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Activation of Nuclear Protein Binding to the Antioxidant/Electrophile Response Element in Vascular Smooth Muscle Cells by Benzo(*a*)pyrene

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This laboratory has previously shown that binding of nuclear proteins to the antioxidant/electrophile response element (ARE/EpRE) participates in deregulation of vascular gene expression by benzo(*a*)pyrene (BaP), a suspected atherogen. In the present study, oligonucleotides representing ARE/EpREs within the c-Ha-ras and glutathione-*S*-transferase (GST-Ya) promoters were employed to evaluate the role of flanking sequences in stabilizing protein:DNA interactions in BaP-treated vascular smooth muscle cells (vSMCs). We also wanted to define promoter-specific patterns of protein recognition to ARE/EpREs in this cell system. In electrophoretic mobility shift assays (EMSA), optimal protein binding to a human Ha-ras ARE/EpRE variant sequence fitted to match the extended mouse(m) GST-Ya ARE/EpRE core (5′-TMAnnRTGAYnnnGCR-3′) was dependent on 5′ nucleic acid sequence. Using immobilized DNA affinity chromatography (IDAC), we identified four nuclear proteins of M_r 62, 60, 50, and 30 kDa that associated specifically with the mGSTYa ARE/EpRE. Photo crosslinking to a BrdU-substituted hHa-ras or mGST ARE/EpRE probe identified specific proteins of M_r 80, 60, 55, 25, 23 kDa or 80, 60, 55, 27, 25, 23 kDa, respectively. Protein:DNA complexes detected using IDAC eluate overlapped with those observed in crude nuclear extracts. Chemical treatments known to modulate ARE/EpRE protein binding in vSMCs did not alter overall protein:DNA affinity and/or sequence recognition to either hHa-ras or mGST-Ya elements. We conclude that nucleotide sequences 5′ to the core ARE/EpRE influence specific binding of nuclear proteins and that multiple proteins bind to ARE/EpREs in a promoter-specific manner in vSMCs.

The onset and progression of atherosclerosis in response to atherogenic chemicals is a complex process that involves increased vascular smooth muscle cell (vSMC) proliferation and loss of differentiation (1). During the early stages of atherosclerotic lesion formation by benzo(*a*)pyrene (BaP), vSMCs undergo a switch from a quiescent to a proliferative phenotype characterized by loss of contractile function and enhanced mitogen sensitivity (2). Although mechanisms of BaP-induced phenotypic transition are not fully understood, transcriptional upregulation of c-Ha-ras via an antioxidant/electrophile response element (ARE/EpRE) located in the 5′-regulatory region of the gene is involved in the atherogenic response (3, 4). To date, only limited information is available on the protein:ARE/EpRE interactions that govern regulation of c-Ha-ras in response to BaP and other oxidants.

Based on patterns of xenobiotic inducibility of the rat glutathione-*S*-transferase (GST-Ya) gene in HepG2 cells, Pickett and co-workers first defined the core sequence of the ARE/EpRE as 5′-RTGACnnnGC-3′ (5). Sequence alignment, functional characterization and mutational analysis aided in identifying ARE/EpREs in the regulatory regions of several other stress-responsive genes in HepG2 cells (6). From these studies, it was concluded that sequences flanking the ARE/EpRE core directly influenced basal and inducible gene expression and consequently, the ARE/EpRE core sequence was redefined as 5′-TMAnnRTGAYnnnGCR-3′.

