Mechanisms of Radiation-Induced Gene Responses[1]

Gayle E. Woloschak and Tatjana Paunesku

Center for Mechanistic Biology and Biotechnology
Argonne National Laboratory
9700 South Cass Avenue
Argonne, Illinois 60439-4833

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ABSTRACT

In the process of identifying genes differentially expressed in cells exposed to ultraviolet (UV) radiation; we identified a transcript having a 25-bp region that is highly conserved among a variety of species including *Bacillus circulans*, pumpkin, yeast, *Drosophila*, mouse, and man: 5′ AAGTGTTCTGCATAAGTGGCTTCC 3′. When in the 5′ region (flanking region or UTR) of a gene, the sequence is predominantly in +/+ orientation with respect to the coding DNA strand; while in the coding region and the 3′ region (UTR), the sequence is most frequently in the –/+ orientation with respect to the coding DNA strand. In two genes, the element is split into two parts; however, in most cases, it is found only once but with a minimum of 11 consecutive nucleotides precisely depicting the original sequence. The element is found in a large number of different genes with diverse functions (from human *ras* p21 to *B. circulans* chitosanase). Gel shift assays demonstrated the presence of a protein in HeLa cell extracts that binds to the sense and antisense single-stranded consensus oligomers, as well as to double-stranded oligonucleotide. When double-stranded oligomer was used, the size shift demonstrated an additional protein-oligomer complex larger than the one bound to either sense or antisense single-stranded consensus oligomers alone. It is speculated either that this element binds to protein(s) important in maintaining DNA in a single-stranded orientation for transcription or, alternatively that this element is important in the transcription-coupled DNA repair process.

INTRODUCTION

During the past decade, many studies have identified genes induced in response to DNA-damaging agents such as UV and ionizing radiation (Anderson and Woloschak 1992; Boothman *et al.* 1991; Fornace *et al.* 1988, 1989; Fornace, 1992; Herrlich *et al.* 1992; Hallahan *et al.* 1989; Libertin *et al.* 1994; Martin *et al.* 1993; Munson and Woloschak 1990; Panozzo *et al.* 1991; Peak *et al.* 1991; Ramsamooj *et al.* 1992; Ronai *et al.* 1988; Stein *et al.* 1989a, b; Valerie *et al.* 1988; Woloschak and Chang-Liu 1990, 1991, 1992, 1995; Woloschak *et al.* 1990a, b, c, 1994, 1995a, b, c; Sakakeeny *et al.* 1994). The collective contribution of these studies has led to the implication of several different transcription or regulatory elements as playing a key role in the immediate early response, including p53, AP-1, NF-κB, and others (Hallahan *et al.* 1991; Kastan *et al.* 1991; Nelson and Kastan 1994; Sun *et al.* 1995; Brach *et al.* 1991; Angel *et al.* 1987; Andalibi *et al.* 1993; Kharbanda *et al.* 1995; Datta *et al.* 1992, 1993; Mohan and Meltz 1994; Prasad *et al.* 1994; Sahijdak *et al.* 1994; McKenna *et al.* 1991), and the identification of nuclear and nonnuclear events as playing essential roles in the actual induction process (Stein *et al.* 1989a, b; Uckun *et al.* 1992, 1993; Simon *et al.* 1994; Devary *et al.* 1993; Hayashi *et al.* 1993; Koong *et al.* 1994). For some of these transcription factors, target genes in the transcription factor regulon have been identified (Brach *et al.* 1993; Dominquez *et al.* 1993; Engstrom *et al.* 1993; Finco and Baldwin 1993; Kunsch and Rosen 1993; Stein *et al.* 1989b); for example, NF-κB and AP-1 activation contributes to the induction of HIV-LTR following UV exposure (Zmudzka and Beer 1990; Schreck *et al.* 1991, 1995; Biswas *et al.* 1993; Kretzchmar *et al.* 1992; Perkins *et al.* 1993; Angel *et al.* 1987; Schmid *et al.* 1991; Stein *et al.* 1989a, b). In addition, AP-1 and NF-κB sites have been found in a large number of UV- and ionizing-radiation-

