

Ultraviolet A (320–380 nm) radiation causes an alteration in the binding of a specific protein/protein complex to a short region of the promoter of the human heme oxygenase 1 gene

A.L.T.O.Nascimento⁺, P.Luscher and R.M.Tyrrell*

Swiss Institute for Experimental Research, ch. des Boveresses 155, CH-1066 Epalinges, Switzerland

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ABSTRACT

Ultraviolet A (320–380 nm) radiation strongly stimulates expression of the human heme oxygenase 1 gene as a consequence of an enhancement in transcription rate (1). We have used a 147 bp fragment of the promoter of this gene as a probe for DNA binding activity in nuclear extracts prepared from untreated and UVA treated populations of cultured human skin fibroblasts. Analysis using gel electrophoresis mobility shift assays clearly demonstrates the appearance of a strong binding activity unique to UVA-treated extracts that is formed in the absence of *de novo* protein synthesis. Footprint analysis defines a binding region from –41 to –50 bp that partially overlaps with a region known to constitutively bind upstream stimulatory factor (USF). Further analysis using synthetic oligonucleotides and gel retardation has confirmed that the crucial sequence for binding the protein present in both control and UVA-treated extracts lies within a 26 bp sequence that includes the core USF binding site. UVA radiation appears to lead to a modification of the USF complex (or closely related proteins(s)) to give a structurally modified protein/protein complex which protects only the upstream half of a region originally defined by footprinting of the promoter with USF.

INTRODUCTION

Stress proteins are induced in eukaryotic cells as a result of a variety of treatments including those that cause heat shock, growth arrest and DNA damage (for recent reviews see 2 and 3). Recently we have proposed (4, 5) that the induction of the catabolic enzyme, heme oxygenase, could be a general mammalian stress response that occurs in particular after oxidising treatments, including UVA (320–380 nm) radiation.

Activation of expression of the human heme oxygenase gene by various inducing agents is clearly due to an enhancement in

transcriptional activity (1). Transient expression analysis indicates that oxidant-responsive elements lie within 147 bp of DNA sequence in the proximal promoter region of the gene (6). In the present study we have used gel retardation assays and DNase 1 footprinting to localise a region within the promoter of the heme oxygenase gene that binds a modified protein/protein complex present only in extracts from UVA treated cell populations.

MATERIALS AND METHODS

Cell culture and treatment with UVA

The normal human skin fibroblast line FEK4 was derived from a foreskin explant in this laboratory and cultured routinely as a monolayer as described (7). To prepare for experiments, fibroblasts were plated (1×10^6 cells/dish) in 20 cm diameter Falcon tissue culture dishes and grown to 75 percent confluency prior to treatment with UVA radiation. To treat cells, medium was removed and reserved, and cells were rinsed with phosphate-buffered saline (PBS) plus calcium and magnesium and irradiated through this buffer at room temperature. UVA radiation was provided by a Uvasun 3000 lamp (Mutzhas, Munich, Germany) which emits wavelengths between 330 and 450 nm with a broad peak between 360 and 410 nm. After irradiation, the cell monolayers in both treated and sham-irradiated dishes were washed twice and the reserved medium was added back. Incubation was then continued at 37°C for 1 h prior to preparation of nuclear extracts.

Genomic DNA cloning

The genomic DNA library used for screening was kindly donated by Dr M.Noll (Basle) and had been prepared by inserting partial digests of human DNA into the SalI site of the λ EMBL3 replacement vector. The library was screened for human heme oxygenase DNA fragments using the *in situ* plaque hybridization technique developed by Benton and Davis (8). The probes employed were human heme oxygenase cDNA fragments (either the large EcoRI fragment from clone 2/10, bps –32 to 932 or

* To whom correspondence should be addressed

⁺ Present address: Inst. de Quimica, Dept. de Bioquimica B12 TNF, C.P. 20780, 01498 Sao Paulo SP, Brazil

clone 1/149, bp 967 to 1475, see ref. 4) that had been labelled with [α - 32 P] dATP by random oligonucleotide-primed synthesis and a 17 mer oligonucleotide based on the 5' untranslated region of the mRNA (bp -7 to -22) which had been end-labelled using T4 polynucleotide kinase. In this way a clone was isolated containing a 15 kb insert including sequence upstream of the mRNA cap site of the gene. After preliminary restriction mapping, a 1.44 kb fragment was excised from this clone and inserted between the EcoR1 and Xho1 sites of the Bluescript SK⁻ plasmid by directional cloning. This clone (HUHO5'1416 [SK⁻]) contains 1416 bp of upstream sequence and runs 24 bp into the untranslated region of the gene. The entire sequence is described elsewhere (6). The first 540 bp upstream of the Xho1 site are in close agreement with those described by Shibahara *et al.* (9).