The homology between c-Ha-ras ARE/EpRE and the extended consensus ARE/EpRE sequence is variable (6), especially in the proximal flanking regions. Therefore, the present studies were conducted to evaluate the effects of ARE/EpRE flanking sequences on the formation of ARE/EpRE:protein complexes relative to the mGST-Ya ARE/EpRE (5′-TAATGGTGACAAAGCAACTT-3′). We also sought to define promoter-specific patterns of protein recognition to ARE/EpREs in this cell system. We present evidence that nucleotides immediately upstream of the ARE/EpRE core region of the mGST-Ya promoter are required for optimal protein binding in nuclear extracts of BaP-treated

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TABLE 1

ARE/EpRE Oligonucleotide Constructs Used

ARE/EpRE	EMSA probe
mGST-Ya	TAATGGTGACAAAGCAACTT
hHa-ras	**CCTG**GGTGACAGAGCGAGA**C**
mut hHa-ras	**TAAT**GGTGACAGAGCGAGA**T**

Note. The hHa-ras ARE/EpRE (hHa-ras) sequence was constructed in genomic context, while the mutant hHa-ras ARE/EpRE (mut hHa-ras) sequence was modified (changes in bold) to fit the extended mGST-Ya ARE/EpRE (mGST-Ya) sequence.

vSMCs and that nuclear proteins associate with this sequence in a promoter-specific manner.

MATERIALS AND METHODS

Enzymes and reagents. Restriction endonucleases and DNA modification enzymes (Promega, Madison, WI) were used according to manufacturer's instructions. Benzo(*a*pyrene 3,6-quinone was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories (c/o Midwest Research Institute, Kansas City, MO). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Oligonucleotides. Oligonucleotides were synthesized with a Perceptive Biosystems DNA Synthesizer (DNA Technologies Core Facility, Center for Environmental and Rural Health, Texas A&M University). Oligonucleotide probes were generated by annealing complementary oligonucleotides, filling in the ends with dNTP's and Klenow, and end-labeling with [γ-^{32}P]-ATP and T4 polynucleotide kinase. Crosslinking oligonucleotides were generated as described for EMSA except that Klenow fragment was used for second-strand synthesis using [α-^{32}P]-dCTP and brominated dUTP for labeling and crosslinking sites, respectively. A listing of oligonucleotides used in this study is shown in Table 1.

Cell culture/chemical treatments. Primary cultures of mouse vSMCs (aortic) were isolated as described previously (7) and maintained in Media 199 (Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (Gibco) (100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B) at 37°C in a 5% CO_2 atmosphere. Passage-17 vSMCs were treated with BaP (3 μM in DMSO) or vehicle for 3 h.

Nuclear extracts and electrophoretic mobility shift assays (EMSA). Tissue culture plates (Becton Dickinson, Lincoln Park, NJ) were washed twice in cold HEGD (25 mM HEPES, 1 mM DTT, 1.5 mM EDTA, 10% glycerol, 0.5 mM PMSF, pH 7.6) before harvesting. Cells were lysed by dounce homogenization (30 strokes) and nuclei were pelleted at 5,000 × g. Nuclear protein was extracted by resuspending nuclei in HEGD + 0.5 M KCl (HEGDK) and incubating on ice for 1 h. Samples were centrifuged at 12,000 × g for 10 min at 4°C and the supernatant was removed and stored at −80°C. Protein was measured by the method of Bradford. EMSA incubations included 5 μg of vSMC nuclear protein in HEGDK with 2 mM DTT, 40 ng poly(dI/dC) and 20 μg BSA. Samples were incubated with 10 fmol of [γ-^{32}P]ATP-labeled probe at room temperature for 20 min and then loaded onto a 7% non-denaturing polyacrylamide gel (40 acrylamide:1 bisacrylamide) and electrophoresed in 0.5× TBE buffer at 25 mA for 30 min. Gels were dried at 80°C for 30 min and exposed to film for 18 h without an intensifying screen.

