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JOURNAL FOR SUBMISSION

12/3/96

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For submission to *Environmental Health Perspectives*

ANH/CMB/CP--91543
CONF-961150--1

Radiation-Induced Gene Responses

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Running Title: Radiation-induced gene responses

Key Words: radiation-induced genes; radiation; transcriptional regulation; transcription factors; DNA-damaging agents

Abbreviations: LDH — lactate dehydrogenase
UV — ultraviolet
E-UV — element regulated by UV
dd-RT-PCR — differential display-reverse transcriptase-polymerase chain reaction

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Felicia King for outstanding secretarial assistance and Drs. David Grdina and Frank Collart for critical review of the manuscript.

This work was supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38 and NCI Grant #CA73042. This publication was also made possible by grant number ES07141-02 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

ABSTRACT

In the process of identifying genes that are differentially regulated in cells exposed to ultraviolet (UV) radiation, we identified a transcript that was repressed following the exposure of cells to a combination of UV radiation and salicylate (a known inhibitor of NF- κ B). Sequencing of this band determined that it had identity to lactate dehydrogenase, and Northern blots confirmed the initial expression pattern. Analysis of the sequence of the LDH 5' region established the presence of NF- κ B, Sp1, and two Ap-2 elements; two partial AP-1, one partial CRE, and two halves of E-UV elements were also found. Electromobility shift assays were then performed for the AP-1, NF- κ B, and E-UV elements. These experiments revealed that (1) binding to NF- κ B was induced by UV, but this was repressed with salicylic acid; (2) UV did not affect AP-1 binding, but salicylic acid inhibited it alone or following UV exposure; and (3) E-UV binding was repressed by UV, and salicylic acid had little effect. Since the binding of no single element correlated with the expression pattern of LDH- β , it is likely that multiple elements govern UV/salicylate-mediated expression.

INTRODUCTION

During the past decade, many studies have identified genes induced in response to DNA-damaging agents such as UV and ionizing radiation (1–29). The collective contribution of these studies has led to the implication that several different transcription or regulatory elements play key roles in the immediate early response, including p53, AP-1, NF- κ B, and others (30–43), and to the identification of nuclear and nonnuclear events as playing essential roles in the actual induction process (15,16,44–49). For some of these transcription factors, target genes in the transcription factor regulon have been identified (16,50–54); for example, NF- κ B and AP-1 activation contributes to the induction of HIV-LTR following UV exposure (15,16,35,55–61). In addition, AP-1 and NF- κ B sites have been found in a large number of UV- and ionizing-radiation-induced genes (7,42,62–65). Nevertheless, the precise pathway following transcription factor activation by UV or ionizing radiation (or both) has not yet been mapped.

MATERIALS AND METHODS

Differential display - reverse - transcriptase polymerase chain reaction (dd-RT-PCR)

The differential display of eukaryotic messenger RNA (mRNA) by means of reverse transcription coupled with the polymerase chain reaction (dd-RT-PCR) is a technique developed by Liang and Pardee (66) in order to separate and, eventually, to clone individual mRNAs differentially expressed in mRNA preparations from similar cells. In our laboratory, however, we developed an improved dd-RT-PCR approach (26) that at the same time allows one to ignore polyT contamination and ensures that contamination with products of random priming by 5' primers will not be detected on the sequencing gel. Briefly, we are using (T)₁₂XY end-labeled primer for the PCR under conditions similar to the original, except for the use of higher concentrations of dNTPs.

Purification and sequencing of bands from dd-RT-PCR

Differentially expressed cDNAs were extracted from the dried sequencing gel for reamplification. Bands of interest were located and released from the dried gel by cutting through the film. Pieces of dried gel carrying the band of interest were soaked along with the 3MM paper (used as backing) in 100 μ l of H₂O for 10 min at room temperature and then were boiled for 15 min. After a 2-min spin in the microcentrifuge, the supernatant was transferred to a clean microcentrifuge tube and mixed with 0.10 volume of 3 M sodium acetate, 0.05 volume

of glycogen (10 mg/ml stock), and 4 volumes of ethanol. The mixture was placed at -80°C for 30 min and centrifuged for 10 min at $+4^{\circ}\text{C}$. The pellet was dissolved in 10 μl of distilled H_2O and stored at -20°C . The band was reamplified twice.