induced genes (Hiscott *et al.* 1993; Messer *et al.* 1990; Lacoste *et al.* 1990; Hallahan *et al.* 1989; Sahijdak *et al.* 1994; Singh and Lavin 1989). Nevertheless, the precise pathway following transcription factor activation by UV or ionizing radiation (or both) has not yet been mapped. The purpose of these experiments is to identify the subset of the NF-κB regulon that is inducible by UV and the subset of UV-induced genes that is contained within the NF-κB regulon. The identification of this NF-κB-proximal step in the UV-induced response pathway will lead to further elucidation of mechanisms of UV-mediated late effects such as apoptosis, DNA repair, or mutation fixation.

MATERIALS AND METHODS

Differential-display real-time polymerase chain reaction (dd-RT-PCR)

Differential display of eukaryotic messenger RNA (mRNA) by means of the polymerase chain reaction (PCR) is a technique developed by Liang and Pardee (1992) in order to separate and, eventually, to clone individual mRNAs differentially expressed in mRNA preparations from similar cells; however, in our laboratory, however, we developed an approach (Woloschak *et al.* 1995a) 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 $(T)_{12}XY$ 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

Bands were extracted from the dried sequencing gel for reamplification. Bands of interest were located and marked by needle punches or 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 µl of H_2O 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 volumes of 3 M sodium acetate, 0.05 volumes of glycogen (10-mg/ml stock), and 4 volumes of EtOH. The mixture was placed at –80 °C for 30 min and centrifuged for 10 min at +4 °C. The

pellet was dissolved in 10 µl of distilled H_2O and stored at –20 °C. The band was reamplified twice.

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 can be compared to those available in the GenBank for identification of the gene.

EMSA binding

Gel mobility shift assays were performed by using the consensus element, which was labeled γ-^{32}P-ATP in a T4 polynucleotide kinase reaction. For a 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.* (1989), Davis *et al.* (1986), and Lederer *et al.* (1996). The reactions were done in the presence of poly (dI-dc) nonspecific inhibitor and with 3.5 µg of unlabeled crude nuclear protein extract. The assays were set up to use lysate from equal numbers of cells for each experiment. The free oligonucleotides were resolved from protein-DNA complexes by Tris-acetate polyacrylamide gel electrophoresis (Jones *et al.* 1985). The DNA bands were resolved by autoradiography (Lo *et al.* 1991). NF-κB-1 and NF-κB-2 can be purchased in purified form from Promega Biotech.

RNA analyses

For all genes, we verified induction following UV exposure by dot blots and 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) (Woloschak *et al.* 1990a, b, c). RNA was stored as an ethanol precipitate at –20 °C.

Large-scale dot blot screening of differentially expressed bands was performed as described (Woloschak *et al.* 1988, 1995a) and as shown in preliminary results. In brief, DNA to be probed (PCR products) was spotted in excess on nitrocellulose filters and hybridized to ^{32}P-γ-labeled RNA extracted from unexposed and UV-exposed cells.

For Northern blot analysis, RNA was separated by using formaldehyde agarose gel electrophoresis as described previously (Woloschak *et al.* 1990a, b, c). Northern transfers were performed as described (Woloschak *et al.* 1990a, b, c). The blots were hybridized to ^{32}P nick-translated or oligo-labeled DNA probes. 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 µg/mL sonicated denatured herring sperm DNA at 43 °C. Prior to hybridization, all labeled probes were heat-denatured at 90 °C for 5 min. After hybridization, nonspecific binding was reduced by washing the blot three times for 1 h each at 65 °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-µg/mL herring sperm DNA (sonicated; denatured), and 0.1% SDS, followed by three more washings for 1 h each at 65 °C in 1.5 mM sodium citrate (pH 7.4), 15 mM NaCl, 50-µg/mL herring sperm DNA (sonicated;

denatured), and 0.1% SDS. The blot was then dried and exposed to x-ray film at –70 °C with intensifying screens.