Immobilized DNA affinity chromatography (IDAC). Equimolar amounts of oligonucleotides corresponding to the mGST-Ya ARE/EpRE (5′-GATCTAATGGTGACAAAGCAACTT-3′) and its complementary sequence (0.5 mg) were annealed and phosphorylated with polynucleotide kinase to provide a site-specific sequence suitable for affinity chromatography. Double-stranded DNA was purified and multimerized as described in Current Protocols in Molecular Biology (8). DNA was then coupled to *N*-hydroxysuccinamide (NHS)-activated agarose Hi-trap column (Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Ethanolamine (0.5 M) was used to block remaining NHS groups and the column equilibrated with Buffer Z (25 mM HEPES, 1 mM DTT, 20% glycerol, 0.1% NP-40, pH 7.6). vSMC nuclear extracts were equilibrated with Buffer Z in the presence of 2 ng/μl poly dI/dC before loading onto the column, followed by incubation on ice for 30 min. Protein was eluted by an increasing salt gradient (0–2.0 M KCl) and was separated on an 8% resolving SDS-PAGE gel.

UV protein crosslinking. Binding conditions and oligonucleotides were the same as those described for EMSA, except that 5 μg of nuclear protein was incubated with 10–15 mol BrdU-substituted crosslinking probe. Samples were incubated at room temperature for 30 min prior to UV exposure (254 nm) for 30 min on ice and were separated on a 10% SDS-PAGE gel, mounted onto filter paper and dried. [γ-^{32}P]ATP oligonucleotide was applied directly to the Rainbow protein molecular weight standards using a razor blade to create a discrete reference band for autoradiography. Gels were exposed to film for 24–36 h at room temperature without intensifying screen.

RESULTS

The binding profiles of vSMC nuclear extracts to wild type hHa-ras and mGST-Ya ARE/EpRE oligonucleotides are shown in Fig. 1 (lanes 2 and 7, respectively). Striking differences in the number and intensities of shifted complexes were observed between the two oligonucleotides. Nuclear protein binding to the mGST-Ya ARE/EpRE sequence yielded two protein:

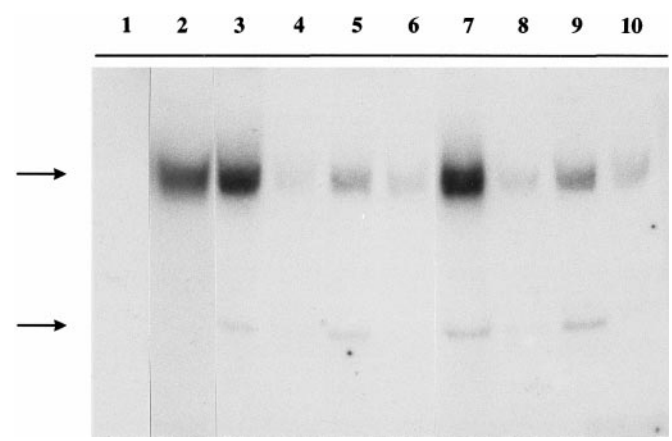

FIG. 1. EMSA competition analysis of mouse (m)GST-Ya and mut human (h)Ha-ras ARE/EpRE oligonucleotide probes using 5 μg of BaP-treated nuclear extracts. 50 ng of each competitor was used (specific and non-specific) to evaluate binding. A 20 min preincubation with competitor was allowed for competition reactions. EMSA reactions resolved on a non-denaturing 7% polyacrylamide gel. Oligonucleotides used are designated as probe/competitor. Lane 1, free probe (mut hHa-ras); lane 2, hHa-ras; lane 3, mut hHa-ras; lane 4, mut hHa-ras/mut hHa-ras; lane 5, mut hHa-ras/non-specific DNA; lane 6, mut hHa-ras/mGST-Ya ARE; lane 7, mGST-Ya; lane 8, mGST-Ya/mGST-Ya; lane 9, mGST-Ya/non-specific DNA; lane 10, mGST-Ya/mut hHa-ras.