Nuclear extracts

Nuclear extracts were prepared as described by Dignam *et al.* (10). Final protein concentrations were measured according to Bradford (11).

Preparation and labelling of DNA fragments and oligonucleotides

The plasmid HUHO5'1416 (SK⁻) was digested with Sma1 and Xho1 restriction enzymes to give a 147 bp fragment spanning from bp -123 of the HO gene through to 24 bp into the untranslated region of the mRNA. The fragment was then isolated by preparative gel electrophoresis and labelled at one end by using the Klenow fragment of DNA polymerase (Boehringer) with (32 P) dCTP at the Xho1 site. Oligonucleotides (for details see Fig. 3) were either synthesised at ISREC (Epalinges) or by Genosys Biotechnologies, Inc. (Texas). They were then annealed according to Mihm *et al.* (12) and labelled with the Klenow fragment as described above for the 147 bp fragment. A double-stranded 26mer oligonucleotide containing a 10 bp NFkB binding site was originally described by Zabel *et al.* (13). The 108 bp TK fragment for competition studies contained all of a modified HSV-TK promoter and was obtained by cutting the EcoR1-HincII fragment from pJJio (14).

Gel mobility shift assay

Gel mobility shift assays were performed according to Offord and Beard (15) with some minor modifications. Standard binding reaction mixtures (20 μ l) contained 2-8 μ g of nuclear protein extract [dialyzed against buffer D of Dignam (10)] plus buffer D up to 12 μ l, 1 mM dithiothreitol, 8 mM mgCl₂, 1 μ g of poly (dI-dC) and either 1.6 fmol labelled 147 bp fragment or 14 fmol labelled oligonucleotides. In later experiments, 4 μ g of (dI-dC) was used in each reaction mix since this considerably reduced the appearance of bands attributable to non-specific binding. Protein-DNA binding reaction mixtures were then incubated at room temperature for 20 min and analyzed by electrophoresis through a 5% polyacrylamide gel at 10 v/cm and at room temperature in 1 \times Tris-borate-EDTA buffer. The gel was then dried and subjected to autoradiography. For competitive binding studies, the reaction mix was incubated with unlabelled competitor for 10 min before the addition of labelled probe.

DNase 1 footprinting

The DNase 1 footprinting procedure was a modified version of that previously described (15). The protein-DNA binding reaction mixture (28 μ l) contained 0.5 ng of labelled 147 bp fragment, 20 to 60 μ g of nuclear protein extract, 0.7 mM

dithiothreitol, 7mM MgCl₂, 1 to 6 μ g poly (dI-dC) and, Dignam buffer D (10) to a total volume of 16 μ l. The reaction mix was incubated for 20 min at room temperature. Just prior to the addition of DNase 1 (Boehringer) the magnesium concentration was raised to 15 mM in tubes containing protein. DNase 1 was then added (the amount was empirically determined but was of the order of 0.07 or 0.7 U in the absence and presence of protein, respectively) and incubation was continued for 1 min at room temperature. The digestion was stopped by addition of a solution containing 0.1 M NaCl, 0.01 M EDTA, 1% sodium dodecyl sulfate, 0.4 μ g/ μ l t-RNA and 1.4 μ g/ μ l proteinase K in 0.1 M Tris pH 7.9. After 45 min at 37°C, the reaction mixtures were extracted with chloroform and the DNA precipitated at -70°C with 2.5 volumes of ethanol. The precipitate was resuspended in formamide loading buffer (Boehringer), heated at 95°C, and analyzed by electrophoresis through an 8% polyacrylamide/7 M urea sequencing gel containing 10% formamide (16). Reference lanes for sequence localization were obtained by carrying out a Maxam and Gilbert (17) G or G+A reaction on the same fragment.

