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Identification of Benzo[*a*]pyrene-Inducible *Cis*-Acting Elements Within *c-Ha-ras* Transcriptional Regulatory Sequences

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SUMMARY

Previous studies in this laboratory have demonstrated that transcriptional deregulation of c-Ha-ras expression is associated with the induction and maintenance of proliferative vascular smooth muscle cell (SMC) phenotypes by benzo[*a*]pyrene (BaP). We examined previously undescribed *cis*-acting elements within the proximal 5′ regulatory region of c-Ha-ras (−550 to +220) for their ability to influence BaP-induced transcription in murine SMCs. BaP-inducible DNA binding activity was demonstrated at a site located −30 relative to the major start site cluster at +1 that exhibits extensive homology to a consensus aryl hydrocarbon response element (AHRE), as well as a site located at −543 that contains a consensus electrophile response element (EpRE). *In vitro* cross-linking studies revealed the specific interaction of 104- and 96-kDa proteins with the putative AHRE and of an 80-kDa protein with the EpRE. The use of monoclonal antibodies to the aryl hydrocarbon receptor transcription factor in competition electrophoretic mobility shift assays indicated this protein is specifically induced by BaP to interact at the AHRE within the *c-Ha-ras* 5′ regulatory region. Transient transfection with an *Ha-ras* promoter construct containing the putative AHRE but lacking the EpRE linked to the chloramphenicol acetyl transferase reporter gene, followed by challenge with BaP (0.3, 3.0, and 30 μM), revealed transcriptional activation that was not statistically significant. However, insertion of an oligonucleotide composed of the EpRE immediately upstream of basal sequences at −330 was associated with strong activation of transcription by BaP. These data indicate that *c-Ha-ras* gene expression is modulated by BaP via a complex mechanism that likely involves interactions among multiple regulatory elements. We conclude that c-Ha-ras expression is regulated by BaP at the transcriptional level, a response that may constitute an epigenetic basis of atherogenesis.

Ras genes encode proteins that exhibit guanine nucleotide binding and GTP hydrolytic activity and function as membrane-associated proteins that couple receptor tyrosine kinases to nuclear events associated with growth and differentiation (for a review, see Ref. 1). Mutation of ras proteins in codons 12, 13, or 61 activates the protein to the oncogenic form present in ≈20% of human tumors. However, recent studies have demonstrated a significant role for ras proteins in human cancers in the absence of mutational activation; persistent signaling through the ras/raf/mitogen-activated protein kinase pathway has been identified as a contributing factor in renal (2), mammary (3), and epidermal (4) tumors, as well as vascular atherosclerotic lesions (5). Ras protein coding sequences are highly conserved among higher eukaryotes. Regulatory sequences are less so, but the binding sites for several *trans*-acting factors seem to be conserved within the 5′ region, including multiple Sp1 sites (GC-II, -III, and -IV), an NF-1 site, and an Ha-ras element (6).

Studies in this laboratory as well as others have implicated altered ras signaling with derangements of growth and differentiation of vascular SMCs. Stable transfection of the Ha-ras oncogenic EJ clone in vascular SMCs induces a transformed phenotype (7). Transfection of a *trans*-dominant negative mutant of *Ha-ras* that interferes with normal protein function into rat arteries subjected to balloon catheterization significantly reduced restenosis, confirming the key role of Ha-ras in SMC proliferation *in vivo* (8). Furthermore, abnormal expression of c-Ha-ras has been implicated in the induction and maintenance of a proliferative phenotype in aortic SMCs (9), a seminal event in the development of atherosclerotic lesions.

Polycyclic aromatic hydrocarbons have been investigated for many years as contributors to atherogenesis and carcinogenesis. BaP, a persistent environmental contaminant, is a

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ABBREVIATIONS: SMC, smooth muscle cell; BaP, benzo[*a*]pyrene; BRE, benzo[*a*]pyrene responsive element; AHRE, aryl hydrocarbon receptor response element; AHR, aryl hydrocarbon receptor; EpRE, electrophile response element; CAT, chloramphenicol acetyl transferase; ARNT, aryl hydrocarbon receptor nuclear translocator; FBS, fetal bovine serum; mAb, monoclonal antibody; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AP-1, activator protein-1; SDS, sodium dodecyl sulfate; SSPE, standard saline/citrate/EDTA; TBE, Tris/borate/EDTA.

member of this class of compounds and has been demonstrated to initiate and/or promote atherogenesis in experimental animal models (10). BaP is generated by the incomplete combustion of organic material such as coal and petroleum tars and is prevalent in the exhaust of internal combustion engines and tobacco smoke. Toxicity often results from conversion of the parent compound to electrophilic forms by mixed-function oxidases (11). BaP is also capable of binding to the AHR (12) to induce translocation to the nucleus, where the receptor acts as a transcription factor (for a review, see Ref. 13). The relative contributions of metabolism versus receptor-dependent pathways to BaP toxicity remain to be defined.

Early work in this laboratory demonstrated that BaP enhances c-Ha-ras expression in rat aortic SMCs, an effect that was inhibited by α-naphthoflavone, an AHR antagonist (14). Subsequent studies demonstrated that this effect was the result of altered transcriptional activity and suggested the AHR may play a significant role in the response (15). Here, we continue this investigation and report the identification of two BaP-inducible *trans*-acting factor binding sites within the 5′ regulatory region of c-Ha-ras. A BRE was previously identified using nuclear extracts of rat aortic SMCs (15). This element was defined at −30 overlapping the GC-IV element and subsequently shown to serve as a binding site for the AHR. We therefore refer to this element as the Ha-ras AHRE. A perfect consensus EpRE located at −543 was defined as functionally critical for BaP induction of this gene. An unidentified 80-kDa protein was induced to bind the EpRE in response to BaP challenge. These results suggest a mechanism by which BaP can induce *Ha-ras* transcription in the absence of mutations to influence vascular SMC proliferation.

Materials and Methods

Enzymes and reagents. Nucleic acid restriction and modification enzymes were used according to suppliers' [Promega (Madison, WI) or New England Biolabs (Beverly, MA)] recommendations. Chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted.

Oligonucleotides. Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer (Oligonucleotide Synthesis Services, Department of Biochemistry and Biophysics, Texas A & M University). Complementary oligonucleotides composing the consensus EpRE motif (16) were annealed and phosphorylated with ATP and T4 polynucleotide kinase. This created a phosphorylated oligonucleotide with *Hin*dIII-compatible ends of sequence 5′-AGCTCCTGGGTGACAGAGCGAGAAGCT-3′ for use in cloning (the consensus EpRE motif is underlined). The same double-stranded oligonucleotide was filled in using the Klenow fragment of DNA polymerase I and end-labeled using [γ-^{32}P]ATP (New England Nuclear Research Products, Boston, MA) and T4 polynucleotide kinase for EMSA. An oligonucleotide containing severe mutations within the EpRE sequence (5′-CCTGGGGAGAAGAAAGAGAGGTAC-3′) (EpREWo) was used to further define the binding specificity of BaP-induced protein complexes on the EpRE (16). Oligonucleotides composing the Ha-ras AHRE motif were prepared in a similar fashion, resulting in a sequence of 5′-GATCCGCGGGGCGGGGCGTGCGCAGGCCCGGATC-3′, with the AHRE motif underlined. The random oligonucleotide was designed to contain no known *trans*-acting factor binding sites, sequence 5′-GATCTTCATGCAGCCTTTGAGCTGCACTGATC-3′. The consensus AHRE oligonucleotide was derived from sequences from −968 to −997 of the human *CYPIA1* gene, yielding a sequence of 5′-GATCTCCGGTCCTTCTCACGCAACGCCTGGGCGATC-3′, with the AHRE motif underlined. The Ha-ras AHRE cross-linking probe was synthesized by annealing the BRE/GCBOT oligonucleotide (5′-GATCCGGGCCTGCGCACGCCCCGCCCCGCG-3′) with the BRE/GCPRIMER (5′-CGCGGGGCG-3′) and synthesizing the opposite strand in a Klenow reaction containing 2 mM dATP, 2 mM dGTP, 2 mM bromodeoxyuridine-UTP, 30 mM DTT, and 100 μCi of [α-^{32}P]dCTP. EpRE cross-linking probe was prepared similarly, using ARETOP (5′-AGCTCCTGGGTGACAGAGCGAGA-3′) and AREPRIMER (5′-TCTCGCTC-3′), with the EpRE motif underlined. Labeled oligonucleotides were purified using Sephadex G-25 spin-columns (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Antibodies. The mAb rpt1 was the kind gift of G. Perdew (Department of Veterinary Science, Pennsylvania State University, State College, PA) (17).