RESULTS AND DISCUSSION

The experiments to identify UV-inducible genes that are NF-κB-dependent (but p53-independent) take advantage of previous studies by several groups documenting that κB binding is inhibited in HeLa cells by the addition of salicylate to the medium (Ghosh and Kopp 1995; Woloschak *et al.* 1995d). HeLa cells (which lack functional p53) were untreated or 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) (see Table 1). Concentrations and exposures are as previously published by our group in studies demonstrating that salicylate inhibits UV- and *cis*Pt-mediated HIV-LTR transcription (Woloschak *et al.* 1995d). Bands were selected by dd-RT-PCR using primers and were sequenced using protocols previously published by our group (Woloschak *et al.* 1995a). These bands were compared with sequences in GenBank and dbEST (database for expressed sequence tags); identities as determined by the search are listed in Table 1. Bands listed in Table 1 for which "Features" are listed were confirmed to show the reported expression patterns (features) by screening dot blots or Northern blots (Woloschak *et al.* 1995a). The genes marked by and asterisk in Table 1 were further confirmed to be UV-induced and salicylate-inhibited by Northern blot. These bands were obtained by using different arbitrary primers and different anchored dT primers. In analyzing all experiments using a large number of primer pairs (for which we have not yet sequenced all differentially expressed bands), approximately 2500 bands are evident on the total gels, suggesting that nearly 25% of the expressed genome in HeLa cells is represented. Table 1 presents many genes analyzed in this

experimental set from the same experimental and cell conditions to demonstrate the large number of primer sets studied to date.

Table 1. Identification of Human Genes Expressed Differentially

Band	Primers[+]	Features	Identity
T100	T_{12}VA/380-1		None
T101	T_{12}VA/380-1		STS UT930 (69% over 51 nt)
T102	T_{12}VA/380-1		L-lactate dehydrogenase H chain (95% over 200 nt)
T103	T_{12}VA/380-1		None
J1	T_{12}VA/R3		mt NADH-ubiquinone reductase (24k)
T20	T_{12}VC/R2		ND
T21	T_{12}VC/R2		ND
T22	T_{12}VC/R2		ND
T23	T_{12}VC/R2		ND
T24	T_{12}VC/R2		ND
T25	T_{12}VC/R2		cDNA clone c - OubO3 (77% over 39 nt)
J2	T_{12}VA/R3		ND
J3	T_{12}VA/R3		ND
J4	T_{12}VA/R3		ND
J5[*]	T_{12}VA/R3	↑UV/sa ↑sa	cDNA R06677 (60% over 203 nt) SP-100 (83% over 39 nt)
J6	T_{12}VA/R3		ND
T30	T_{12}VC/R1		ND
T31	T_{12}VC/R1		ND
T32	T_{12}VC/R1		ND
T33	T_{12}VC/R1		Plant ribosomal protein S19 (69% over 58 nt)
T34	T_{12}VC/R1		EST/09855 (68% over 45 nt)
T35	T_{12}VC/R1		Glycogen phosphorylase (67% over 231 nt)
J20	T_{12}VG/375-2		EST 396613 (67% over 46 nt)
J21[*]	T_{12}VG/375-2		Human ribosomal protein S23 (58% over 246 nt)
J22	T_{12}VG/375-2		ND
J30	T_{12}VG/380-1		ND
J31	T_{12}VG/380-1		None
T1	T_{12}VA/TCE		ND