RESULTS

Protein binding to the 147 bp proximal promoter fragment

At least certain of the genetic elements responsible for the strong stimulation of transcription of the human heme oxygenase gene by a variety of agents appear to reside in the 147 bp proximal promoter region of the gene (6). Nuclear extracts were prepared from both untreated fibroblast populations and from cell populations which had been treated with 2.5×10^5 Jm² of broad band UVA radiation. The extracts were then mixed with the 147 bp Sma1-Xho1 fragment (cut from HUHO5'1416[SK⁻]) end-labelled with 32 P and analysed by the gel retardation assay (Fig. 1). The fragment consistently bound to a protein present in the control extract (C1) but a quite distinct faster migrating complex (A1) was always observed after mixing the fragment with extracts derived from UVA-irradiated cells (Figs 1a, b and c). The band labelled A2 was also unique to extracts from irradiated cells and was present in most but not all independently prepared extracts. Both the control band (C1) and the induced bands (A1, A2) are competed out by a 50-fold excess of unlabelled fragment (Fig. 1b). The control band is also slightly weakened by the addition of excess unlabelled TK promoter fragment or a 26 mer oligonucleotide containing the NFkB binding site (Fig. 1b) as well as by increasing the concentration of poly d(I-C) (Fig. 1c). However the retarded bands arising from extracts derived from the UVA treated cells are entirely unaffected by addition of non-specific inhibitor (Fig. 1b) and only slightly reduced by the addition of poly d(I-C) (Fig 1c), an effect which appears to be largely due to a reduction in non-specific binding. Neither the bands arising from the control or the treated extracts are affected by the presence of cycloheximide (Fig. 1d) at a concentration which reduces residual protein synthesis to below 5 percent. These results are clear evidence that UVA radiation leads to the modification of a protein or protein complex in such a way that it can bind strongly to a region of DNA within the proximal promoter region of the heme oxygenase gene.

DNase 1 footprint analysis of the 147 bp proximal promoter fragment

To further define the region of the heme oxygenase promoter that binds to specific proteins, we have subjected the 147 bp fragment to DNase1 footprint analysis. Prior to DNase 1