Plasmids. pRASCAT1 was the kind gift of E. Keller (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY). This vector consists of human *c-Ha-ras* sequences from −330 to +220 replacing the SV40 promoter of pSV2CAT (18). pRASCAT1 was subjected to partial *Hin*dIII digestion followed by blunting with Klenow to remove the 3′ *Hin*dIII site downstream of the c-Ha-ras start site and create pRASCAT2. The consensus EpRE oligonucleotide was subcloned upstream of pRASCAT2 in both orientations to create plasmids pARERC2A and pARERC2B. pRASCAT2 was linearized with *Hin*dIII, followed by ligation of the phosphorylated oligonucleotide using T4 DNA ligase and ATP. Random insertion orientation allowed both clones to be isolated from the same reaction. The structure of each vector was verified by sequence analysis of double-stranded plasmid DNA.

Cell culture. Primary cultures of SMCs were isolated from inbred C57BL/6 mouse aorta and maintained under standard conditions as described previously (19). Cells were grown in Media 199 (GIBCO, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (GIBCO). Subcultures were prepared by trypsinization (GIBCO) of subconfluent primary cultures. Synchronization of cultured SMCs in G_0 was achieved by serum deprivation (0.1% FBS/72 hr) as described previously (14). Cells were challenged with BaP as described in individual figure legends. The concentration of DMSO vehicle in all experiments was <0.1%.

RNA isolation and Northern analysis. Total RNA was extracted according to a modified version of the procedure of Chomczynski and Sacchi (20). Cells were seeded at 100 cells/mm^2 onto 100-mm tissue culture dishes and growth-arrested by serum deprivation for 72 hr in 0.1% FBS. Cultures were stimulated with 10% FBS in the presence or absence of BaP and processed at the desired time points after synchronous cell cycle entry. RNA was extracted using Trireagent (Molecular Research Center, Cincinnati, OH) and precipitated with isopropanol (Fisher Scientific, Pittsburgh, PA). RNA concentrations were determined spectrophotometrically, and 5–10 μg was loaded onto a 1.2% agarose/1 M formaldehyde denaturing gel and electrophoresed in 1× buffer A (20 mM Na_2HPO_4, pH 6.8, and 2 mM cyclohexanediaminetetraacetate) at 50 V for ≈3 hr. Samples were transferred from the gel to a nylon membrane (Amersham, Arlington Heights, IL) by upward capillary action for ≥24 hr before cross-linking using a Stratagene (La Jolla, CA) UV cross-linker (4 min at 254 nm). The membrane was prehybridized at 42° for 24 hr in hybridization buffer containing 45% formamide, 6× SSPE (0.75 M NaCl, 0.05 M Na_2HPO_4, 5 mM EDTA, pH 7.4), 10% dextran sulfate, 1% SDS, and 100 μg/ml sheared herring testes DNA. The membrane was hybridized sequentially to a 1.3-kilobase-pair c-Ha-ras cDNA probe purchased from Oncor (Gaithersburg, MD) and a 1.6-kilobase-pair β-tubulin probe isolated from plasmid pβTUB by *Eco*RI digestion followed by gel isolation. Each probe was radiolabeled using a High Prime random-primed labeling kit (Boehringer-Mannheim) and 50 μCi of [α-^{32}P]dCTP (New England Nuclear). The membrane was washed twice with 0.1× SSPE plus 0.2% SDS at room temper-

ature (5 min) and 0.1× SSPE/0.2% SDS at 42° for 45 min before exposure to Kodak X-Omat film at −80° for 4–18 hr. Signal intensities were quantified using a Sharp JX-330 scanning laser densitometer. Ha-ras bands were normalized to β-tubulin bands to correct for recovery and loading differences between samples. Normalized values were then expressed as a percentage of vehicle-treated control at 1 hr after challenge.

Transient transfection and analysis. Cells were seeded at 100 cells/mm^2 onto 60-mm plates and allowed to recover for 24 hr before transfection. Cells were transfected using a cationic liposome formulation available from InVitrogen (pFx-6; San Diego, CA). For each plate, 11 μg of DNA (9 μg of CAT vector and 2 μg of β-galactosidase control) and 33 μl of pFx-6 were added to separate tubes containing 1 ml of Optimem-I (GIBCO). Cells were washed twice with Optimem-I, and DNA and lipid were mixed gently and overlaid onto the cells for 6 hr at 37° (5% CO_2). After this incubation, the transfection mix was replaced with standard growth medium. Cells were allowed to grow an additional 36 hr and then challenged with chemicals added directly to the growth media. Transfected cells were harvested 24 hr after challenge, and cellular extracts were prepared by five freeze/thaw cycles (liquid nitrogen/37°). Total cellular protein concentration was determined according to the method of Bradford. CAT activity was determined according to the method of Gorman (18), using 5–20 μg of protein in an overnight incubation at 37°. β-Galactosidase concentrations were determined using a commercially available enzyme-linked immunosorbent assay kit (Boehinger-Mannheim) according to the manufacturer's instructions. CAT activity was normalized to β-galactosidase protein concentration driven from the pcDNA/HIS/lacZ vector (InVitrogen) to control for variations in transfection efficiency. Normalized CAT activities were then expressed as a percentage of vehicle-treated control expression for individual experiments and averaged. Statistical significance of the results was assessed with use of the Mann-Whitney *U* test. In all cases, the 0.05 level of probability was accepted as significant.

Nuclear extract preparation and EMSA. Nuclear extracts were prepared as described previously (21). Briefly, cells were washed twice in ice-cold buffer B (25 mM HEPES-Cl, pH 7.6, 1 mM DTT, 1.5 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) and scraped from the plates. Cells were transferred to a Dounce homogenizer and lysed with ≈30 strokes. Nuclei were pelleted at 5000 × *g* at 4° in a variable speed microfuge, and the supernatant was discarded. Nuclei were resuspended in 30 μl of buffer C (same as buffer B plus 0.5 M KCl) and incubated on ice for 1 hr to extract nuclear proteins. Nuclear ghosts were removed by centrifugation at 12,000 × *g* in a microfuge, and the supernatant (nuclear extract) was used immediately or stored frozen at −80°. Protein concentration was determined according to the method of Bradford. For Ha-ras AHRE EMSA reactions, 5 μg of nuclear extract was incubated with 10 fmol of double-stranded, ^{32}P-labeled oligonucleotides at room temperature for 20 min. Binding reactions were performed in 0.3× buffer C supplemented with 2 mM DTT, 20 μg of bovine serum albumin, and 40 ng of poly(dI/dC) in a total volume of 20 μl. Loading dyes were added (50% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol, 10 mM Tris-C, pH 7.9), and reactions were loaded onto 7% nondenaturing polyacrylamide gels run in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3). Ha-ras EpRE binding reactions were performed in similar fashion, except 50 ng of sonicated herring testes DNA and 1 ng of random oligonucleotides were used in place of poly(d/IdC), and the gels were run in 1× Tris/glycine/EDTA (10 mM Tris·HCl, 76 mM glycine, 0.4 mM EDTA, pH 8.4). For competition experiments in which cold wild-type EpRE sequence (EpREWt) was compared with EpREWo, 50 ng of poly(dI/dC) and 1 ng of EpREWo in 0.5× TBE were used. Gels were dried and exposed to Kodak X-Omat film for autoradiography at room temperature without an intensifying screen for 12–18 hr.

