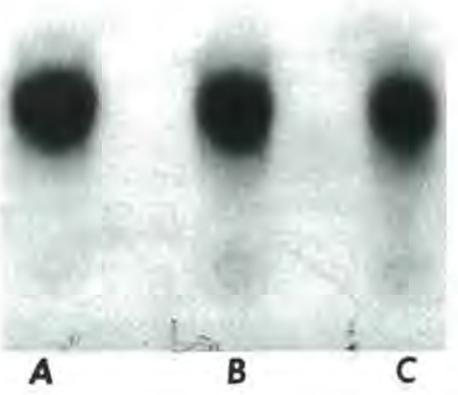


Figure 3. This figure represents the rechromatography of the dGMP and adduct no. 7 from the mtDNA. Spots from Fig. 3 that correspond to adduct no. 7 in the dGMP and adduct no. 7 of the mtDNA were extracted and rechromatographed. Lane A represents the dGMP adduct. Lane B represents a mixture of dGMP and mtDNA adduct no. 7. Lane C represents mtDNA adduct no. 7 alone.

only two were detected in the mtDNA (Fig. 2). However, the chromatographic mobility of these adducts was not completely different as demonstrated in the kidney. Two of the adducts in the nDNA of the liver cochromatographed with the mtDNA adducts (Fig. 3). The relative intensities of the adducts correlated with the concentrations (Table II). The major nDNA relative adduct level was $1.53 \times 10^{-5}$ RAL. The total relative adduct level in the mtDNA was higher than the total relative adduct level in the nDNA. The total nDNA relative adduct level was $4.5 \times 10^{-5}$ RAL and the total adduct level in the mtDNA was $2.53 \times 10^{-4}$ RAL. The ratio of mtDNA adducts to nDNA adducts is greater than five fold in the liver.

To identify one of the of DES quinone DNA adducts, dGMP was reacted with chemically synthesized DES quinone. It was demonstrated that DES quinone formed several dGMP adducts. When compared to the *in vivo* mitochondrial chromatographs adduct no. 7 from the dGMP map migrated similarly (Fig. 2). These two adducts were further analyzed by rechromatography and cochromatography. As assayed by seven different solvent systems and three different stationary phases the two adducts migrated similarly (a representative photograph using one solvent and solid stationary phase is shown in Fig. 3). This indicates that these adducts are the same with respects to chromatographic mobility.

Before carrying out DNA polymerase arrest assay using COIII gene as a template, we first ascertained that DES quinone binds to the COIII gene. DES quinone was reacted with the COIII gene for 4 h, the gene was extracted, and analyzed by

Table II. RALs for mtDNA and nDNA in DES-treated liver.[a]

| Adduct no. | nDNA | mtDNA |
|---|---|---|
| 1 | $1.53 \times 10^{-5}$ | - |
| 2 | $2.13 \times 10^{-5}$ | - |
| 3 | $4.41 \times 10^{-6}$ | - |
| 5 | $1.36 \times 10^{-5}$ | $1.72 \times 10^{-4}$ |
| 7 | $9.64 \times 10^{-6}$ | $8.03 \times 10^{-5}$ |
| Total adducts | $4.42 \times 10^{-5}$ | $2.53 \times 10^{-4}$ |
| mtDNA/nDNA | 5.72 | |

[a]This table demonstrates the RALs for each of the adducts in Fig. 2. The spots in Fig. 2 were counted and the RALs were calculated (10).

$^{32}$P-postlabeling analysis. The analysis revealed that the DES quinone generated several adducts. The chromatographic mobility of these adducts was similar to that of the dGMP that was reacted with DES quinone (Fig. 4). The COIII gene not reacted with DES quinone did not reveal any adducts.

To determine the sites of reaction of DES quinone with mtDNA, both unreacted and DES quinone reacted COIII gene were used as templates for polymerase based arrest assay. This analysis showed that there were no stop sites in the unreacted COIII gene. As demonstrated by the termination of T7 DNA polymerase one base prior to the adducted sites there were several stop positions in the COIII gene that was reacted with DES quinone. The effect of DES quinone on the arrest of polymerase was dose dependent because the intensities of the stop bands were greater in the lane where the COIII gene that was reacted with a higher concentration of DES quinone (Fig. 5). When these stop sites were compared to the control sequence it was found that 58 out of 70 stop sites were dGMP sites. Therefore, 84% of the adducts generated by DES quinone were dGMP adducts. These results confirmed our results with $^{32}$P-postlabeling. Upon further analysis it was determined that in 20 out of 24 GCC sequences the guanine bases were adducted. It appears from this data that the DNA adduct sites formed by DES were predominantly at guanines, and GCC sequences in the COIII gene was more attacked by DES quinone for adduct formation than other sequences in COIII.

We also examined the influence of DES treatment on the expression of COIII gene. Short-term treatment of animals to DES for 8 and 15 days did not produce any difference in the expression of the COIII gene (data not shown). However, in the tumor samples the expression of the COIII was increased by several folds compared to the controls (Fig. 6).

