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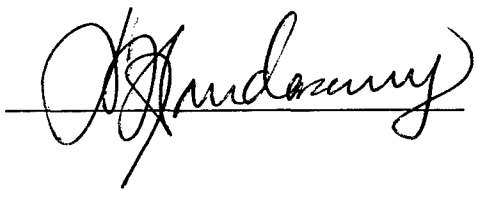

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Our previous studies in pubescent female rats suggested that nitrosomethylurea (NMU) mediated epigenetic effects on DNA conformation within the Ha-ras promoter contributes to mammary carcinogenesis. The DNA region affected by NMU includes a transcriptional element identical to Ha-ras response element (HRE) found in human Ha-ras promoter. To identify the trans-acting factors that bind to the HRE sequence in mammary cells, we employed sequence specific DNA affinity chromatography and amino acid sequencing by tandem mass spectroscopy. CARG box-binding factor A (CBF-A) is the major protein species in mammary cells that bind specifically to the rat and human HRE sequence with high affinities. Transient transfection assays using reporter plasmids verified that mutations within the HRE that disrupt binding of CBF-A, also reduced the activity of the rat Ha-ras promoter. We demonstrated a correlation between HRE binding activity, induction of Ha-ras mRNA expression and cell cycle progression following serum stimulation in the mammary carcinoma cell line. Our results further showed that CBF-A activity is regulated by post-translational modifications. Further results indicated increased CBF-A activity in chemically induced rodent tumors, suggesting an plays an important role in the regulation of the Ha-ras promoter during *in vivo* carcinogenesis.

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## Table of Contents

## Introduction

Our previous finding suggested a new paradigm for non-mutagenic mammary carcinogenesis . We demonstrated that the activating *Hras1* mutations found in NMU-induced tumors arise as background mutations within cells of the developing gland (14), and that NMU enhances the phenotypic penetrance of these mutations by initiating alterations in DNA conformation (41). We identified a cell-type-specific, DNA structure or conformational switch (CS) within the *Hras1* promoter of normal rat mammary cells (RMCs) *in vivo*. The analogous structure was also detected in the promoter of the human *Hras* gene. Our results further demonstrated that depending upon hormonal status of the animals, RMCs switch between states where the structural feature is present or absent from the *Hras1* promoter. In the F344 and SD rat strains, which are sensitive to mammary carcinogenesis, a carcinogenic dose of NMU initiated the loss of this structure from the *Hras1* promoter of RMCs. By contrast, the same exposure failed to disruption the promoter structure in Cop rats, which are highly resistant to mammary carcinogenesis. NMU also failed to promote the outgrowth of pre-existing *Hras1* mutants present in the mammary epithelium of resistant Cop rats. Phenotypic analysis of resistant (F344 X Cop)F1 progeny further indicated that the suppression of CS disruption was mediated by one or more suppressors expressed in RMCs of Cop rats. Together our results suggest that NMU-induced alterations in DNA conformation promote the outgrowth of pre-existing mutants by irreversibly deregulating expression of *Hras1*, thereby increasing the phenotypic penetrance of conditional oncomutations. Analysis of the DNA sequence comprising the CS region suggested the presence of an *ets*-like transcription factor responsive element (57). When we compared the binding of proteins from a variety of cell types to this response element, we detected the formation of a major complex with mammary cell extracts that was distinct from that formed in other cell types. Together these results supported a model in which CS disruption unmasks the *ets*-like responsive element contained within the CS structure, and thereby allows increased binding of transcription factors and elevated expression of responsive genes. The overall goals of these studies were to i) identify and clone the mammary cells specific transcription factor that forms a complex with the *ets*-like responsive element present in the CS switch region, and ii) determine its role in mammary carcinogenesis.

## Body of Report

The work on this project has proceeded as expected and without significant problems. We have successfully completed Technical Objectives 1 & 2. Technical Objective 3 not completed because we were able to obtain a polyclonal antibody against the rat CBF-A protein from other investigators. Instead we plan to develop a monoclonal antibody against the human CBF-A protein, which we are in the process of cloning. Technical Objective 4 is essentially complete.

The Ras proteins are closely related set of genes which encode membrane-associated proteins involved in cell proliferation and differentiation. The Ras proteins belong to the family of small GDP/GTP-binding protein that transduce signals from activated cell surface, tyrosine kinase receptors to the nucleus by activating a cascade of second messengers within the cytoplasm. Inactive forms of

Ras (GDP-bound form) can be reactivated by binding to free GTP in an exchange reaction catalyzed by guanine nucleotide exchange factors (GEFs). The active GTP-bound form is converted to the inactive GDP-bound form by an inherent GTPase activity, which is greatly stimulated by interaction with GTPase activating proteins (GAPs). Ras proteins mediate their effects on normal cell growth by regulating the levels or activities of key regulators of cell cycle progression. Ha-ras is essential for induction of cyclin D1 gene, suppression of the CDK inhibitor and subsequent Rb phosphorylation (25, 48).