DNA complexes, while the hHa-ras ARE/EpRE only generated a less abundant, slower-migrating species corresponding to that seen with mGST-Ya ARE/EpRE. A mutant hHa-ras ARE/EpRE oligonucleotide (mut hHa-ras), constructed to include the "TMA box" present in the 5′ region of many ARE/EpREs was also tested for vSMC nuclear protein binding. The sequence added to the functional hHa-ras core ARE/EpRE conferred a binding profile comparable to that of the mGST-Ya ARE/EpRE (compare Fig. 1, lanes 2, 3 and 7). These results show that 5′ sequence information from the mGST-Ya ARE/EpRE (5′ TAAT 3′) affords the hHa-ras element added affinity to vSMC nuclear proteins.

The specificity of protein:DNA interactions was tested in competition EMSAs (Fig. 1, lanes 4–6 and 8–10). The low mobility complex seen with any of the constructs was ablated when using either mut hHa-ras or mGST-Ya ARE/EpRE as competitor (compare Fig. 1, lanes 3, 4 or 7, 8). Human Ha-ras ARE/EpRE was effectively competed by both mut hHa-ras and mGST-Ya ARE/EpRE oligonucleotides (not shown). Although random oligonucleotide competed for some of the binding (Fig. 1, lanes 5 and 9), this sequence was later shown to exhibit high affinity for vSMC nuclear proteins (3). The higher-mobility ARE/EpRE:protein complex was competed away effectively by both specific competitors, but was unaffected by excess non-specific oligonucleotide (compare Fig. 1, lanes 4, 5 and 6 or lanes 8, 9 and 10). Therefore, competition tests revealed that a portion of the low mobility complex binds non-specifically to the ARE/EpREs, while the association between the ARE/EpREs and components of the high mobility complex are specific.

Immobilized DNA affinity chromatography was employed to isolate ARE/EpRE-binding proteins. The immobilized DNA corresponding to the mGST-Ya ARE/EpRE associated with several proteins of M_r of 62, 60, 50, and 30 kDa (not shown). The mobility of mGST-Ya ARE/EpRE was retarded by affinity column eluate in a concentration-dependent manner (Fig. 2A), and the reconstituted ARE/EpRE binding complex was comparable to the complex seen in nuclear extracts from BaP-treated vSMCs (Fig. 2B, lanes 2 and 3). The reconstituted complex was not competed by excess poly dIdC (Fig. 2B, lane 4), but was effectively competed by increasing amounts of mGST-Ya ARE/EpRE oligonucleotide (Fig. 2B, lanes 5–7).

ARE/EpRE:protein photo-crosslinking experiments were then performed to compare binding profiles of vSMC nuclear proteins to mGST-Ya and hHa-Ras ARE/EpREs and to resolve the monomeric DNA-binding proteins involved. We also wanted to determine if chemical treatments known to increase expression of ARE/EpRE-controlled genes can modulate overall affinity and/or sequence-specificity of nucleic acid:amino acid contacts. Six proteins associated with the mGST-Ya ARE/EpRE and were resolved from