Table 1. Identification of Human Genes Expressed Differentially

Band	Primers[+]	Features	Identity
T2	T_{12}VA/TCE		ND
T3	T_{12}VA/TCE		ND
T4	T_{12}VA/TCE		ND
T5	T_{12}VA/TCE		ND
T6	T_{12}VA/TCE		ND
T7	T_{12}VA/TCE		ND
T8	T_{12}VA/TCE		ND
T9	T_{12}VA/TCE		ND
S1	T_{12}VA/R2		ND
S2	T_{12}VA/R2		ND
S3	T_{12}VA/R2		ND
S4	T_{12}VA/R2		ND
S5	T_{12}VA/R2		ND
S6	T_{12}VA/R2		ND
P1	T_{12}VA375-2		ND
P2	T_{12}VA/375-2		ND
P3	T_{12}VA/375-2		ND
P4	T_{12}VA/375-2		ND
P5	T_{12}VA/375-2		ND
I1	T_{12}VT/R2		None
I2	**T_{12}VT/R2[*]**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo**	**Mitochondrial-specific single-stranded DNA binding protein (M94536)**
I3	**T_{12}VT/R2[*]**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo; I3=I2**	**Mitochondrial-specific single-stranded DNA binding protein (M94536)**
I4	T_{12}VT/R2		Human U2 snRNP spec. A′ protein (69% over 75 nt)
I5	T_{12}VT/R2		ND
I6	T_{12}VT/R2		ND
I7	T_{12}VT/R2		Human cDNA clone 73459 (83% over 37 nt)
I8	T_{12}VT/R2		Human cDNA clone 41132 (81% over 172 nt)
C1	**T_{12}VT/LTK3[*]**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo**	**Human cDNA clone 125698 (RO7494) conserved sequence in 5′ UTR in NF-κB p49 (see Table 2)**
C2	T_{12}VT/LTK3		ND
C3	**T_{12}VT/LTK3[*]**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo**	
D1	T_{12}VT/R1		ND

Table 1. Identification of Human Genes Expressed Differentially

Band	Primers+	Features	Identity
D2	T_{12}VT/R1		ND
D3	T_{12}VT/R1		Rabbit endopeptidase/(67% over 211)
D4	T_{12}VT/R1		ND
L1	T12VC/375-2		ND
Y1	**T12VT/375-2***	**↑UV ↓UV/sa ↑*Cis*Pt ↓*Cis*Pt/sa n.c. UV/indo**	**Human subclone 10-b2 (292095) (65% over 49 nt) *S pombe* cosmid C12C2 (254 b) (71% over 39 nt)**
U1	T12VG/375-2		Human ribosomal 60S protein L32
Q2	**T12VT/Ltk3**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo**	**None**
R1	T12VT/375-2		ND
R2	**T12VT/375-2**	**↑UV ↓UV/sa ↑*cis*Pt ↓*cis*Pt/sa n.c. UV/indo R2=Y1**	**Human subclone 10-b2 (292095) (65% over 49 nt) *S pombe* cosmid C12C2 (254 b) (71% over 39 nt)**
L1	T12VC/375-2		ND

Bold = have criteria to be included in the studies proposed here (i.e., ↑ UV ↓ UV/sa, etc.).
* = bands confirmed by Northern blots to show the expression pattern indicated in features column.
n.c. = no change.
+ = primer sequences are defined in Table 3, Research Design.
ND = search through databases not yet complete.
None = no homology found in database.

In the analysis of band C1, we obtained some very interesting information regarding a consensus sequence in the C1 transcript. Table 2 provides a partial sequence of band C1, which meets the criteria for the experiment proposed here (i.e., it is UV-induced and UV/salicylate repressed). The portion that bears high homology to a 25-bp sequence conserved in 3′/5′ UTRs or 5′ flanking regions of several human genes is shown for a large number of genes identified in the GenBank database (including NF-κB p49 subunit, TcR-C-δ, β-globin gene, stromelysin, *ras* p21, superoxide dismutase, etc.; see Table 2). Interestingly, this consensus sequence is also highly conserved among species, being found in mammalian genes as well as in *Caenorhabditis elegans*, *Limulus* (horseshoe crab), and even plant species. Two have been found in prokaryotes