UV cross-linking. Equilibrium binding reactions were set up as described above with the use of 5 μg of nuclear extract and 10^5 CPM cross-linking probe. The reaction was allowed to attain equilibrium for 10 min at room temperature, followed by UV irradiation at 254 nm for 30 min on ice at a distance of 1–2 cm from the source (Stratalinker 1800; Stratagene). Loading dyes were added (100 mM Tris-Cl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol), and the samples were boiled for 5 min and run on denaturing SDS gels (6–8% resolving) along ^{14}C-labeled molecular mass markers (Amersham). Gels were dried and exposed to Kodak X-Omat film with an intensifying screen for 1–4 days at room temperature. For antibody inhibition studies, nuclear extracts were incubated with mAbs for 10 min at room temperature, followed by the addition of the probe and continuation of the binding reactions as described above.

Results

Previous work in this laboratory has demonstrated overexpression of the *c-Ha-ras* protooncogene in rat vascular SMCs treated with BaP *in vitro* after mitogenic stimulation and synchronous cell cycle entry (14). The kinetic profile of the ras activation response was suggestive of a transcriptional mechanism, so experiments were conducted to define the molecular basis of the response. A schematic depiction of the proximal *c-Ha-ras* regulatory region is shown in Fig. 1 and illustrates the approximate locations of important *trans*-acting factor binding sites (6). These regulatory elements are conserved among higher eukaryotes, including humans, and afforded us the opportunity to study human *c-Ha-ras* regulatory elements in a murine-based model that is more amenable to genetic manipulation.

Northern analysis of *c-Ha-ras* expression in response to BaP challenge was performed using cultured mouse SMCs. Cycling cultures synchronized in G_0 by serum deprivation were released into the cell cycle in the presence or absence of 0.3, 3.0, and 30 μM BaP and harvested at 1, 2, 3, 5, and 8 hr after challenge. Total cellular RNA was analyzed sequentially for c-Ha-ras and β-tubulin expression. Representative data from this experiment are shown in Fig. 2, *top*. Band intensities were quantified by densitometry and are presented graphically in Fig. 2, *bottom*. Ras expression in control cells varied as the cells synchronously entered the cell cycle, exhibiting a 50% increase at 5-hr after serum stimulation relative to the expression observed at 1 hr (*open bars*). Cells challenged with BaP exhibited an additional 50% enhancement of ras mRNA above vehicle-treated controls at all time points and concentrations examined, confirming previous observations using a more restricted time scale (14). The reduced signal in cells treated with 30 μM BaP for 1 hr is most

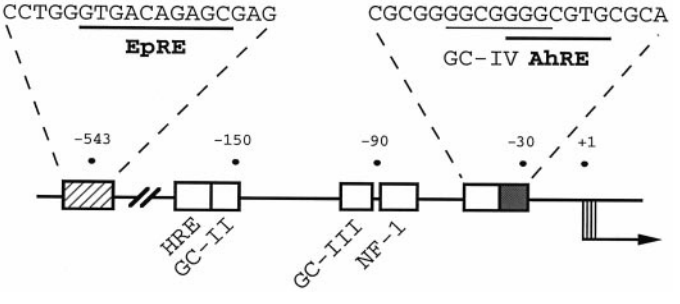

Fig. 1. Schematic illustration of proximal 5′ regulatory sequences of *c-Ha-ras*. *Solid line*, DNA sequence. *Open boxes*, previously identified *trans*-acting factor binding sites. *Shaded box*, Ha-ras AHRE. *Hatched box*, EpRE. The sequence surrounding these two elements is expanded for reference. *Arrow*, major start site cluster. [Approximate locations as defined by Lu *et al*. (6).]

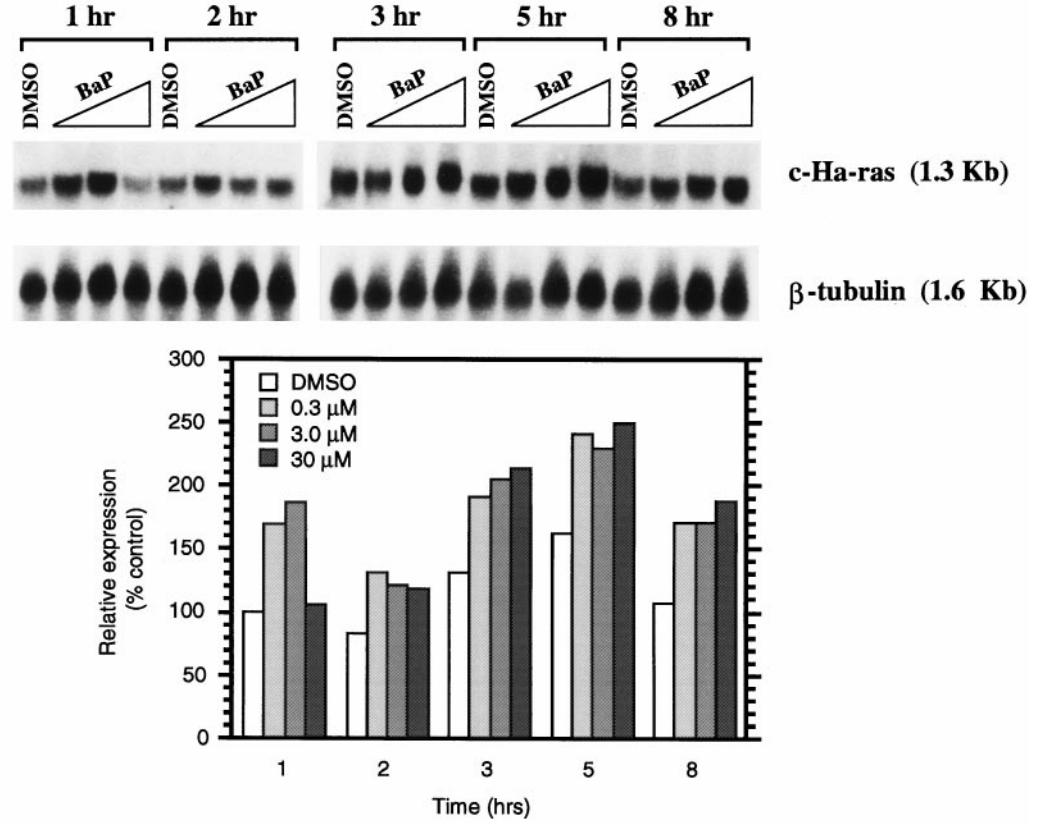

Fig. 2. Northern analysis of c-Ha-ras expression in mouse vascular SMCs. *Top*, low-passage mouse SMCs were synchronized in the G_0 phase of the cell cycle by serum deprivation for 72 hr, followed by challenge with 0.3, 3.0, or 30 μM BaP for the times indicated (*above the figure*). *Bottom*, band intensities were quantified using scanning laser densitometry. A ≈50% increase in c-Ha-ras message was observed at all time points and at nearly all concentrations examined relative to vehicle-treated controls. Similar results were obtained in three separate experiments. *DMSO*, dimethylsulfoxide.

likely due to severe isolated cytotoxicity in this sample and is not typically observed.

We previously identified a potential binding site for the AHR near the major start site cluster of the human *c-Ha-ras* by using nuclear extracts derived from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-challenged rat SMCs (15). This element was termed the Ha-ras AHRE based on homology to the consensus AHRE sequence, the binding site for the AHR. We investigated the capacity of BaP to induce protein binding to the Ha-ras AHRE in nuclear extracts derived from mouse SMCs using an EMSA. Cycling cultures of mouse SMCs were challenged with 3 μM BaP or DMSO for 3 hr, followed by nuclear extract preparation and EMSA. It is apparent that several protein complexes can form on this oligonucleotide; however, only two (labeled C1 and C2) exhibit enhanced binding due to BaP challenge (Fig. 3, *top*). The binding of these two complexes was specific as reflected by oligonucleotide competition of the binding equilibrium by oligos of identical but not random sequence. The remaining bands varied little if at all due to BaP challenge or oligonucleotide competition and thus were judged to represent nonspecific interaction of nuclear proteins with the probe. Due to the high degree of homology between the Ha-ras AHRE and the consensus binding site for the AHR, we investigated the capacity of BaP to induce binding to a consensus AHRE derived from the human CYPIA1 promoter (22). Enhanced binding to this sequence was also observed using extracts derived from SMCs challenged with 3 μM BaP for 3 hr (Fig. 3, *bottom*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, the prototypical ligand for the AHR, also induced binding to both of these elements (data not shown and Ref. 15). Because BaP is a recognized ligand for the AHR complex and the Ha-ras AHRE possesses a high degree of homology to the consensus AHRE, these studies implicated the AHR in inducible interactions within c-Ha-ras regulatory sequences.