Sequencing was done with the PRIZM dyedeoxy sequencing kit according to the manufacturer's instructions. Sequencing electrophoresis was carried out by first dissolving the dried sample in 4 μl of a 5:1 mixture of deionized formamide and 50 mM EDTA (pH 8.0). Immediately before loading, the sample was heated to 90°C and then run on a standard sequencing gel prepared for use with a DNA sequencer (Applied Biosystems 373A). Gene sequences were compared to those available in the GenBank using the BLAST search program (available at BLAST@NCBI.NLM.NIH.GOV).

EMSA binding

Electromobility shift assays (EMSA) were performed by using the consensus- containing oligonucleotides that were labeled with $\gamma[^{32}\text{P}]\text{ATP}$ in a T4 polynucleotide kinase reaction. For a nonspecific competitor (negative control), we used an irrelevant recognition sequence (Sp1). All binding conditions for proteins (1 μg) were similar to those described by Schreiber *et al.* (67). The reactions were done in the presence of sonicated salmon sperm DNA as a nonspecific binding inhibitor and with 0.8–5 μg of unlabeled crude nuclear protein extract. The assays were set up to use lysate from equal amounts of protein for each experiment. The free oligonucleotides were resolved from protein-DNA complexes by Tris-borate polyacrylamide gel

electrophoresis (70). The DNA bands were resolved by autoradiography on a PhosphorImager.

RNA analyses

For all genes, we verified expression patterns by Northern blots. RNA was routinely purified in our laboratory by isolation in guanidine isothiocyanate, extraction from phenol, and precipitation from 3 M sodium acetate, pH 6.0, (22–24). RNA was stored as an ethanol precipitate at $-20\text{ }^{\circ}\text{C}$.

For Northern blot analysis, RNA was separated by using formaldehyde-agarose gel electrophoresis as described previously (22–24). Northern transfers were performed as described (22–24). The mass probe was an end-labeled 18S rRNA 30-nt-long probe; the LDH probe was labeled from dd-RT-PCR amplified with $\alpha[^{32}\text{P}]\text{dCTP}$ as the sole source of dCTP. Hybridization conditions were 50% deionized formamide, 0.75 M NaCl, 75 mM sodium citrate, 25–50 mM sodium phosphate (pH 6.5), 0.2% sodium dodecyl sulfate (SDS), 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 50 $\mu\text{g}/\text{mL}$ sonicated denatured herring sperm DNA at $43\text{ }^{\circ}\text{C}$. Prior to hybridization, all labeled probes were heat-denatured at $90\text{ }^{\circ}\text{C}$ for 5 min. After hybridization, nonspecific binding was reduced by washing the blot three times for 1 h each at $65\text{ }^{\circ}\text{C}$ in 45 mM sodium citrate (pH 7.4), 0.45 M NaCl, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 $\mu\text{g}/\text{mL}$ herring sperm DNA (sonicated, denatured), and 0.1% SDS, followed by three more washings for 1 h each at $65\text{ }^{\circ}\text{C}$ in 1.5 mM sodium citrate (pH 7.4), 15 mM NaCl, 50 $\mu\text{g}/\text{mL}$ herring sperm DNA (sonicated, denatured), and

0.1% SDS. The blot was then dried and exposed (to x-ray film at $-70\text{ }^{\circ}\text{C}$ with intensifying screens) on the PhosphorImager.

RESULTS AND DISCUSSION

Previous studies by several groups have shown that NF- κ B binding is inhibited in HeLa cells by the addition of salicylate to the medium (71,72). HeLa cells (which lack functional *p53*) were either untreated or were exposed to UV, UV plus salicylate (UV/sa), salicylate (sa), *cis*-Pt, *cis*-Pt plus salicylate (*cis*Pt/sa), vinblastine (vin), vinblastine plus salicylate (vin/sa), UV plus indomethacin (UV/indo), or *cis*Pt plus indomethacin (*cis*Pt/indo). Concentrations and exposures were as previously published by our group in studies demonstrating that salicylate inhibits UV- and *cis*Pt-mediated HIV-LTR transcription (72). Bands were selected by dd-RT-PCR using primers and sequencing protocols previously published by our group (26).

The sequence of one dd-RT-PCR band (T102) is presented in Table 1. It was found to have identity with the human lactate dehydrogenase β -chain (LDH- β) 3' region. Northern blots of the LDH- β probe hybridized to RNAs derived from control cells and from cells exposed to UV or *cis*-Pt, with or without salicylic acid, revealed that LDH- β mRNA was not affected by any exposures except when UV and salicylate or cisplatin and salicylate were combined; these treatments caused a marked reduction in LDH- β mRNA accumulation (Table 2).