## Discussion

The present work for the first time has demonstrated that DES can covalently bind to mitochondrial DNA *in vivo*; mitochondrial DNA was 5-6 times more susceptible to the covalent attack by DES than nuclear DNA in both the liver and kidney of hamsters; the DNA adduct sites formed by DES were predominantly at guanines; and the termination of DNA polymerase progression one base prior to the adducted sites suggested that DES mediated modifications can stop replication of mitochondrial genes.

Considering the unstable nature of DES reactive intermediates and the presence of an ample quantity of nucleophilic molecules in the main organelle of metabolic activation (endoplasmic reticulum) and the cytoplasm capable of scavenging reactive intermediates, it seems unlikely that highly reactive and short-lived DES genotoxic metabolites will traverse the distance from endoplasmic reticulum to mitoplasts. It has been previously shown that mitochondria have cytochromes P450 and have the metabolic activation system to metabolize polycyclic aromatic hydrocarbons (7). Also, our recent study revealed that mitochondria are capable of catalyzing oxidation and reduction reactions of stilbene estrogen, and stilbene estrogen reactive metabolites are able to covalently bind to mtDNA, providing support to the

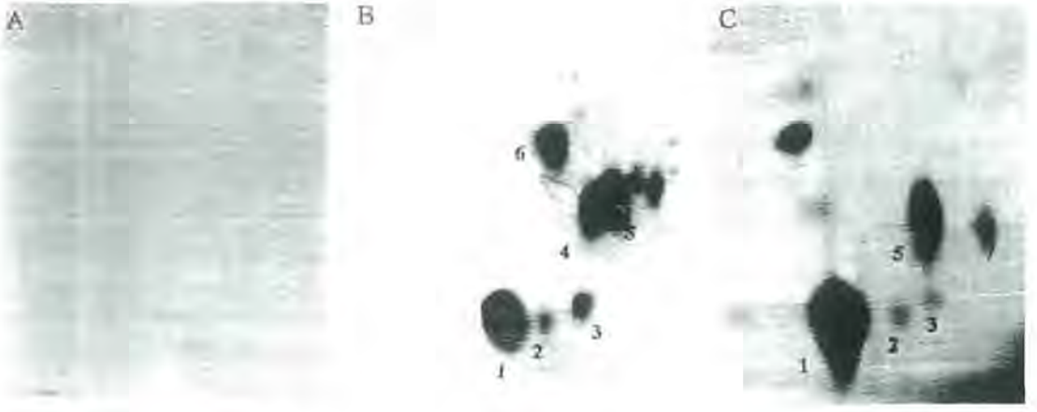


Figure 4. The two dimensional chromatography of DES quinone adducts that was generated by reacting the COIII with DES quinone. Panel A illustrates the chromatography of unreacted COIII gene. Panel B illustrates the chromatography of the dGMP reacted with DES quinone. Panel C illustrates the chromatography of the COIII gene reacted with DES quinone.


B

9210ATGACCCACC,AATCACATGC,CTATCATATA,GTAAAACCCA,GCC
ATGACCCTAACAGGGG,CCCTCTCAGC,CCTCCTAATG,ACCTCCGGCC,
TAGCCATGTG,ATTTCACTTCCACTCCATAA,CGCTCCTCAT,ACTAGGC
CTA,CTAACCAACA,CACTAACCAT,ATACCAATGATGGCGCGATG,TAAC
ACGAGA,AAGCACATAC,CAAGGCCACC,ACACACCACC,TGTCCAAAAA
GGCCTTCGAT,ACGGGATAAT,CCTATTTATT,ACCTCAGAAG,TTTTTTT
CTT,CGCAGGATTTTTCTGAGCCT,TTTACCACTC,CAGCCTAGCC,CCTA
CCCCCC,AATTAGGAGG,GCACTGGCCCCGAACAGGCA,TCACCCCGC
T,AAATCCCCTA,GAAGTCCCAC,TCCTAAACAC,ATCCGTATTACTCGCA
TCAG,GAGTATCAAT,CACCTGAGCT,CACCATAGTC,TAATAGAAAA,CA
ACCGAAACCAAATAATTC,AAGCACTGC/T,TATTACAATT,TTACTGGGT
C,TCTATTTTAC,CCTCCTACAA,GCCTCAGAGT,ACTTCGAGTC,TCCCTT
CAC,CATTTCCGAC,GGCATCTACG,GCCTCAACAT,TTTTTGTAGC,CAC
AGGCTTC,CACGGACTT,CACGTCATTA,TTGGCTCAAC,TTTCCTCACT,
ATCTGCTTCA,TCCGCCAACT,AATATTTCA,CTTTACATCC,AAACATCA
CT,TTGCCTTCGA,AGCCGCCGCC,TGATACTGGC,ATTTTGTAG,ATGTG
G/TTTG,ACTATTTCTG,TATGTCTCCA,TCTATTGATG,AGGGTCTT 9990

Figure 5. A, sequence analysis of the COIII genes which had been reacted with two different concentrations of DES quinone. Lanes 1-3 represents the COIII gene that was polymerized without any dideoxynucleotides. Lane 1, unreacted COIII gene. Lane 2, the COIII gene reacted with 1.0 mg/ml DES quinone. Lane 3, the COIII gene reacted with 0.25 mg/ml of DES quinone. Lanes denoted C, T, A, G represents sequencing reactions of the unreacted COIII gene with the corresponding nucleotides (cytosine, thymine, adenine, and guanine respectively). The actual gene sequence is complementary to the sequence shown in B. B, sequence of COIII gene showing obstruction of replication highlighted by underlining.