To further characterize the *trans*-acting factor or factors interacting at the Ha-ras AHRE, approximate molecular masses were obtained through UV cross-linking experiments. Incubation of a bromodeoxyuridine-substituted probe with nuclear extracts derived from cycling mouse SMCs treated with 3 μM BaP for 3 hr followed by UV irradiation catalyzed the covalent cross-linking of the bromodeoxyuridine residue to amino acids within any *trans*-acting factor in close proximity, presumably within the DNA binding domain. Two bands were induced by BaP challenge (Fig. 4, *top*). The complex labeled C1 migrated with a molecular mass corresponding to 104 kDa, whereas the complex labeled C2 migrated as a 96-kDa species. Both bands were competed effectively by a nonradioactive oligonucleotide of identical sequence but not by an oligonucleotide of random sequence, confirming their specificity for the Ha-ras AHRE.

To directly determine whether the AHR complex was induced to bind this sequence in BaP-treated mouse SMCs, the effect of an mAb to the AHR on the binding equilibrium was determined (Fig. 4, *bottom*). Nuclear extracts from BaP-treated mouse SMCs were incubated with 45 and 90 μg of mAb rpt1 (α-AHR) or goat serum IgG for 10 min at room temperature before the addition of Ha-ras AHRE cross-linking probe. This preincubation was performed to allow association of the antibody with the AHR present in the nuclear extract. Association of the mAb with an epitope of the receptor involved in DNA binding would have the potential to sterically disrupt those interactions, preventing the AHR complex from binding the Ha-ras AHRE. Alternatively, rec-

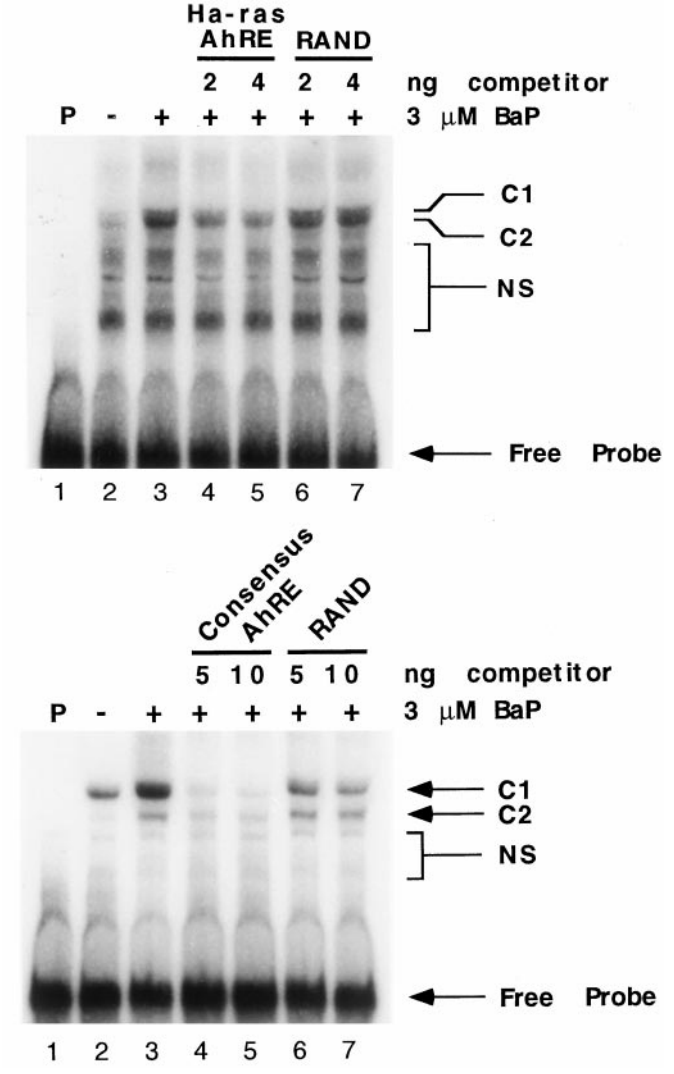

Fig. 3. BaP-induced binding to the Ha-ras AHRE or consensus AHRE elements. Nuclear extract (5 μg) was incubated with 10 fmol of end-labeled Ha-ras AHRE (*top*) or consensus AHRE (*bottom*) for 20 min at room temperature. Protein/DNA complexes were resolved on 7% non-denaturing polyacrylamide gels. Specific complexes were identified by competition using the indicated amount of specific (*Ha-ras AHRE* or *consensus AHRE*) or nonspecific (*RAND*) oligonucleotides. *Lane 1*, *P*, reactions performed in the absence of extract. *Top*, +, Nuclear extracts isolated from 3 μM BaP-treated cells. *Arrows and brackets*, specific complexes (*C1 and C2*), nonspecific complexes (*NS*), and free probe.

ognition of an epitope allowing association with DNA would have no apparent effect on cross-linking results because the mAb×protein interaction would be disrupted under the conditions used to run the gel. The mAb was thus used as a functionally neutralizing agent specific for the AHR in solution.

Products of the reaction were visualized as described above. Inclusion of 45 or 90 μg of mAb rpt1 in the binding reaction resulted in the disappearance of both the 104- and 96-kDa bands, whereas goat IgG was unable to compete at these concentrations. Inclusion of goat IgG in the binding reactions actually increased apparent binding, which is likely due to nonspecific effects. These experiments demonstrate that the 96- and 104-kDa bands are components of an AHR complex that can be induced by BaP to bind the c-Ha-ras AHRE.

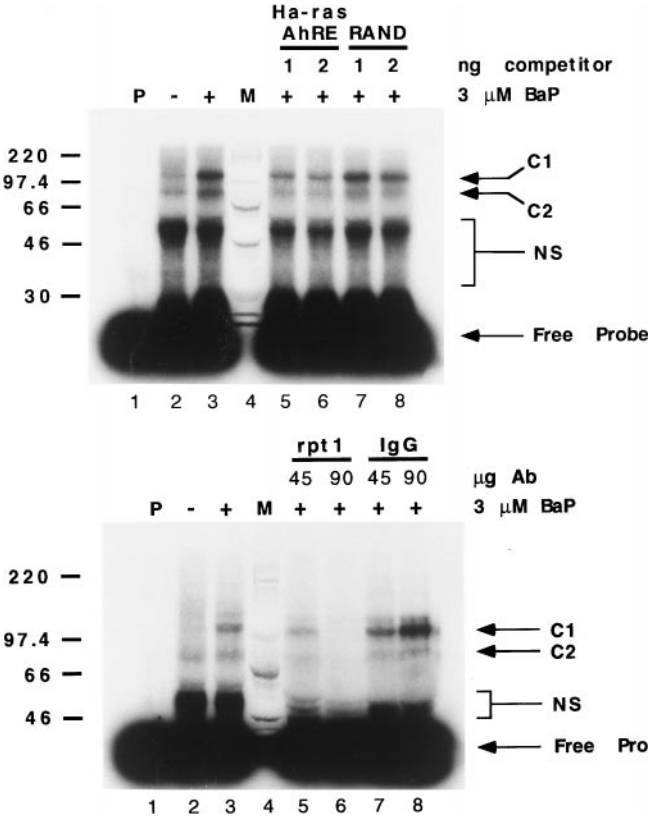

Fig. 4. UV cross-linking to the Ha-ras AHRE oligonucleotide. Equilibrium DNA-binding reactions using a bromodeoxyuridine-substituted Ha-ras AHRE oligonucleotide were performed as described in the text. *Top*, specificity of the binding reaction was assessed by oligonucleotide competition using 1 or 2 ng of unlabeled (*Ha-ras AHRE*) or random (*RAND*) oligos. Reactions were separated on an 8% resolving SDS gel. *Bottom*, mAbs to the AHR (*rpt1*) or goat (*IgG*) as a negative control were included in binding reactions to identify specific bands. Then, 45 or 90 μg of each antibody was preincubated with nuclear extract 10 min at room temperature before the addition of probe (*above lanes*). Reactions were separated on a 6% resolving SDS gel. *Right*, specific induced complexes (*C1* and *C2*), nonspecific complexes (*NS*), and free probe. *Left*, bands corresponding to molecular mass standards (*M*) are shown in kDa.