although this finding requires more extensive analysis. This suggests an important regulatory role for the sequence. The element is "split" in two genes — soybean PcP carboxylase and human CD36A antigen. This consensus appears to be highly conserved across the evolutionary tree. It is of significance that one gene bearing this sequence in the 5′-UTR is the NF-κB p49 subunit; however, the orientation of the sequence relative to the coding region, differs if it is in the 5′ UTR or 5′ flanking region (+/- or -/+) or if it is in the 3′ UTR (-/- or +/+). This difference suggests a functional significance to the location of the element. It is noteworthy than many genes bearing the element are induced by UV exposure in different cell systems (NF-κB, stromelysin, superoxide, dismutase, Band C1, and *ras* p21). (Note that calculations determining the chance occurrence of sequences have shown that on the basis of chance alone, this sequence would be found not more than once in the entire human transcribed sequence database.) A search of dbEST reported over 80 transcripts bearing this element.

The sequence used for gel retardation assays is shown at the bottom of Table 2. This oligo was used in + or sense (shown) or in antisense orientation (or in both) in standard mobility shift assays. The results (shown in Fig. 1) demonstrate the following: (1) NF-κB does not bind to the element; (2) the element binds a protein or proteins from HeLa cell extracts in sense or antisense single-stranded orientation or in double-stranded form; (3) this binding is not competed out with cold κB or Sp1 binding sites but is competed out with cold consensus oligonucleotide; (4) binding for the double-stranded form uses different or additional proteins than binding of the single-stranded forms. A single gel shift experiment revealed that while NF-κB binding is induced with UV, binding of HeLa cell extract proteins to the consensus element is repressed with UV. This is precisely the sort of element that we propose to find in the experiments outlined here. The function of this element is not yet known, although it is possible that the

element is important for maintaining a single-stranded conformation, for transcription-coupled repair, or as a repressor element for UV-induced responses.

Table 2. The 25-nt Consensus Sequence in Sequences from Nonredundant Databases.

Gene	Sequence	Location	Orientation
Band C1	AAAGTGTTCTGCATAAGTGGCTTCC	3′ UTR	-/+
Limulus coagulation inhibitor type 92	AAAGTCTTCTGCATAAGAGGATACC	coding region	-/+
Mouse EST clone 92	GGACTGTTCTGCATAGCTGGCTTAA	mRNA	+/+
human cDNA clone 125698	AAAGTGTTCTGCATAAGTGGCTTCC	3′ UTR	-/+
human NF-κB p49 subunit*	TATCATTTTTGCATAAGTGGTTTCA	5′-UTR	-/+
human mt NAD(P)-dependent malate enzyme	CTTGGGTTCAGCATAAGTGGCTTGC	coding	-/+
human clone 178950	TTTGTGTTGTGANTAAGTGGCTTCT	5′-UTR	+/+
C. elegans cosmid T13C2 (LDL-receptor related protein)	CACTTGTTCTGCATAAGAGGTTTGC	coding region	-/+
ras p21 (human GTPase-activating protein)	AATCTGTTCAGCATAAGTGGCCTAC	coding region	+/+
Mus choline acetyltransferase	CCAGGGTTCTGCATAAGCAGCTGCC	5′ flanking	+/+
soybean PcP carboxylase at 2914	CCAGTGTTCTGCATGCCAGCAGCAA	coding region	-/+
soybean PcP carboxylase at 1221	CTCAACATTGGTGAAAGTGGCTTCC	coding region	-/+
rat leucine-rich protein (LR PR1)	ACAGTATTCTGCATAAGTGTGTCTG	coding region	-/+
human superoxide dismutase*	TAAGTGTTCTGCCTGCTTGGCCTCC	5′-UTR	-/+
rabbit stromelysin	AATATCTTCTCCATAAGTGGCTGCA	5′ flanking	-/+
human CD36A antigen at 1527	TTTTCGTTTCACCAAAGTGGCTTCC	intron	-/+
human CD36A antigen at 2468	GACATGTTCTGCATATTTCTGAAA	intron	-/+
human TcR-δ	GCAGTGAACTGCATAAGTGGGTTAT	intron betweeen TcR-V-δ and TcR-∝	+/+
duck hepatitis virus polymerase	TGTATGTTCTGCATAAGTTGTTTGG	coding region	-/+
human RFG (RET/PTC3 *fusion gene in* carcinoma)	AGGAGCCTCTGTATAAGTGGCTTCT	coding region	+/+
H. influenzae Rd ribonucleoside-diphosphate reductase 1 α-chain	AATGCCTTTTGCATAAGTGGTTTCA	coding region	-/+
rat salivary proline-rich protein (RP4)	TAATTGTTTTCTTTAAGTGGCTTCC	5′ flanking	-/+
human cDNA clone 200187	AAGATGTTCTGCATACTTGGCTCTC	5′ UTR	+/+
Turnip crinkle virus avirulent satellite RNA F	TGGGTGTTCTGCATTAGTTGCGTAG	?	-/+
Turnip crinkle virus virulent satellite RNA C	TGGGTGTTCTGCATTAGTTGCGTAT	?	-/+
rat cDNA clone Y159 (EST)	CCACTGTTCTGCATACTTGGCTATC	mRNA	-/+