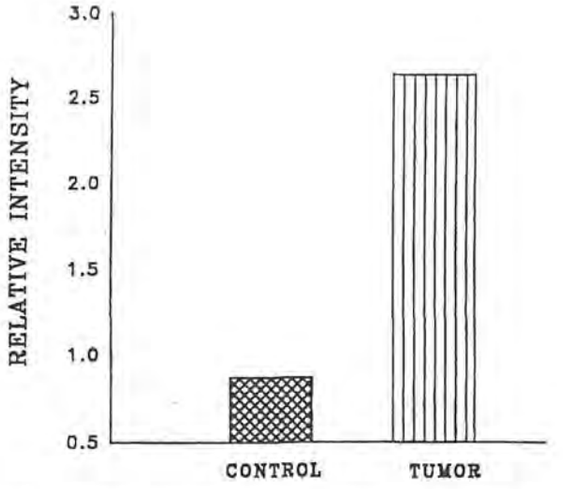


Figure 6. The relative intensity of the mRNA levels of the COIII gene in age-matched control kidneys and DES-induced kidney tumors. RNA was isolated from control and tumor samples, run on 1.5% agarose gel and transferred on nitrocellulose paper. The filter was probed with labelled COIII cDNA. The relative intensities were calculated after scanning autoradiogram of COIII gene by densitometry. The relative intensity of the background was equivalent to 0.5.

concept that in the cell the metabolic activation of stilbene estrogens to genotoxic metabolites can also occur in mitochondria (9). The fact that in the cell the activation of DES occurs in the mitoplasts may be very important because certain DES metabolites may have been prevented from reacting with mtDNA by selective trapping or transport out of the cell. This may also explain the possible mechanism by which DES metabolites covalently bind to mtDNA *in vivo*.

DES is a renal carcinogen in the Syrian hamster (1). Recently, structural abnormalities in the mitochondria of DES-treated renal cells have been reported (14). The mitochondria of tumor cells are frequently structurally and functionally different from those isolated from normal cells (15,16). Mitochondrial genome appears to be more susceptible to the attack of some metal and chemical carcinogens (7), and several mitochondrial genes have been reported to undergo more frequent changes in some cancer patients, early ageing and neuromuscular disease (17,18). The formation of mtDNA-DES adducts and arrest of DNA polymerase just prior to adducted base as observed in this study suggest that these adducts can arrest DNA polymerase progression. T7 DNA polymerase is a robust enzyme because it lacks 3'->5'-exonuclease activity and is highly processive (12,13). Thus, it appears that the arrest sites we observed reflected the actual site of synthesis termination and could also interfere with replication by mammalian polymerases *in vivo*. DES-mtDNA adducts may produce instability in mitochondrial genome presumably by affecting transcription and replication and may provide one possible explanation for the carcinogenic effects of DES.

Cytochrome oxidase (CO) is a vital component of the mitochondrial electron transport chain involved in ATP synthesis. Previous studies have shown that some subunits of CO gene are highly expressed at the mRNA level in rat

hepatoma cells (19). In this study we observed that COIII was upregulated in tumors compared to controls. The mechanism of upregulation of COIII is not clear. Whether the over-amplification of the COIII is the result of DES adduction to COIII gene, a tumor cell phenotype, change in oxygen level, or other unknown factor(s), can not be ruled out. Cancer development is frequently accompanied by an increase of energy expenditure. As one of the electron driven proton pumps of oxidative phosphorylation, CO plays an important role in energy homeostasis (20). To meet the demand of the high energy requirement, cancer cells need a high respiration rate, which could account for the elevated transcripts of some respiratory mitochondial proteins in tumors, including COIII as observed in this study.

Thus, the study presented here shows that mtDNA appear more susceptible to formation of DES adducts than nuclear DNA *in vivo*; the distribution of adduct is sequence dependent and preferentially formed in G's surrounded by C's. It also provides evidence that DES-mediated modifications in mtDNA can arrest DNA polymerase progression. These reports of modifications *in vivo* that have been correlated with arrest in gene replication *in vitro* suggest that DES-induced DNA polymerase arrest can play a significant role in the induction of instability in mitochondrial genome. Additionally, this observation may be of significant importance in providing insight into the mechanism(s) of estrogen-induced carcinogenesis. Studies are in progress to determine the structure of the DES adducts derived from the reaction of DES quinone with COIII gene.

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