To address the functional relevance of our DNA binding studies, transient transfection of mouse SMCs was performed using a CAT reporter construct (pRASCAT2) containing a fragment of the human c-Ha-ras promoter from −330 to +220 (relative to the major transcription start site cluster at +1) (6). The Ha-ras AHRE element is located at −30. Cycling mouse SMCs were transfected with pRASCAT2, followed by challenge with 0.3, 3.0, or 30 μM BaP for 24 hr. Cell extracts were prepared and CAT activity was determined according to the method of Gorman (18). Although the concentrations examined induce a ≈50% increase in Ha-ras expression as determined by Northern analysis, transcription from pRASCAT2 was not activated to a significant extent (Fig. 5). Because additional regulatory elements exist 5′ of −330 in rat Ha-ras sequences (23), subsequent experiments were conducted to investigate the possibility that pRASCAT2 did not possess all the DNA sequence elements required for transcriptional activation by BaP.

If pRASCAT2 does not contain all required DNA binding sites for response to BaP, then inclusion of additional regulatory sequences should restore the response of the construct

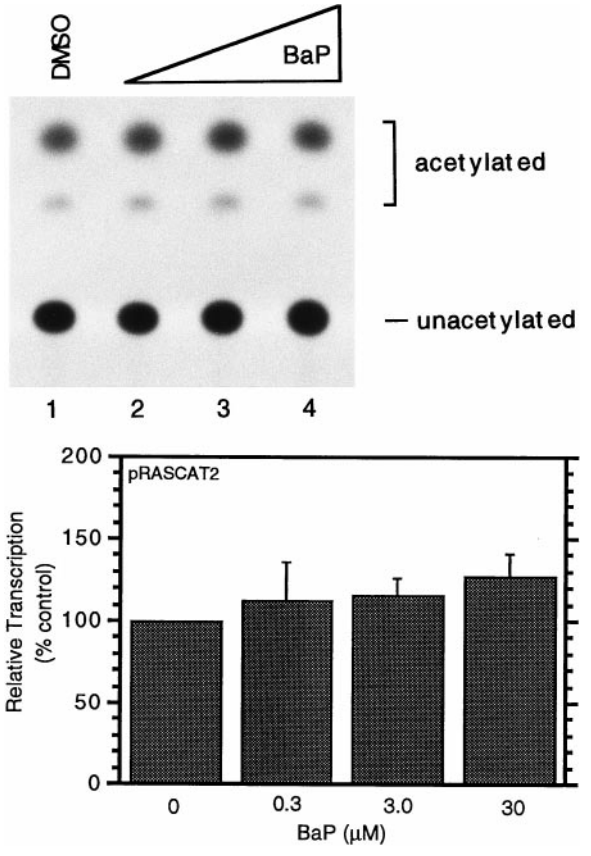

Fig. 5. Response of pRASCAT2 to BaP challenge. *Top*, representative data from mouse SMCs transiently transfected with pRASCAT2 and then challenged with BaP for 24 hr. *Lane 1*, vehicle-treated control. *Lane 2*, 0.3 μM. *Lane 3*, 3.0 μM. *Lane 4*, 30 μM. Right, acetylated and unacetylated forms of chloramphenicol. *Bottom*, CAT activity is shown as normalized activity ± standard error relative to a cotransfected vector constitutively expressing β-galactosidase. Values shown are relative to dimethylsulfoxide (*DMSO*)-treated controls and represent the average of three trials. Statistical significance was not observed between BaP- and vehicle-challenged cultures at the 0.05 level using the Mann-Whitney *U* test.

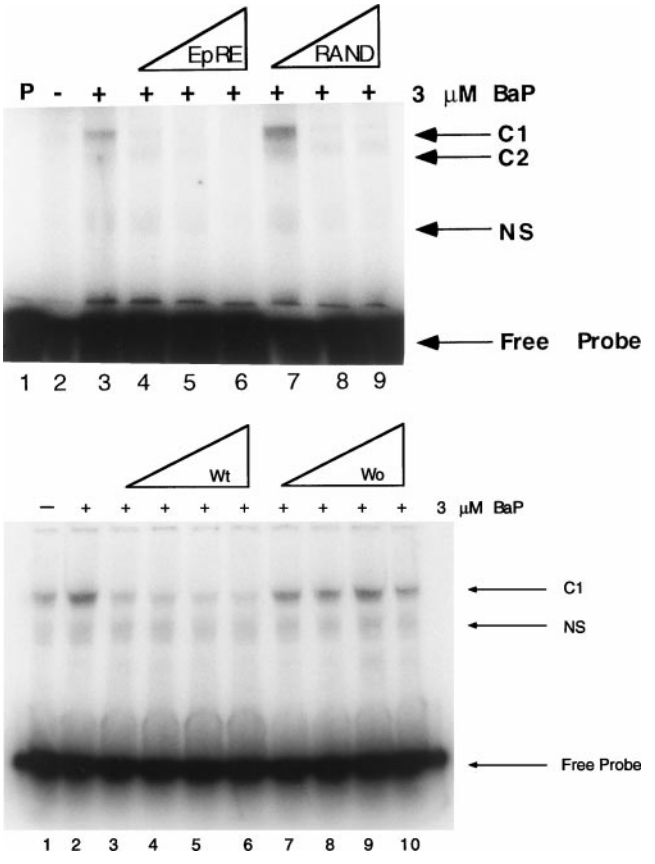

Fig. 6. BaP-induced binding to the EpRE element. Cycling cultures of mouse SMCs were challenged with 3.0 μM BaP for 3 hr, followed by nuclear extract preparation. Protein (5 μg) was incubated for 20 min at room temperature with 10 fmol EpRE and separated on 7% nondenaturing gels. *Top*, specificity was assessed by oligonucleotide competition using EpRE or random (*RAND*) oligonucleotides. In these experiments, gels were run in 1× Tris/glycine/EDTA using 50 ng of sonicated herring testes DNA and 1 ng of random oligonucleotide in place of poly(dI/dC) to allow resolution of two BaP-induced complexes. *Lanes 4 and 7*, 5 ng. *Lanes 5 and 8*, 10 ng. *Lanes 6 and 9*, 20 ng. *Top*, +, extracts isolated from BaP-treated cells. *Arrows to the right*, specific complexes (*C1 and C2*), nonspecific complexes (*NS*), and free probe. *Bottom*, specificity was assessed by oligonucleotide competition using wild-type EpRE sequence (*Wt*) or mutated sequence (*Wo*). In these experiments, the use of 50 ng of poly(dI/dC) and 1 ng of EpREWo in 0.5× TBE resolved a single BaP-induced complex. *Lanes 3 and 7*, 25 ng. *Lanes 4 and 8*, 50 ng. *Lanes 5 and 9*, 75 ng. *Lanes 6 and 10*, 100 ng. *Top*, +, extracts isolated from BaP-treated cells. *Arrows to the right*, specific complexes (*C1 and*, *C2*), nonspecific complexes (*NS*), and free probe.

to chemical challenge. A computer-aided analysis of the 5′ sequences of *c-Ha-ras* revealed a perfect consensus for an EpRE located at −543 (16). Our initial efforts focused on this element because BaP is readily metabolized by phase I enzyme systems present within the vasculature to various hydroxylated and epoxidated metabolites that can induce oxidative stress and may activate oxidant-responsive *trans*-acting factors (24). A similar paradigm has been demonstrated to be operative in the rat NADPH:quinone reductase gene promoter as well as the rat glutathione-*S*-transferase Ya and P promoters (25). Furthermore, recent work has implicated the AHR as a component of the protein complex interacting at the EpRE present within the mouse glutathione-*S*-transferase Ya promoter (26).