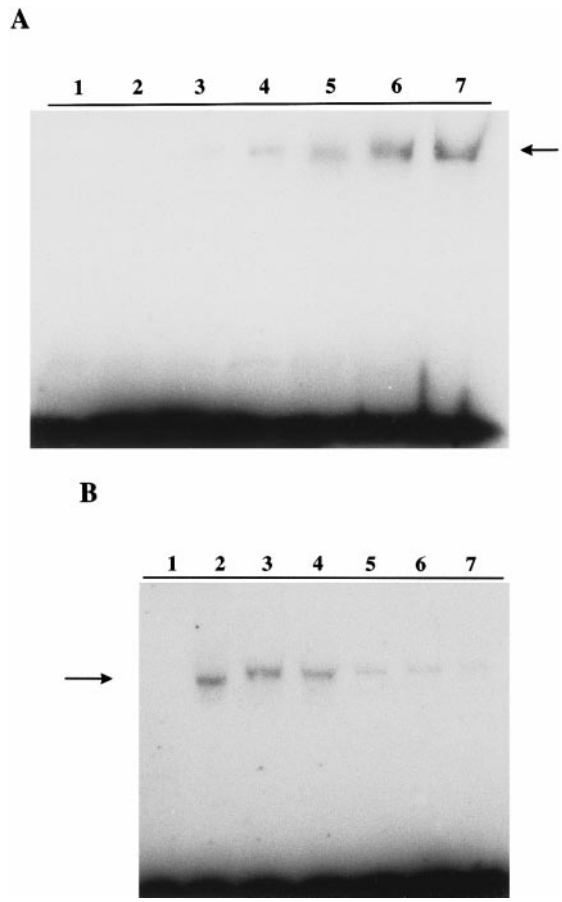

FIG. 2. EMSA reconstitution and competition of mouse(m) GST-Ya ARE/EpRE affinity column eluate. (A) EMSA reconstitution using mGST-Ya ARE/EpRE probe and mGST site-specific DNA-affinity eluate. Lane 1, free probe; lanes 2–7, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 μg of affinity eluate added to incubations. (B) Competition of affinity eluate by increasing amounts of unlabeled mGST-Ya ARE/EpRE: Oligonucleotides used are designated probe/competitor. Lane 1, free probe; lane 2, mGST with BaP (3 μM) nuclear extract; lane 3, mGST with affinity eluate; lane 4, mGST/dIdC; lane 5, mGST/10 ng GST; lane 6, mGST/25 ng GST; lane 7, mGST/50 ng GST.

vSMCs nuclear extracts (Fig. 3, lane 2). Binding to the hHa-ras ARE/EpRE was comparable to mGST ARE/EpRE, except that the relative intensities of individual complexes were promoter-specific (Fig. 3, lane 3). The most prominent complexes for mGST-Ya ARE/EpRE were M_r 80, 60, 55, 27, 25 and 23 kDa versus M_r 80, 60, 25, and 23 kDa for hHa-ras ARE/EpRE. Challenge of vSMCs with BaP (3 μM) or BaP 3,6-quinone (0.03 μM) at concentrations known to activate ARE/EpRE signaling (3, 4) did not alter protein binding to either ARE/EpRE probe (Fig. 3, lanes 8 and 9). Binding profiles of the mGST-Ya affinity eluate in photo-crosslinking experiments identified the 80 kDa protein as the most prominent band. As noted for binding affinity profiles in EMSA, the affinity of the 80 kDa protein was considerably greater for the mGST-Ya ARE/EpRE than for the hHa-ras sequence. All of the complexes identified