The 5' flanking region of the LDH- β gene was analyzed using sequence information available from GenBank. A summary of these results is presented in Figure 1, showing the presence of complete sites for NF- κ B, Sp1, and AP-2 elements as well as partial sites for AP-1 and CRE elements. In addition, an element recently defined in our laboratory called E-UV (73) was also found to be present, but with several base pair changes relative to the consensus

sequence. This element has been found to be split in several different genes and appears to be split in LDH- β as well.

Gel shift analyses of the AP-1, NF- κ B, and E-UV sequences were performed using extracts of cells exposed to UV, salicylic acid, UV/salicylic acid, etc. An example of one such gel shift is presented in Figure 2, in which E-UV (Fds = full-length, double stranded) and AP-1 elements were examined. The results (Table 2) revealed that no single element showed precisely the same binding pattern as the expression pattern of LDH- β mRNA. Therefore it is likely that several of these elements (and perhaps some working in concert) govern regulation of LDH- β following UV/salicylate exposure. It has been documented in the literature that salicylate inhibits NF- κ B binding to its element. The experiments in Figure 2 demonstrate that AP-1 binding is also inhibited by prior exposure of cells to salicylate. This suggests that UV-induced responses that are affected by salicylate may involve either the NF- κ B or AP-1 pathways.

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FIGURE LEGENDS

Figure 1. Diagram of LDH- β 5' flanking region with sites for complete elements (NF- κ B, Sp1, Ap2) and partial elements (AP-1, CRE, E-UV) indicated.

Figure 2. Gel shifts were performed as described by Schreiber *et al.* (67) and Lederer *et al.* (69). For each experiment, HeLa cell extracts and consensus elements were purchased from Promega Biotech. The + and - strand oligonucleotides of the E-UV elements, annealed to create full-length double-stranded (Fds) sequence, were synthesized on a "gene synthesizer" (Applied Biosystems) according to the manufacturer's conditions. Reactions were performed in the presence of sonicated salmon sperm DNA, nonspecific binding inhibitor, and with 0.8 μ g of HeLa unlabeled crude nuclear protein extract, or 5 μ g HeLa nuclear extract purchased from Promega Biotech (comm. extract). Free oligonucleotides were resolved from protein-DNA complexes by Tris-borate polyacrylamide gel electrophoresis. The dried gel was exposed on the PhosphorImager screen.