To determine whether BaP could induce protein binding to this element, EMSA was used using nuclear extracts prepared from mouse SMCs treated with 3 μM BaP for 3 hr (Fig. 6, *top*). Two bands were induced by BaP to bind this element (labeled C1 and C2), which appear to differ in their sensitivity to oligonucleotide competition. C1 was efficiently competed by 5 ng of specific competitor but not by 5 ng of random oligonucleotide (compare *lanes 4* and *7*), whereas C2 was competed to a smaller extent by either competitor. Higher concentrations of both specific and random oligonucleotides (10 and 20 ng) efficiently competed complex formation. However, the remaining weak binding activity of C1 and C2 was progressively ablated with increasing concentrations of cold EpRE, whereas residual binding was not affected by additional cold random competitor. To better define the specificity of EpRE/protein interactions in response to BaP challenge, competition experiments were carried out using EpREWo, an oligonucleotide with severe mutations in the EpRE sequence (16). Under the experimental conditions used, the only specific complex resolved was competed away by wild-type sequence in a concentration-dependent manner (25–100 ng), whereas an oligonucleotide of mutant sequence had no significant effect (Fig. 6, *bottom*). Thus, BaP can induce *trans*-acting protein factors to interact specifically with the EpRE.

The functionality of this response was assessed by subcloning an oligonucleotide encompassing this sequence upstream of the basal ras transcriptional control elements present in pRASCAT2 to create pARERC2A. Assay of this vector in transient transfection experiments with mouse SMCs revealed a strong activation of transcription (Fig. 7). The greatest response was achieved with 3.0 μM BaP, resulting in a $\approx$75% increase in transcription. Activation at 0.3 μM was less than that at 3.0 or 30 μM but not statistically different from the higher concentrations. This increase in response to BaP challenge closely parallels the response of c-Ha-ras mRNA expression in these cells observed through the use of Northern analysis. A similar response was observed with pARERC2B, which contains the EpRE in the reverse orientation (data not shown). Thus, it seems that a key element regulating ras transcription in response to BaP is the EpRE.

To begin characterization of the protein or proteins interacting with this element, UV cross-linking was used. Randomly cycling mouse SMCs were challenged with 3 μM BaP for 3 hr, followed by lysis and nuclear extract preparation. Several bands were easily detected using this method; however, only one was induced by BaP and effectively competed by unlabeled EpRE sequences (Fig. 8). The molecular mass of this band was calculated as 80 kDa, which is significantly different than that of the AHR or ARNT proteins. The physical characteristics of this protein seem to be unique and do not match those of other proteins demonstrated to interact with this sequence in various other systems. We therefore termed this protein EBP (electrophile response element binding protein).

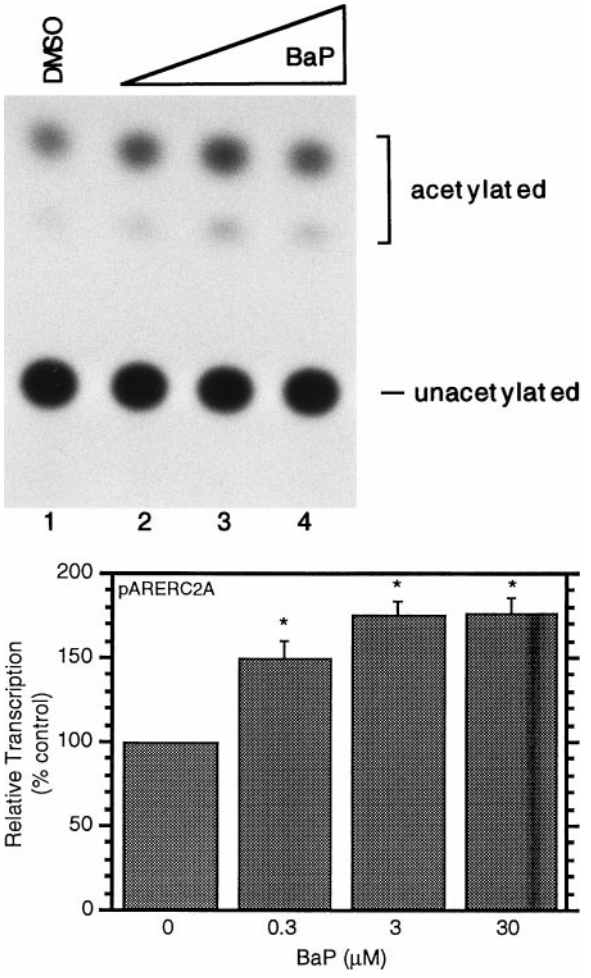

Fig. 7. Response of pARERC2A to BaP challenge. *Top*, representative data from mouse SMCs transiently transfected with pARERC2A and then challenged with BaP for 24 hr. *Lane 1*, vehicle-treated control. *Lane 2*, 0.3 μM. *Lane 3*, 3.0 μM. *Lane 4*, 30 μM. Right, acetylated and unacetylated forms of chloramphenicol. *Bottom*, CAT activity is shown as normalized activity $\pm$ standard error relative to a cotransfected vector constitutively expressing β-galactosidase. Values shown are relative to dimethylsulfoxide (*DMSO*)-treated controls and represent the average of five trials. $*$, Significance at the 0.05 level using the Mann-Whitney *U* test.

Discussion

BaP is commonly generated as a by-product of the combustion of organic materials and is a fairly ubiquitous environmental contaminant. BaP is found in high concentrations in the exhaust of internal combustion engines, cigarette and industrial smoke, and charcoal-broiled foods. Workers in the mining, chemical, transportation, and electrical industries, as well as individuals who actively or passively inhale smoke, may be exposed to considerably high levels of BaP. The lipophilicity of BaP influences its body distribution, with the majority of the parent molecule traveling throughout the systemic circulation in association with plasma lipoproteins (27). Estimates of BaP exposure in nonsmoking adults range from 0.171 to 1.64 μg/day (28). Due to the rapid and nearly complete uptake of BaP by lipoproteins, tissues proximal to areas of exposure receive higher doses than more distal targets. Because the primary route of exposure in humans is the lungs, cells of the vasculature would be expected to experience some of the highest exposures in the body. The range of BaP concentrations used in these experiments varied from 0.3 to 30 μM (0.23–23 μg/dish), a range spanning the Environmental Protection Agency-estimated human burden.

The current results demonstrate that BaP can induce transcriptional activation of the *c-Ha-ras* protooncogene in vascular SMCs. This response is mediated via specific DNA-binding protein or proteins that interact directly with regulatory sequences. Activation of a gene tightly coupled to

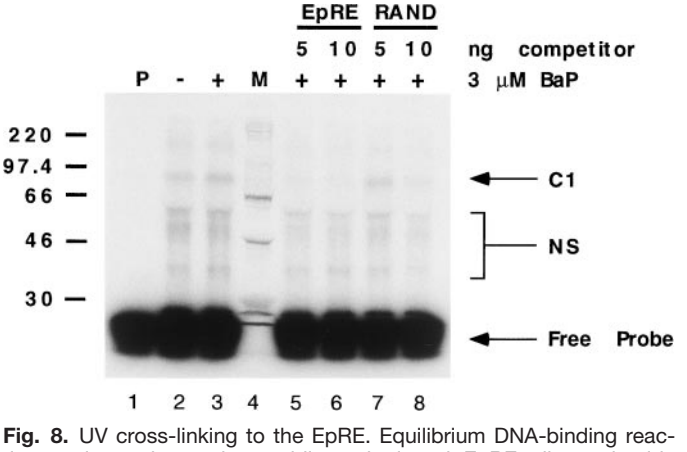

Fig. 8. UV cross-linking to the EpRE. Equilibrium DNA-binding reactions using a bromodeoxyuridine-substituted EpRE oligonucleotide were performed as described in the text. Reactions were run on 6% resolving SDS gels. Specificity of the binding reaction was assessed by oligonucleotide competition using the indicated amounts of unlabeled EpRE or random (*RAND*) oligos. *Arrows to the right*, specific complexes (*C1*), nonspecific complexes (*NS*), and free probe. *Left*, bands corresponding to molecular mass standards (*M*) are shown in kDa.

cell cycle-related events may have significant implications for the atherogenic process because deregulated proliferation of medial SMCs has been implicated in the initiation and progression of atherosclerotic lesions. Previous work in this laboratory has demonstrated that a 2-fold increase in Ha-ras expression is essential for continued cell cycle progression (29). Thus, the up-regulation of Ha-ras expression in response to BaP may have significant impact on the proliferative status of these cells. The data summarized here provide a basis for a mechanism of BaP-induced atherogenesis involving epigenetic deregulation of *c-Ha-ras* gene expression.