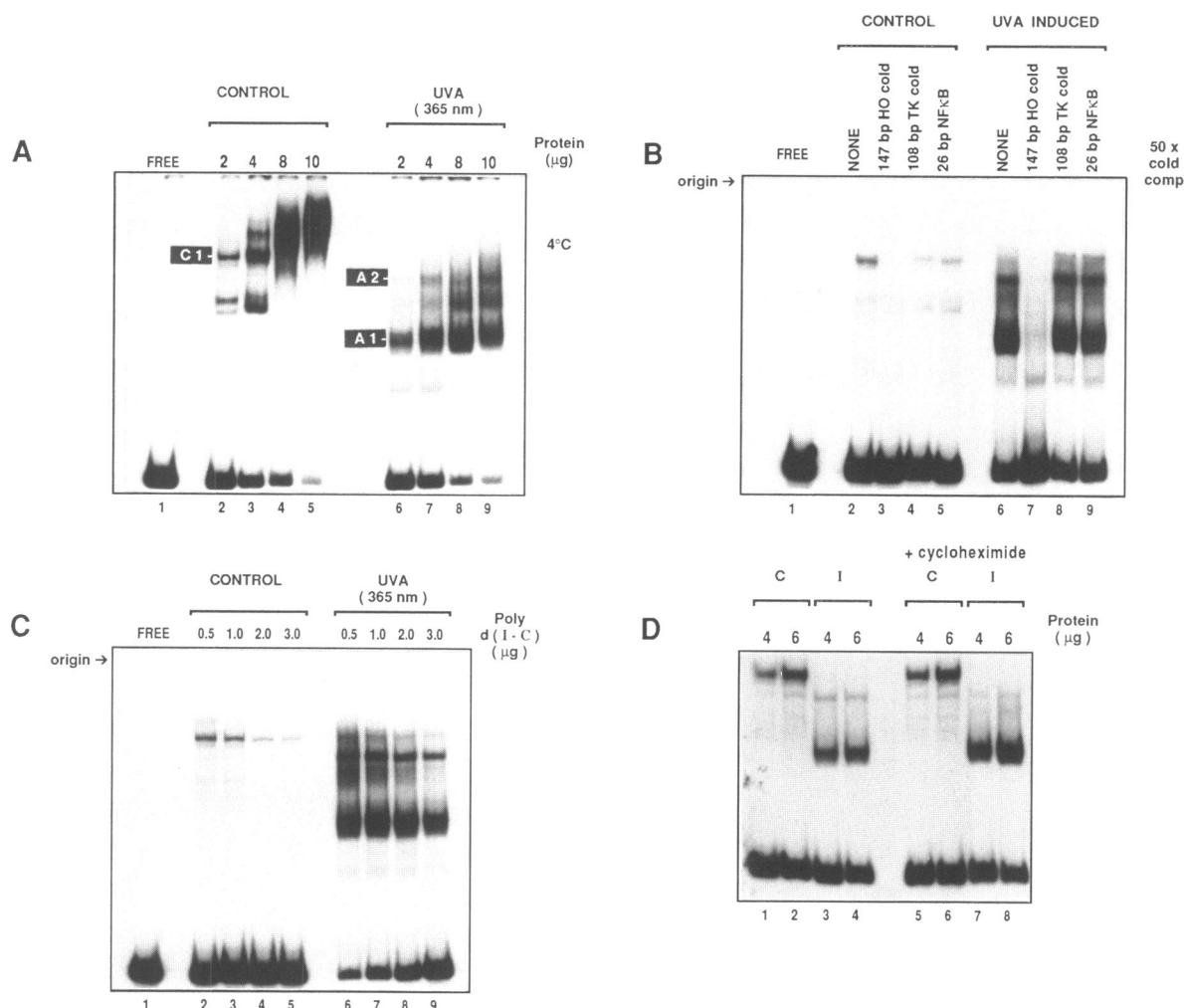


Figure 1. Gel mobility shift assays using the 147 bp HO promoter fragment (Sma1–Xho1) to probe for binding activity in extracts of cultured human fibroblasts, treated (or not) with 250 KJm^{-2} of UVA radiation. **A]** Protein bound as a function of increasing protein concentration. **B]** Protein bound with and without a 50-fold excess of cold competitor. **C]** Protein bound as a function of increasing concentrations of poly d(I-C). **D]** Protein bound with and without the addition of $50 \mu\text{g/ml}$ cycloheximide.

digestion, the 3' end-labelled Sma1–Xho1 fragment was incubated with saturating levels of protein extract derived from control or UVA irradiated cells. DNA sequence profiles were then obtained using either increasing concentrations of protein extract (Fig. 2a) or poly d(I-C) (Fig. 2b). In both experiments, a protected region is observed between bp –41 and –50 when protein extracted from UVA-treated cells is used.

Definition of the protein-binding DNA element

To confirm that a region of the HO promoter corresponding to the protected region is involved in binding to protein(s) present in cell extracts after UVA treatment, we have prepared a series of oligonucleotides that include the footprinted sequence (Fig. 3). They include a 24 mer oligonucleotide (HOPO1) which contains an 18 bp sequence of the promoter from bp –38 to –55 flanked by restriction site linkers. In addition we have prepared an oligonucleotide (USF oligo) containing the minimum sequence required for binding upstream stimulatory factor (USF, see 18) and a larger oligonucleotide (USF/UVA oligo) which includes 24 bp of the HO promoter and spans and flanks the entire region footprinted by both the complex present in extracts derived from

UVA treated cells (here-in referred to as UVAF) and the region footprinted by purified USF (18).

It is clear from a comparison of Figures 4a and b that the 18 bp sequence (–38 to –55) is sufficient to bind to unique proteins present in the extracts prepared from either control or UVA-induced cells and that a factor present in the treated extracts leads to a faster migrating bound complex. Both control and induced bands are reduced to low intensities by sufficient excess cold competitor whereas a similar excess of oligonucleotide containing the NFkB binding site has no effect. We have also shown that when the 147 bp Sma1–Xho1 fragment is used as a probe an excess of the cold oligonucleotide, HOPO1, will prevent the appearance of the DNA binding proteins in extracts from both control and UVA-irradiated populations (Fig. 4a). Although a 1000 fold excess of the oligonucleotide is necessary for near-maximal competition, the results strongly suggest that the proteins bound by the short oligonucleotide fragment (HOPO1) are identical to those for which binding was observed initially with the longer 147 bp promoter fragment (Figs 1, 4a and 4b). However, when oligonucleotides containing a 10 bp sequence corresponding to the UVAF footprint (HOPO4) or plus one