Table 1. Sequence Comparison: Band T102 vs. Human LDH-B

```

T102      200 TCACACNACAATAGTTAATTTTATTTGTTCAAGAGCTCAGATTGCAAGCATTAAACCAAG
           |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
huLDH-B   6 TCACACTACAATAGTTAATTTTATTTGTTCAAGAGCTCAGATTGCAAGCATTAAACCAAG

140 CATAGGCTTTGATTCTGTGAGCCCAATTCACATATTGNAGNAGATCNAAGCNANCTGTG
           |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
66 CATAGGCTTTGATTCTGTGAGCCCAATTCACATATTGAAGAAGATCAAAGCAAACCTGTG

86 ATCCATGTACATGGNTGAAAACCTAAAGGCTCGAGTTAATCACATTGTAGTTTTTAAATTT
           |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||  |||||
126 ATCCATGTACATGGATGAAAACCTAAAGGCTCGAGTTAATCACATTGTAGTTTTTAAATTT

20 CTACANCCTAGANCTCACTA 1
   |||||  |||||  |||||  |||||
186 CTACAGCCTAGAGCTCACTA 205

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T102 vs. huLDH-B
95 % identity

Table 2. Summary of Effects of Various Treatments on LDH- β mRNA and Transcription Factor Binding

	Control	UV	sal	UV/sal	Cis	Cis/sal	vin
LDH- β mRNA	0	0	0	↓	0	↓	0
AP-1 binding	0	0	↓	↓	0	↓	ND
NF- κ B binding	0	↑	0	↓	↑	↓	↑
E-UV binding	0	↓	0	0	↓	0	ND

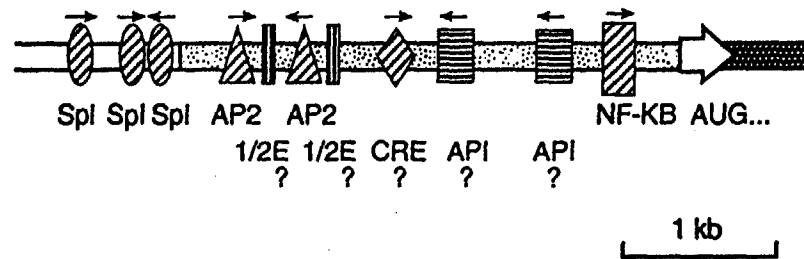
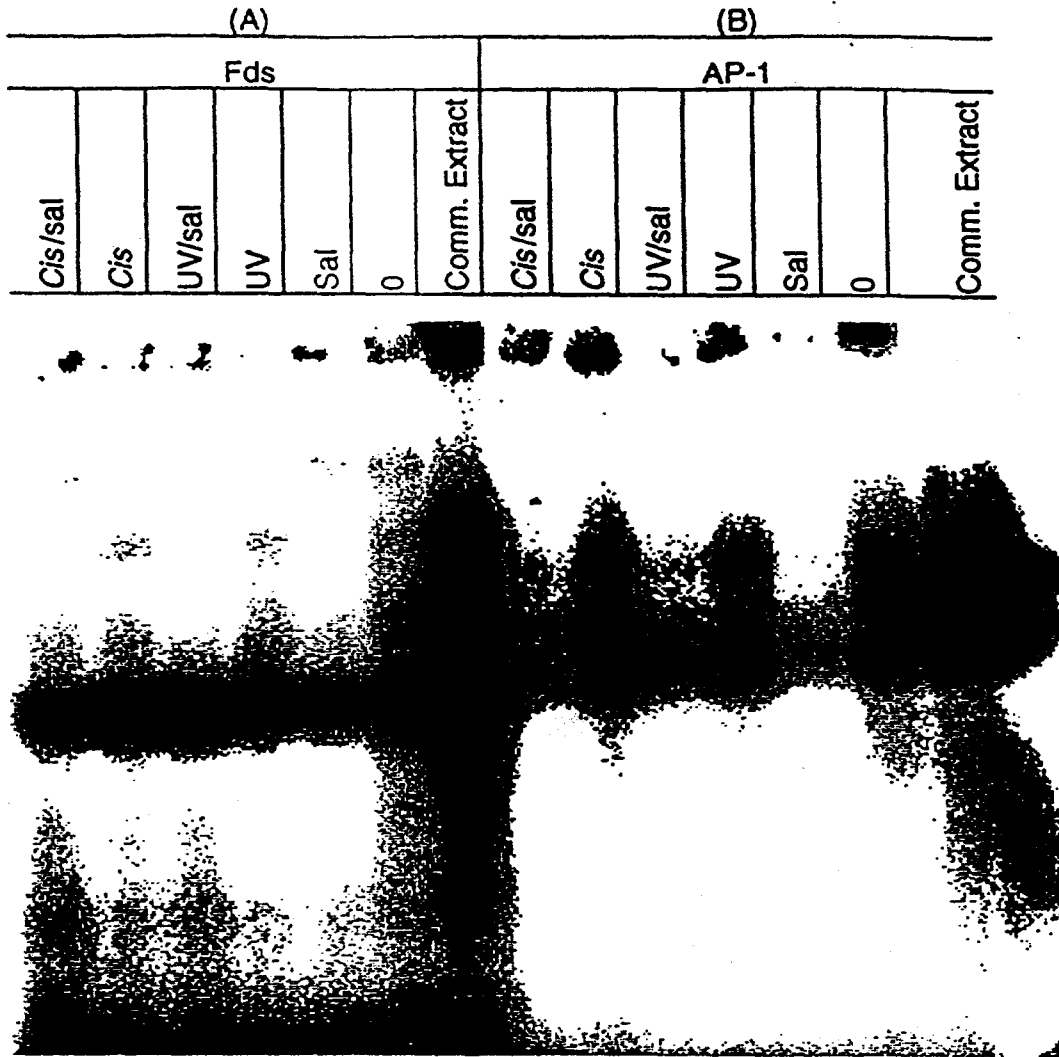


Fig. 1



Gel Shift Assay of HeLa cells nuclear extract after cells were treated with , UV (16 joule/m²/sec), and *cis* platinum (*cis*) 50 μM/ml with or without salicylic acid (5 mg/ml). Labeled Fds (double stranded C1 oligonucleotide) and AP-1, a transcriptional element were reacted with nuclear proteins in a gel shift assay. Protein concentrations were standardized so each sample had 175 mg/ml. Sample volume was 5 μl (final concentration, 0.8 mg).

Fig. 2