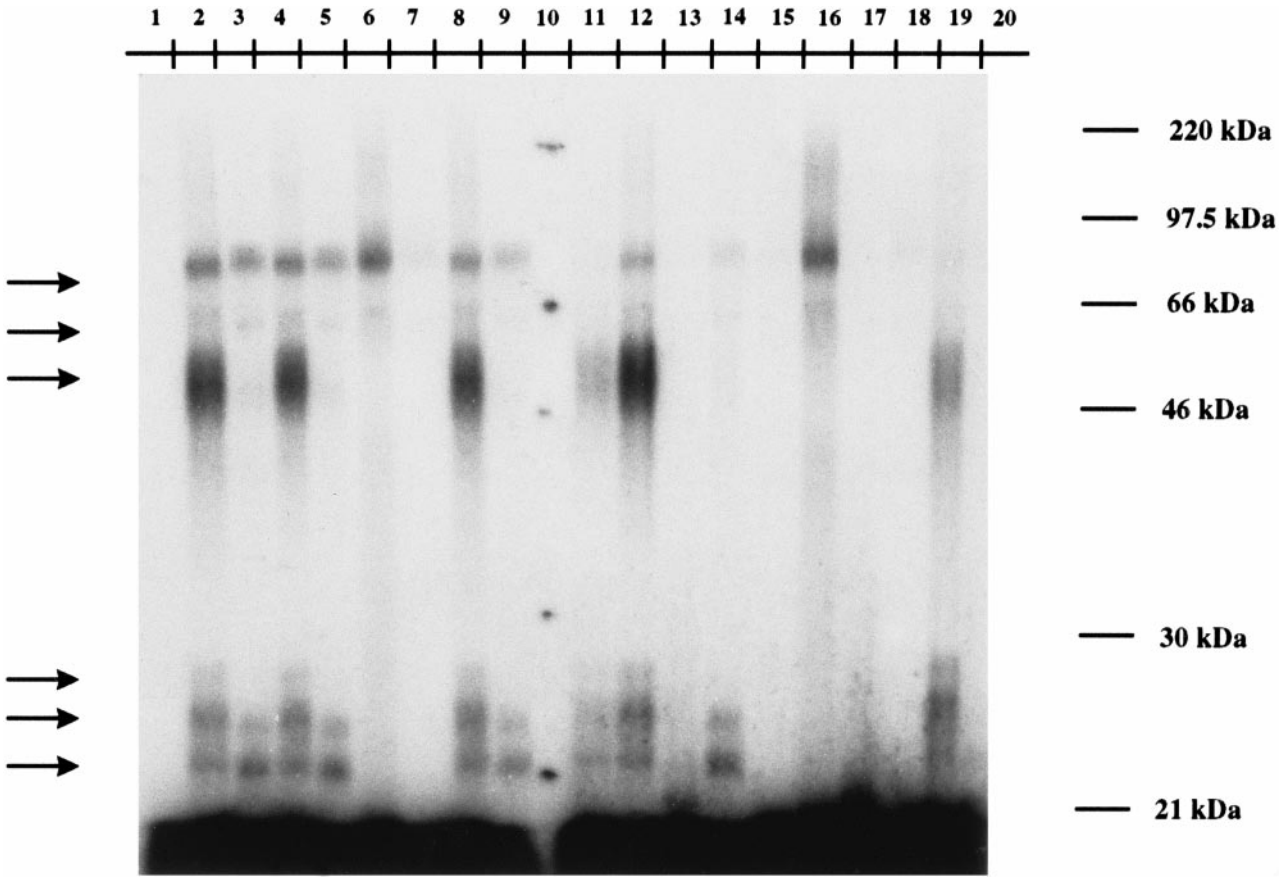

FIG. 3. UV cross-linking of vSMC nuclear proteins and affinity eluate to mouse GST-Ya and human Ha-ras ARE/EpREs. Lanes 1–9 are incubations with vSMC nuclear extracts. Lane 1, free probe; lane 2, DMSO extract with mGST-Ya; lane 3, DMSO extract, hHa-ras; lane 4, 3.0 μM BaP extract, GST-Ya; lane 5, BaP extract, hHa-ras; lane 6, GST-Ya affinity eluate, GST-Ya; lane 7, GST-Ya eluate, hHa-ras; lane 8, 0.03 μM BaP 3,6-quinone extract, GST-Ya; lane 9, quinone extract, hHa-ras. Lane 10, Rainbow molecular weight standard. Lanes 11–20, are competitions with various vSMC extracts are designated probe/competitor. Lane 11, BaP extracts with GST-Ya/GST-Ya; lane 12, BaP with GST-Ya/dIdC; lane 13, BaP with hHa-ras/hHa-ras; lane 14, BaP with hHa-ras/dIdC; lane 15, affinity eluate with GST-Ya/GST-Ya; lane 16, affinity with GST-Ya/dIdC; lane 17, affinity with hHa-ras/hHa-ras; lane 18, affinity with hHa-ras/dIdC; lane 19, 3,6-quinone extract with GST-Ya/GST-Ya; lane 20, 3,6-quinone with hHa-ras/hHa-ras.

were specific in that they were effectively competed by excess oligonucleotide of the same, but not random sequence (Fig. 3, lanes 12–20).