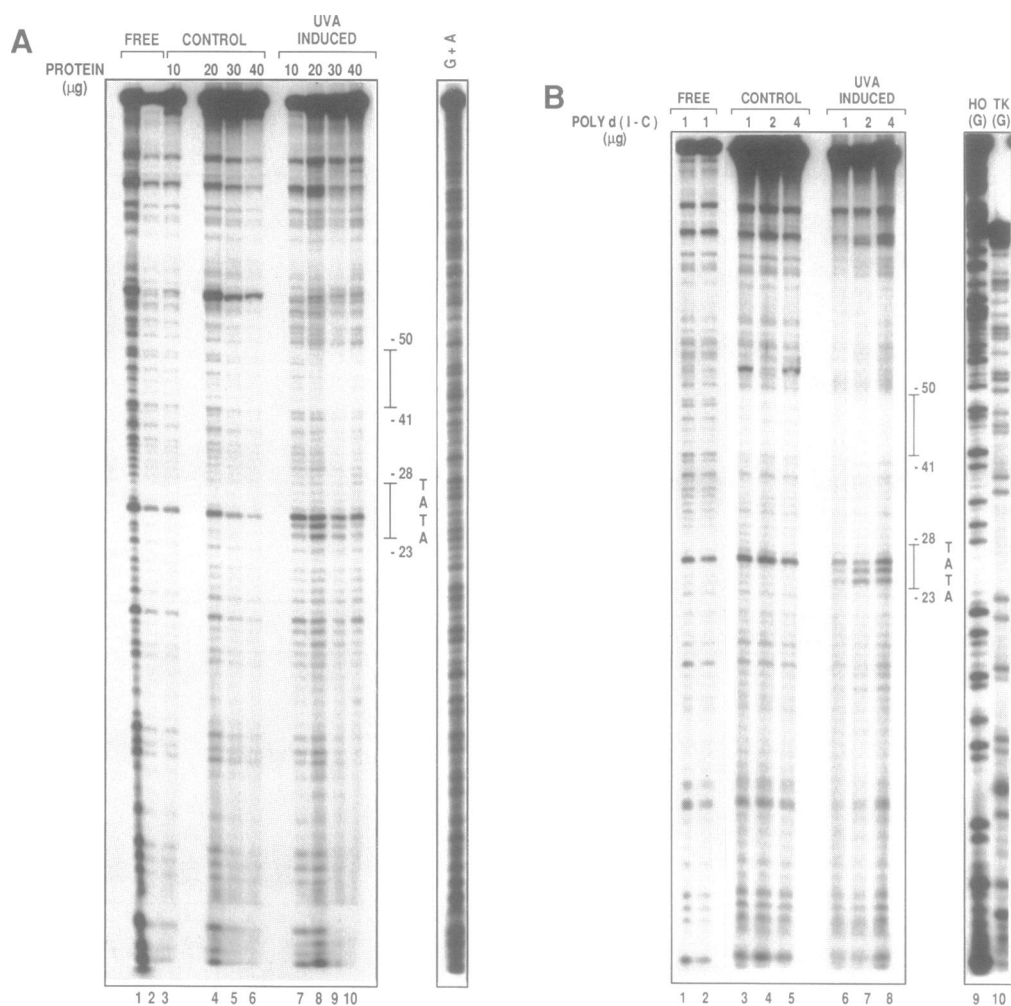


Figure 2. DNase 1 footprint of the 147 bp HO promoter fragment cell extracts treated (or not) with 250 KJm⁻² of UVA radiation. **A)** Results are presented as a function of increasing concentration of protein in the extract. Lane 11 is a reference for sequence localization obtained by carrying out a (G+A) reaction on the same fragment. **B)** Results are presented as a function of increasing poly d(I-C) concentration in the reaction mix using a constant amount (40 µg) of nuclear protein extract. Lanes 9 and 10 are reference lanes for sequence localisation obtained by carrying out a G reaction on either the HO fragment itself or the TK promoter fragment.

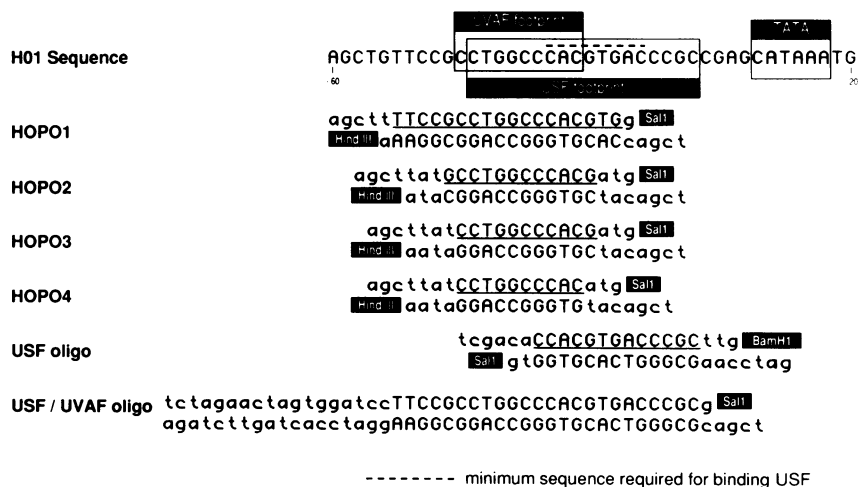


Figure 3. A section of the human HO promoter showing footprinted regions and the structure of oligonucleotides synthesized for this study.