Table 2. The 25-nt Consensus Sequence in Sequences from Nonredundant Databases.

Gene	Sequence	Location	Orientation
rat GTPase-activating protein (homology of *ras* p21)	AATCAGTTCAGCATAAGTGGCCTAC	coding region	+/+
rat ceruloplasmin mRNA	CACAGGATCTGCATAAGTGGGTCCC	coding region	-/+
C. elegans cosmid ZC21	AGAGTGATGTGCATAAGTGGGATGA	5′ UTR	+/+
human cDNA clone HHCPL07 (homologous to epsilon globin)	CCCTTTTTCTGCATAAGGGGCTGTG	mRNA	+/+
Sus scrofa (pig) DNA microsatellite repeat region SO355	ACCTCTTTATGCATAAGTGGCATCA	5′ to the repeat	-/+
pumpkin mRNA for ascorbate oxidase promoter binding protein	TTGATGTTCTGCATATGTGGTTCTT	coding region	-/+
human mRNA for mannose-binding protein C	TGCCAGTTCTGCATAAGTTGATTGA	3′ UTR	-/+
S. cerevisiae CRM1 gene (transcription regulator)	GTTAGCATTTGCATAAGTGGCTTTC	coding region	-/+
P. troglodytes β-globin gene	AAAGTGTTCTGCCGAAGTTTGAATA	5′ flanking	-/+
human β-globin gene	AAAGTGTTCTGCCGAAGTTTGAATA	5′ flanking	-/+
Agrobacterium tumefaciens plasmid pTi 15955 for mannopine utilization	GGAGTGCGCTGCATAAGTGGACCCA	intergenic	+/+
human cDNA GEN-101E02	GTTATGCTCTGCATAAGTGGTAAAG	3′ UTR	-/+
human fetal brain cDNAs:			
·clone 141401 5′	AAACATAAGTTGCAAAAGTGGCTTCC	5′ UTR	+/+
·clone 1335138 5′		5′ UTR	+/+
·clone 129199 5′		5′ UTR	+/+
·clone 131640 5′		5′ UTR	+/+
human cDNA 3′ IB3288	GTTATGCTCTGCATAAGTGGTAAG	3′ UTR	-/+
human aorta cDNA 5′-GEN 259E05	AAAGTGTTCTGTTAATAGTGATAA	5′ UTR	+/+
A. raviloviana (oat) ty1-copia like DNA	AACTTGTATTGCATAAGTGGCTTTG	leader sequence	+/+
oligonucleotide used for gel retardation	TACTAAGTGTTCTGCATATTT		

* Are human cDNA.

Boxed sequences are those which differ when compared to band C1.

Orientation reflects the pattern of the element in relation to the coding region. Band C1 5′ → 3′ is considered the + sequence.