The results of Fig. 2 extend our work in heterologous species to mouse SMCs. A near-identical pattern of expression for c-Ha-ras in response to BaP challenge has been observed in both quail and rat SMCs (14, 19), as well as in rat hepatocytes (30). These findings suggest that the response of *Ha-ras* transcription to BaP extends across species lines and may be a common effect in higher eukaryotes. Our DNA binding data (Figs. 3 and 4) strongly implicate the AHR complex in the deregulation of *c-Ha-ras*, but interaction of this factor with the Ha-ras AHRE seems insufficient on its own to drive the expression of the gene in response to BaP. We believe this is most likely due to functional protein interactions that do not occur in the pRASCAT2 vector but are restored in pARERC2A and pARERC2B. It is important to recognize that the functional significance of the Ha-ras AHRE and any inducible binding observed at this element remain to be established. However, recent experiments using nuclear extracts from the mouse [^{14}Co]S/[^{14}Co]S hepatic cell line have demonstrated the participation of the AHR in protein complexes formed on EpREs present in the regulatory regions of several phase II genes (26). This work suggests that AHR/EpRE-binding protein interactions may be significant in the regulation of stress-responsive genes and may provide an explanation for the two protein complexes observed on the Ha-ras AHRE and EpRE in EMSA (Figs. 3, *top*, and 6). The molecular mass of endogenous AHR derived from the C57BL/6 strain of mice is ≈95 kDa (31), which matches well with one of the specific bands identified in our experiments (Fig. 4). The identity of the 104-kDa band is less clear. Murine ARNT protein is ≈87 kDa and is well conserved across species lines (17). Thus, it is unlikely that the 104-kDa band corresponds to ARNT. However, the AHR identified in the C3H mouse strain is ≈104 kDa, which may suggest that the mice used to isolate primary vascular SMCs in our experiments have a heterogeneous background despite inbreeding, as has been observed in recent studies using B6C3F1 hybrid splenocytes (32). Alternatively, the 104-kDa band could result from modifications of the protein specific to vascular SMCs, such as alterations in phosphorylation status or other post-translational modifications. Nevertheless, the BaP-inducible binding activity observed at the Ha-ras AHRE coupled with evidence of protein/protein interactions between the AHR and EpRE-binding protein implicate the AHR in transcriptional regulation of the *c-Ha-ras* gene. Experiments to assess directly the contribution of AHR-bound Ha-ras AHRE to transcription are under way.

It is apparent that a key control element within the *c-Ha-ras* 5′ regulatory sequences is the EpRE located at −543 in the genomic context of the gene. The Ha-ras EpRE (5′-GTGACAGAGC-3′) is a perfect match to the core binding sequence of the mouse glutathione-*S*-transferase EpRE (5′-GTGACNNNGC-3′) (16). However, little homology exists between the two elements in surrounding sequences. In particular, no AP-1-like element or ETS homology is present upstream of the Ha-ras EpRE. Although the binding sequence resembles a canonical AP-1 12-*O*-tetradecanoylphorbol-13-acetate-response element, studies by Nguyen *et al.* (33) demonstrate that AP-1 is not the primary factor recognizing this element. Furthermore, the molecular mass of the protein induced to bind this element in our nuclear extracts is 80 kDa (Fig. 8), which is inconsistent with the molecular mass of c-*jun*, the DNA-binding subunit of AP-1 (39 kDa) (34). However, it is very similar to the newly identified YABP protein partially purified from HepG2 nuclear extracts (35). YABP is a heterodimer of 28- and 45-kDa subunits that binds the mouse glutathione-*S*-transferase Ya EpRE with high affinity. Both subunits cross-link to the core EpRE sequence (36); however, YABP binding is noninducible to this element. It has therefore been suggested that YABP is constitutively bound to the EpRE and other factors are responsible for *trans*-activation of transcription. In contrast, EBP is an 80-kDa monomer that is strongly inducible by BaP to interact with the EpRE of *c-Ha-ras*, and it may be directly involved in mediating transcriptional events (Figs. 6–8). Thus, EBP may represent a cell type-specific variant of YABP that is inducible by planar hydrocarbons, or a completely separate protein. A more complete definition of the protein interacting at the c-Ha-ras EpRE awaits purification and cloning.

The role of the EpRE in inducing transcription of genes involved in combating oxidative stress raises provocative questions about its function in the regulation of *c-Ha-ras* transcription in response to BaP. It is intriguing to speculate on common mechanisms for transcriptional induction of stress-responsive genes. BaP itself may not be the ultimate initiator of transcriptional deregulation, but rather oxidative stress induced within the cell during bioactivation is responsible. However, it is also clear that BaP and its metabolites are ligands for the AHR (12), and the observed binding activity of this complex to *c-Ha-ras* regulatory sequences may further implicate two previously unrelated signaling pathways converging on *ras* in the regulation of aortic SMC proliferation. Preliminary experiments to address the capacity of BaP to alter cellular redox status indicate that BaP depletes cellular glutathione levels, a key molecule in maintaining redox homeostasis.[1] Most importantly, treatment of mouse SMCs with the γ-glutamyl cysteine synthetase inhibitor L-buthionine-*S*,*R*-sulfoximine resulted in a dose-responsive activation of transcription from pARERC2A (data not shown). Thus, cellular redox status seems to be critical in the regulation of *c-Ha-ras* transcription.

BaP has been characterized as both an initiator and a promoter of atherogenesis (10). Treatment of SMCs with BaP results in DNA adduct formation (37), disruption of DNA replication (19), and alterations in protein kinase C activity (38). These pleiotropic effects are indicative of the complex nature of cellular responses elicited by this chemical. The current studies suggest an epigenetic mechanism by which BaP could contribute to a deregulated proliferative phenotype in vascular SMCs. Unregulated progression of SMCs through the G_1 phase of the cell cycle by asynchronous ras expression may create a state of genetic instability which

[1] K. Kerzee and K. S. Ramos, unpublished observations.

coupled to the inhibition of DNA repair by BaP may set the stage for atherogenesis. Expression of viral ras proteins in mouse fibroblasts leads to chromosomal derangements, including multicentric and acentric chromosomes, micronuclei and aberrant mitoses (39). Thus, it is clear that ras signaling plays a central role in SMC growth and differentiation, and disruption of the normal pattern of signaling can have severe implications, including heritable changes in the pattern of gene expression in affected cells. Of significance within the context of this promotional mechanism is a report by Blaes *et al.* (40) demonstrating that a spontaneously initiated population of cells resides within the vasculature. The capacity of BaP to alter expression of *c-Ha-ras* may lead to disruptions in intracellular signaling that allow a preexisting initiated population to proliferate in an uncontrolled fashion. This promotional mechanism may prove to be a critical component of the proliferative response elicited by this hydrocarbon in vascular SMCs.