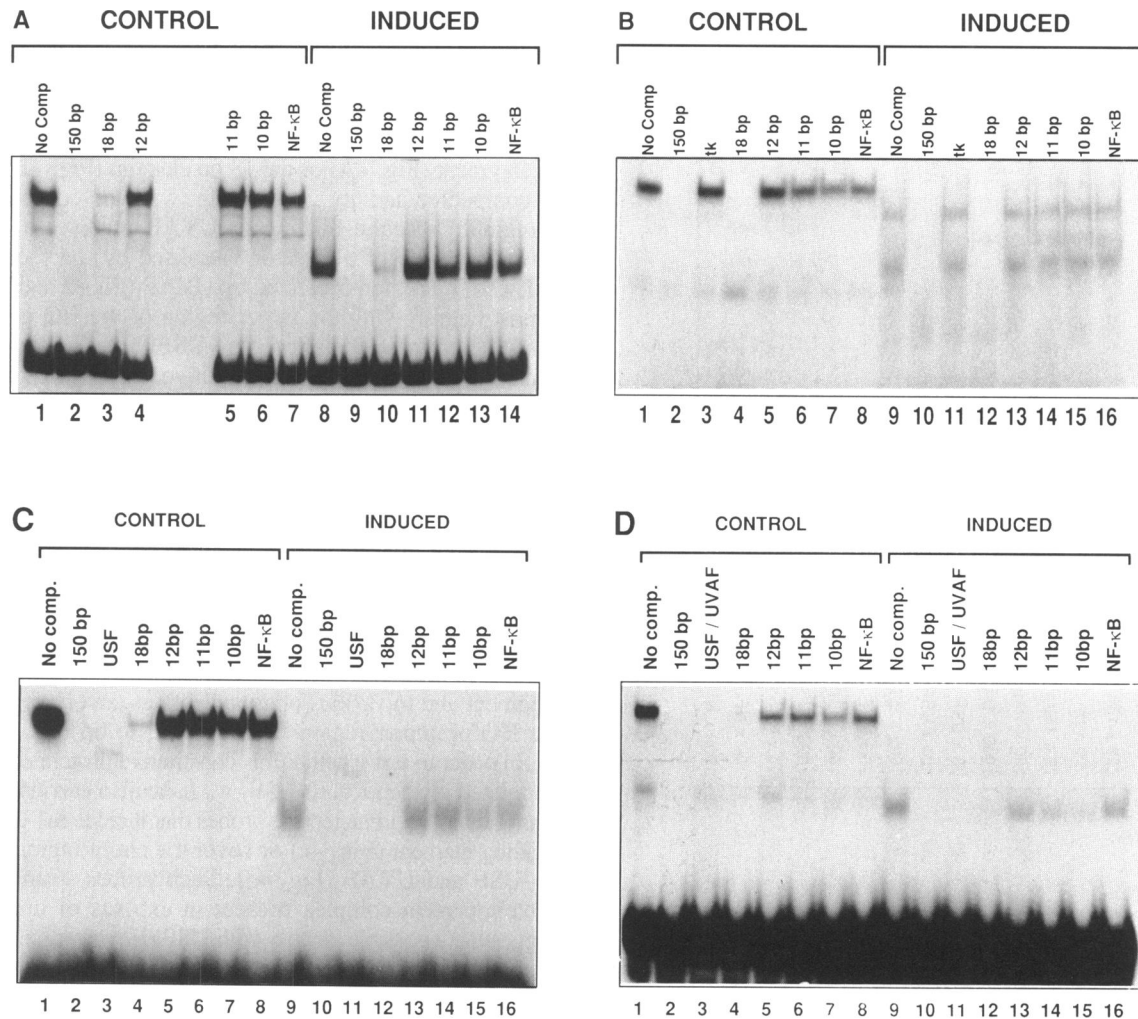


Figure 4. Gel mobility shift assays using A) the 147 bp HO promoter fragment (Sma1–Xho1) or B) the oligonucleotide HOPO1 (Fig. 3) or C) the oligonucleotide USF or D) the oligonucleotide USF/UVAF to probe for binding activity in extracts of cultured human fibroblasts, treated (or not) with 250 KJm⁻² of UVA radiation. Reactions were competed with a series of non-radioactive competitor fragments including a 200-fold excess of the corresponding cold fragment the 147 bp (Sma 1–Xho 1) fragment or a 1000-fold excess of double-stranded oligonucleotides containing 18 bp (HOPO1), 12 bp (HOPO2), 11 bp (HOPO3) or 10 bp (HOPO4) of the HO promoter sequence (see Fig. 4).

(HOPO3, Fig. 3) or two (HOPO2, Fig. 3) flanking bases are used as competitor, they are completely without effect on the proteins bound by either the 147 bp fragment or the HOPO1 oligonucleotide.