FIGURE LEGEND

Figure 1. Gel shifts were performed as described by Schreiber *et al.* (1989) and Lederer *et al.* (1996). For each experiment, HeLa cell extracts, purified NF-κB p50 protein, Sp1, and NF-κB consensus elements were purchased from Promega Biotech. + and - strand oligonucleotides of the "25-bp consensus element" were synthesized on the "gene synthesizer" (Applied Biosystems) according to the manufacturer's conditions. Reactions were performed in the presence of poly (dI-dc) nonspecific inhibitor and with 10 μg of HeLa unlabeled crude nuclear protein extract (Promega Biotech). Free oligonucleotides were resolved from protein-DNA complexes by Tris-borate polyacrylamide gel electrophoresis. The dried gel was exposed on the Phosphorimager screen.

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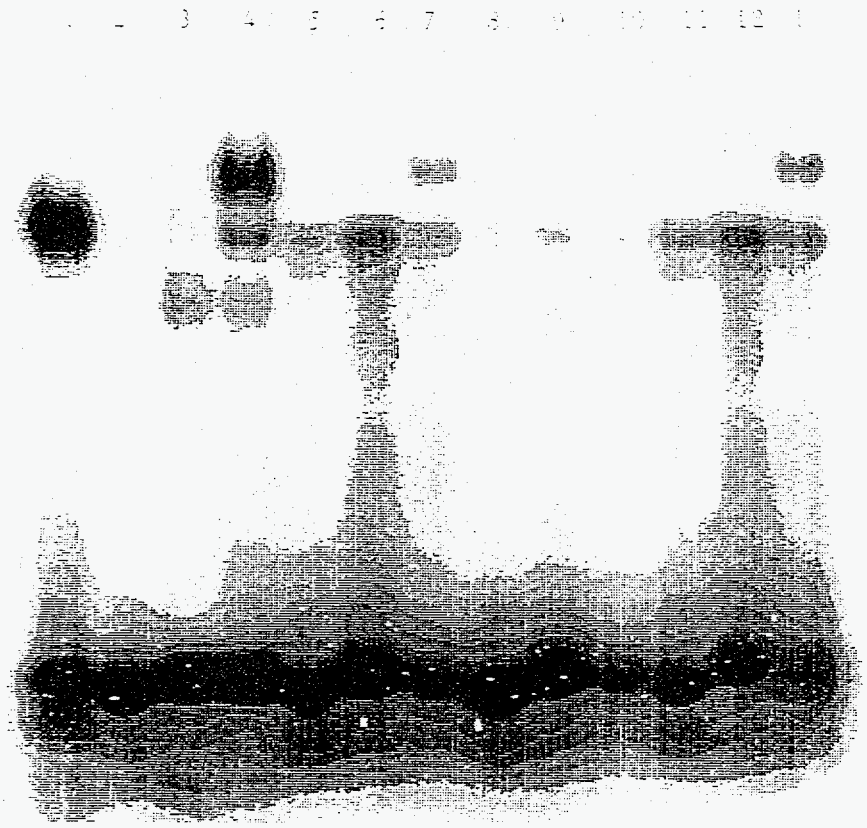

EMSA for Detection of Proteins Binding to the Consensus Element.

Lane	Labeled-oligo	Extract	Cold Competitor	Lane	Labeled-oligo	Extract	Cold Competitor
1	NF-κB	purified NF-κB	0	8	+	HeLa	+
2	ds	purified NF-κB	0	9	–	HeLa	–
3	NF-κB	HeLa	0	10	ds	HeLa	ds
4	Sp1	HeLa	0	11	+	HeLa	Sp1
5	+	HeLa	0	12	–	HeLa	Sp1
6	–	HeLa	0	13	ds	HeLa	Sp1
7	ds	HeLa	0				

Labeled oligonucleotide in lanes 5–13 is +–strand, –strand or double-stranded consensus sequence.