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References

1. Lowy, D. R., and B. M. Willumsen. Function and regulation of ras. *Annu. Rev. Biochem.* **62:**851–891 (1993).
2. Oka, H., Y. Chatani, R. Hoshino, O. Ogawa, Y. Kakehi, T. Terachi, Y. Okada, M. Kawaichi, M. Kohno, and O. Yoshida. Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res.* **55:**4182–4187 (1995).
3. Mangues, R., J. M. Kahn, I. Seidman, and A. Pellicer. An overexpressed N-ras protooncogene cooperates with N-methylnitrosourea in mouse mammary carcinogenesis. *Cancer Res.* **54:**6395–6401 (1994).
4. Jhappan, C., H. Takayama, R. B. Dickson, and G. Merlino. Transgenic mice provide genetic evidence that transforming growth factor a promotes skin tumorigenesis via H-ras-dependent and H-ras-independent pathways. *Cell Growth Differ.* **5:**385–394 (1994).
5. Ramos, K. S., R. C. Bowes III, X. Ou, and T. J. Weber. Responses of vascular smooth muscle cells to toxic insult: cellular and molecular perspectives for environmental toxicants. *J. Toxicol. Environ. Health* **43:**419–440 (1994).
6. Lu, J., W. Lee, C. Jiang, and E. B. Keller. Start site selection by Sp1 in the TATA-less human Ha-ras promoter. *J. Biol. Chem.* **269:**5391–5402 (1994).
7. Sadhu, D. N., M. S. Lundberg, R. C. Burghardt, and K. S. Ramos. c-Ha-*ras*$^{E\text{-}J}$ transfection of rat aortic smooth muscle cells induces epidermal growth factor responsiveness and characteristics of a transformed phenotype. *J. Cell. Physiol.* **161:**490–500 (1994).
8. Indolfi, C., E. V. Avvedimento, A. Rapacciuolo, E. Di Lorenzo, G. Esposito, E. Stabile, A. Feliciello, E. Mele, P. Giuliano, G. Condorelli, and M. Chiariello. Inhibition of cellular ras prevents smooth muscle cell proliferation after vascular injury in vivo. *Nat. Med.* **1:**541–545 (1995).
9. Ramos, K. S., Y. Zhang, D. N. Sadhu, and R. S. Chapkin. The induction of proliferative vascular smooth muscle cell phenotypes by benzo(a)pyrene is characterized by upregulation of inositol phospholipid metabolism and c-Ha-ras gene expression. *Arch. Biochem. Biophys.* **332:**213–222 (1996).
10. Bond, J. A., A. M. Gown, H. L. Yang, E. P. Benditt, and M. R. Juchau. Further investigations of the capacity of polynuclear aromatic hydrocarbons to elicit atherosclerotic lesions. *J. Toxicol. Environ. Health* **7:**327–335 (1981).
11. Lesko, S. A., P. O. P. Tso, S. Y. Yang, and R. Zheng. *Benzo[a]pyrene Radical Involvement in DNA Damage, Cellular Toxicity and Carcinogenesis*. Academic, New York, 401–414 (1982).
12. Bigelow, S. W., and D. W Nebert. The Ah regulatory gene product. Survey of nineteen polycyclic aromatic compounds' and fifteen benzo(a)pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicol. Lett.* **10:** 109–118 (1982).
13. Schmidt, J. V., and C. A. Bradfield. Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* **12:**55–89 (1996).
14. Sadhu, D. N., M. Merchant, S. H. Safe, and K. S. Ramos. Modulation of protooncogene expression in rat aortic smooth muscle cells by benzo-[a]pyrene. *Arch. Biochem. Biophys.* **300:**124–131 (1993).
15. Bral, C. M., and K. S. Ramos. Transcriptional activation of the c-Ha-ras protooncogene in vascular smooth muscle cells by benzo(a)pyrene. *In Vitro Cell. Dev. Biol.* **32**:599–601 (1996).
16. Rushmore, T. H., M. R. Morton, and C. B. Pickett. The antioxidant responsive element. *J. Biol. Chem.* **266:**11632–11639 (1991).
17. Hord, N. G., and G. H. Perdew. Physicochemical and immunocytochemical analysis of the aryl hydrocarbon receptor nuclear translocator: characterization of two monoclonal antibodies to the aryl hydrocarbon receptor nuclear translocator. *Mol. Pharmacol.* **46:**618–626 (1994).
18. Gorman, C. M., L. F. Moffat, and B. H. Howard. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2:**1044–1051 (1982).
19. Ou, X., and K. S. Ramos. Proliferative responses of quail aortic smooth muscle cells to benzo[a]pyrene: implications in PAH-induced atherogenesis. *Toxicology* **74:**243–258 (1992).
20. Chomczynski, P., and N. Sacchi. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159 (1987).
21. Shen, E. S., C. J. Elferink, and J. P. Whitlock, Jr. Use of gel retardation to analyze protein-DNA interactions upstream of CYPIA1 gene. *Methods Enzymol.* **206:**403–408 (1991).
22. Gradin, K., A. Wilhelmsson, L. Poellinger, and A. Berghard. Nonresponsiveness of normal human fibroblasts to dioxin correlates with the presence of a constitutive xenobiotic response element-binding factor. *J. Biol. Chem.* **268:**4061–4068 (1993).
23. Ariazi, E. A., T. A. Thompson, J. K. Burkholder, N. S. Yang, and M. N. Gould. Transcriptional regulatory and response mapping of the rat Ha-ras upstream sequence using primary mammary epithelial cells. *Carcinogenesis* **16:**965–968 (1995).
24. Juchau, M. R., J. A. Bond, and E. P. Benditt. Aryl H-monooxygenase and cytochrome P-450 in the aorta: possible role in atherosclerosis. *Proc. Natl. Acad. Sci. USA* **73:**3723–3725 (1976).
25. Xie, T., M. Belinsky, Y. Xu, and A. K. Jaiswal. ARE- and TRE-mediated regulation of gene expression. *J. Biol. Chem.* **270:**6894–6900 (1995).
26. Vasiliou, V., A. Puga, C.-Y. Chang, M. W. Tabor, and D. W. Nebert. Interaction between the Ah receptor and proteins binding to the AP-1-like electrophile response element (EpRE) during murine phase II [*Ah*] battery gene expression. *Biochem. Pharmacol.* **50:**2057–2068 (1995).
27. Busbee, D. L., J. O. Norman, and R. L. Ziprin. Comparative uptake, vascular transport, and cellular internalization of aflatoxin-B1 and benzo(a)pyrene. *Arch. Toxicol.* **64:**285–290 (1990).
28. Toxicological profile for polycyclic aromatic hydrocarbons. *US EPA* (1990). Publication no. PB 91-181537.
29. Sadhu, D. N., and K. S. Ramos. Cyclic AMP inhibits c-Ha-ras protooncogene expression and DNA synthesis in rat aortic smooth muscle cells. *Experientia* **49:**567–570 (1993).
30. Zhao, W., and K. S. Ramos. Modulation of growth-related gene expression in hepatocytes by aromatic hydrocarbons. *Toxicol. In Vitro,* in press.
31. Poland, A., and E. Glover. Variation in the molecular mass of the Ah receptor among vertebrate species and strains of rats. *Biochem. Biophys. Res. Commun.* **146:**1439–1449 (1987).
32. Williams, C. E., R. B. Crawford, M. P. Holsapple, and N. E. Kaminski. Identification of functional aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator in murine splenocytes. *Biochem. Pharmacol.* **52:**771–780 (1996).
33. Nguyen, T., T. H. Rushmore, and C. B. Pickett. Transcriptional regulation of a rat liver glutathione S-transferase Ya subunit gene. *J. Biol. Chem.* **269:**13656–13662 (1994).
34. Curran, T., C. Van Beveren, and I. M. Verma. Viral and cellular fos proteins are complexed with a 39,000-dalton protein. *Mol. Cell Biol.* **5:**167–172 (1985).
35. Liu, S., and C. B. Pickett. The rat liver glutathione S-transferase Ya subunit gene: Characterization of the binding properties of a nuclear protein from HepG2 cells that has high affinity for the antioxidant response element. *Biochemistry* **35:**11517–11521 (1996).
36. Nguyen, T., and C. B. Pickett. Regulation of rat glutathione S-transferase Ya subunit gene expression. *J. Biol. Chem.* **267:**13535–13539 (1992).
37. Bond, J. A., H.-S. L. Yang, M. W. Majesky, E. P. Benditt, and M. R. Juchau. Metabolism of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in chicken aortas: monooxygenation, bioactivation to mutagens, and covalent binding to DNA *in vitro. Toxicol. Appl. Pharmacol.* **52:**323–335 (1980).
38. Ou, X., T. J. Weber, R. S. Chapkin, and K. S. Ramos. Interference with protein kinase C-related signal transduction in vascular smooth muscle cells by benzo[a]pyrene. *Arch. Biochem. Biophys.* **318:**122–130 (1995).
39. Denko, N., J. Stringer, M. Wani, and P. Stambrook. Mitotic and postmitotic consequences of genomic instability induced by oncogenic Ha-ras. *Somatic Cell Mol. Genet.* **21:**241–253 (1995).
40. Blaes, N., M.-C. Bourdillon, J.-M. Daniel-Lamaziere, J.-J. Michaille, M. Andujar, and C. Covacho. Isolation of two morphologically distinct cell lines from rat arterial smooth muscle expressing high tumorigenic potentials. *In Vitro Cell. Dev. Biol.* **27A**:725–734 (1991).

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