An oligonucleotide containing the minimum sequence for USF binding is also able to bind to a protein complex present in extracts from UVA treated cells (Fig. 4c) suggesting that the binding region is not restricted to bp's –38 to –55. However the slower-migrating complex that it forms with control extracts appears to represent stronger binding. As expected from this result, an oligonucleotide containing the entire sequence from bp –32 to bp –55 (USF/UVAF) also shows a similar binding pattern (Fig. 4d). Again the proteins are competed out by an excess of either the 147 bp fragment or unlabelled fragment itself but not the oligonucleotides with sequence corresponding to the minimal region covered by the UVAF footprint (HOPO's 2, 3 and 4). Thus the region spanning bp –32 to bp –55 is involved in binding both the constitutive factor found in control extracts and the modified factor that arises in UVA treated cells (UVAF). It is also clear that the region determined as the minimum USF

binding sequence is sufficient to bind UVAF (Fig. 4c). To obtain additional information, we have compared the relative strength of the UVAF (HOPO1), USF and USF/UVAF binding fragments as cold competitor in a gel binding assay using the 147 bp fragment as the probe (Fig. 5). Using 20, 100 and 1000-fold molar excess of each competitor it is evident that the USF and USF/UVAF are approximately equivalent in competing for binding of the proteins from either the control or irradiated extract. However, for both extracts a 100-fold excess of HOPO1 is necessary to compete with the same efficiency as a 20-fold excess of the fragments that include the minimal USF binding sequence. It therefore appears likely that despite the shift in footprint pattern (Figs 2 and 3), the primary binding sequence requirements of UVAF are similar to those for USF.

DISCUSSION

The present study has enabled us to define a region in the proximal promoter region of the human heme oxygenase gene which can bind to a specific protein complex that has been

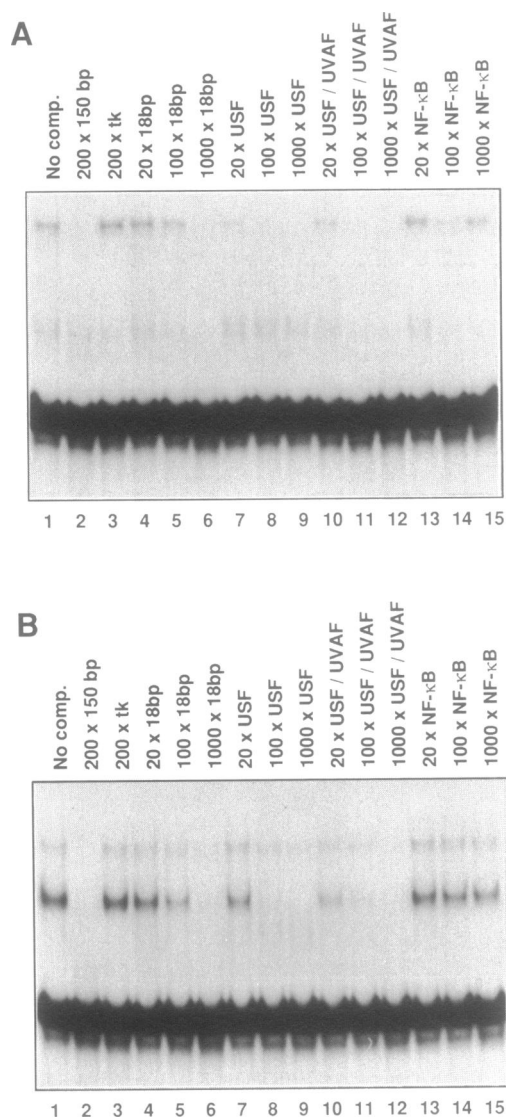


Figure 5. Gel mobility shift assays using the 147 bp HO promoter fragment (Sma1–Xho1) to probe for binding activity in extracts of cultured human fibroblasts from control populations (A) or in populations treated (B) with 250 KJm^{-2} of UVA radiation. A series of DNA fragments and oligonucleotides were used over a range of values of molar excess in order to titrate competitor strength.