DISCUSSION

The results of our study showed that sequence information upstream of the minimal consensus ARE/EpRE sequence is required for optimal protein binding to ARE/EpRE-like sequences in vSMCs. Although molecular determinants for such interactions are not known, the specificity of base pair:amino acid contacts is known to be determined by complex hydrogen bonding between solvent, amino acid side chains, and nucleic acid moieties (9). Consequently, hydration patterns between macromolecular surfaces effected by nucleic acid composition within the "TMA box" region of ARE/EpREs may be critical determinants of the affinity and specificity of protein:DNA interactions (10).

Wasserman and Fahl (6) examined the binding and functional consequences of altering the base composition of the mGST-Ya ARE/EpRE in HepG2 cells. These studies showed that flanking sequences of the originally defined consensus ARE/EpRE were required for increased protein binding. Interestingly, flanking information also regulated inducibility by exogenous oxidants, but molecular determinants of the functional response were not defined. Several proteins have been reported to interact specifically with *cis*-acting ARE/EpREs. For example, Venugopal *et al.* (11) used immunodetection methods and reporter transfection to identify Nrf1, Nrf2, cJun, JunB, and JunD as proteins present in complexes binding to ARE/EpREs in the NQO1 promoter. Studies by Itoh *et al.* (12) showed cMaf, Keap1 and Nrf2 associate specifically with the regulatory region of several ARE/EpRE-containing genes. These observations suggest that multiple ARE/EpRE binding proteins may govern distinct cellular

signaling pathways through this *cis* element, and that differential protein assembly at the ARE/EpRE may result from different signals in different cell types. This interpretation is consistent with the results of our DNA affinity and UV-crosslinking experiments showing that differences in DNA sequence between mGST-Ya and hHa-ras give rise to differential patterns of protein:DNA recognition and complex assembly.

Although our EMSA and DNA affinity chromatography data shed light on some of the critical protein:DNA interactions that govern gene regulation via ARE/EpREs, efforts to correlate findings for different genes, cell-types, and chemical stimuli pose unique challenges. Modulation of protein binding to ARE/EpREs in vSMCs by BaP and BaP 3,6-quinone exhibit promoter-specific characteristics that may be responsible for differential patterns of vascular gene expression during the course of BaP-induced atherogenesis. However, chemical treatments known to modulate protein:ARE/EpRE interactions in vSMCs did not alter the overall affinity and/or sequence recognition for the hHa-ras or mGST-Ya ARE/EpREs, suggesting that BaP and its quinone metabolite influence ARE/EpRE:protein binding in a similar manner. This level of complexity is magnified by the multiplicity of proteins that recognize ARE/EpREs and the possibility that differential protein recognition may account for promoter-, cell- and tissue-specific differences.

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REFERENCES

1. Ramos, K. S., and Parrish, A. R. (1995) *Life Sci.* **57,** 627–635.
2. Ramos, K. S., Zhang, Y., Sadhu, D. N., and Chapkin, R. C. (1996) *Arch. Biochem. Biophys.* **332,** 213–222.
3. Bral, C. M., and Ramos, K. S. (1997) *Mol. Pharm.* **52,** 974–982.
4. Miller, K. P., *et al.* (2000) *Biochem. Pharmacol.*, in press.
5. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) *J. Biol. Chem.* **266,** 11632–11639.
6. Wasserman, W. W., and Fahl, W. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5361–5366.
7. Ramos, K. S., and Cox (1993) *in* Methods in Toxicology: In Vitro Biological Systems (Tyson, C., and Frazier, J., Eds.), pp. 159–168.
8. Ausubel, F. M. (1997) *Current Protocols in Molecular Biology,* 12.10.1–12.10.14.
9. Hard, T., and Lundback, T. (1996) *Biophys. Chem.* **62,** 121–139.
10. Schwabe, J. W. R. (1999) *Curr. Opin. Struct. Biol.* **7,** 126–134.
11. Venugopal, R., and Jaiswal, A. K. (1997) *Oncogene* **17,** 3145–3156.
12. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1998) *Genes Dev.* **13,** 76–86.