modified in response to UVA radiation treatment of cells. The initial localisation of this region is based on two types of experiment. Firstly, DNase 1 footprinting (Fig. 2) has revealed a protected region between bp -41 and bp -50 on the 147 bp Sma1–Xho1 proximal promoter fragment of the human heme oxygenase 1 gene. Secondly, double-stranded oligonucleotides that span all or part of the region to -32 to -50 bp bind strongly to a protein/protein complex (UVAF) that is present only in extracts prepared from UVA-treated cells (Fig. 4 B, C, D). The binding activity is clearly specific since it is not competed away by even a large excess of the NF κ B oligonucleotide of corresponding length or other unrelated DNA's (not shown). However, it is competed out by a relatively small molar excess of the 147 bp Sma1–Xho1 fragment. Furthermore, the cold oligonucleotide that contains sequence from bp -38 to bp -55 (HOPO1) is in turn able to compete for the UVA-activated protein

that binds to the 147 bp fragment (Fig 4A). The protein/protein complex present in extracts prepared from UVA-treated cells and which binds to both the large and small fragments is therefore probably identical. However, the oligonucleotides (HOPOs 2, 3, 4) are unable to compete for binding of UVAF to the 147 bp fragment (Fig. 4A) or the 18 bp element (Fig. 4B). This lack of competitor activity is consistent with the concept (see next paragraph) that the binding of UVAF involves a region that extends outside of the sector visualised by the footprint.

The 10 bp region protected by UVAF (Figs 2 and 3) overlaps almost entirely with the larger region of the HO gene that has been shown to be footprinted by USF (18). Binding to USF is apparently necessary for constitutive expression of the heme oxygenase gene but Sato *et al.* (18) could find no evidence for involvement in inducible expression. USF was originally shown to bind to an upstream element present in the adenovirus late promoter whose core sequence (CACGTGAC) corresponds to the core region that binds to USF in the HO gene (18). The region footprinted by UVAF includes only the first 3 upstream basepairs of this core sequence and the USF footprint extends much further downstream (Fig. 3).

We have also observed that a specific protein/protein complex present in untreated extracts binds to the 147 bp Sma1–Xho1 fragment and to various oligonucleotides covering part or all of the HO promoter region from bp -32 to bp -50 (Fig. 1 and 4). In order to test whether this constitutive protein could actually be related or identical to USF, we have also carried out binding assays using oligonucleotide probes that include the USF minimal binding element (Fig. 4c) or cover the entire region footprinted by USF and UVAF (Fig. 4d). Such probes strongly bind the protein/protein complex present in extracts of untreated cells suggesting a possible identity with USF. However they also bound to the faster migrating complex, present only in extracts from UVA-irradiated cells. Indeed, from competition experiments (Fig. 5) it was evident that fragments containing the minimal USF binding element actually compete considerably more strongly for the binding protein present in both constitutive and irradiated extracts than the oligonucleotide containing only the region footprinted by UVAF. We therefore believe that both the constitutive factor and the modified factor that appear after UVA irradiation are closely related to USF. This has been confirmed in a recent experiment using antibody to the 43 kDa unit of the USF heterodimer (Christian Waltner, unpublished data, this laboratory) which causes both control and induced bands to disappear and the re-appearance of a slower migrating complex (supershift) at identical positions for the two samples.

Overall, these results are consistent with the interpretation that the entire region of the HO promoter spanning at least bp -32 to -50 is involved in binding proteins involved in modulating heme oxygenase gene expression. Basal levels of expression presumably require binding of USF in close proximity to the protein complex involved in forming the RNA polymerase II entry site at the TATA box, a condition known to increase the rate of RNA polymerase II loading (19). Inducing conditions appear to give rise to a modified protein/protein complex which migrates faster through a non-denaturing gel. The complex is also modified in such a way that it now only footprintes the upstream half of the region footprinted *in vitro* by USF. We have no information as to whether the increased mobility and modified binding of the complex derived from the UVA-treated cells is due to a loss of a protein(s) in the complex that binds constitutively or whether the constitutive protein/protein complex undergoes

a conformational change. Such a change may enhance gene expression by facilitating access of the polymerase. In a recent study (20), another inducible gene (type 1 plasminogen activator inhibitor) has been shown to contain a transforming growth factor B1 responsive element which includes two binding sites (one of which is for USF) for transcriptional activation.

Our current evidence would suggest that the binding protein present in extracts from induced cells is a modified version of USF. However, we do not have as yet direct evidence to show that the UVAF binding is actually involved in inducible promoter activity leading to enhanced gene expression. Nevertheless, the protein complex designated as USF, which is a transcription factor, does appear to provide a further example of a protein which is altered by oxidant stress to give a new factor (UVAF) which displays altered binding activity. A previous example was provided by NFκB which is apparently released from the cytoplasmic anchor protein IκB when cells are treated with hydrogen peroxide (H₂O₂) and is thus able to bind to its regulatory sequence which is present in many genes (21). The AP1 protein complex present in nuclear extracts prepared from H₂O₂ (250 μM) treated Hela cells also binds more strongly to a probe corresponding in sequence to the AP-1 binding site of the collagenase gene (22). A comparative study of these responses should provide new insights into the mechanisms by which eukaryotic genes are regulated by oxidant stress.

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