Activated Ras proteins are able to transform a number of immortalized cell lines *in vitro*, and decrease tumor latency and increase tumor frequencies in transgenic animals. However, transformation in these experimental models is usually associated with expression of activated Ha-ras alleles at levels that exceed those observed in most cancers (39). However, numerous studies *in vitro* and *in vivo* suggest that activated rat Ha-ras does not function as a dominant oncogene when expressed at normal levels. When introduced into Rat 1 fibroblasts under the control of its own promoter, mutation activated Ha-ras failed to transform immortalized NIH/3T3 fibroblasts (29). Furthermore, transformed clones arising during passage of these transfected cell populations invariably over-expressed the mutant allele as a result of either gene amplification or transcriptional deregulation. The latter studies are in accord with the observations that mutant ras genes are frequently over-expressed in human tumor (9, 78). A recent study of transgenic animals harboring an inducible Ha-ras transgene demonstrated that continued expression of the oncogene is necessary for the genesis and maintenance of solid tumors *in vivo* (15). A variety of *in vitro* transformation experiments have demonstrated that even wild-type Ras proteins have transforming potential when expressed above normal levels. An *in vivo* correlate of this observation is the finding that over-expression of the normal Ha-ras protein is detectable ~50% of human breast cancers, although ras gene mutations are rare in these tumors (18). Taken together, these studies support the hypothesis that deregulated expression of the mutant or wild type Ras may be important for cancer development and maintenance *in vivo*. Understanding the mechanisms underlying ras deregulation therefore has implications for diagnosis and therapeutic intervention.

The Ha-ras proto-oncogene is constitutively expressed in all cell types, but can be induced in response to a number of mitogenic stimuli (53). The rat and human Ha-ras promoters have been cloned and a number of regulatory elements were identified (21, 47). The Ha-ras promoter in both species is G+C rich and lacks a TATA box, features characteristic of constitutively expressed "housekeeping" genes. Six GC boxes, two NF-1 binding sites and two potential AP-2 sites were identified within upstream regulatory region of human Ha-ras. In addition, two copies of HRC (Ha-ras conserved sequence) and an HRE-I (Ha-ras element I) were identified in the human promoter (47). The individual GC boxes appear to have different effects on the promoter activity: only GC II, which binds SpI, shows a positive effect on Ha-ras promoter activity. The NF-I elements themselves have weak effects on the promoter activity. Deletion of the NF-I binding site along with the HRE and GC-II site decreases transcription by 2.5-fold in the context of the whole promoter. Overall, the rat and human Ha-ras promoters are highly conserved, sharing similar regulatory elements located in the similar positions within the promoter relative to the start site. Only the HRE site present in the human promoter, which is thought to be responsive to the *ets* family of transcription factors (47), was not previously reported to have a counterpart in the rat promoter.

Our previous studies of NMU induced mammary carcinogenesis in the rat may involve carcinogen mediated effects on the Ha-ras promoter (41). Here we demonstrate that the region of the promoter affected by NMU includes a positive transcriptional element identical to Ha-ras response element (HRE) found in human Ha-ras promoter, albeit in the inverted and complementary orientation. We demonstrate that CBF-A (CArG Box Binding Protein-A) protein, originally defined by its ability to interact with CArG box binds to both the human and rat HRE elements. CBF-A binds to the rat and human Ha-ras HRE with higher affinity than the CArG box originally described as the recognition site for this protein. Furthermore, we failed to detect any *ets* transcription factor binding to the HRE elements. These results indicated that in mammary cells, CBF-A is the major protein that binds to recognition sequences commonly accepted as *ets* binding sites. CBF-A binding was correlated with increased Ha-ras promoter activity in mammary cells and there was a direct correlation between the presence of the HRE binding activity, induction of Ha-ras mRNA expression and cell cycle progression following serum stimulation in rat mammary carcinoma cells. Taken together, our results suggested that CBF-A mediated transactivation may play an important role in Ha-ras deregulation during carcinogenesis in rodents and humans.

CArG binding factor A (CBF-A) was first discovered by screening an expression library for proteins able to bind the CArG box DNA sequence (42). The CArG box promoter element was initially described in a genes with muscle tissue specific expression and immediate-early genes(13, 49, 50, 82). For example, it was shown that binding of transcription factors to the CarG element within the dystrophin gene promoter is regulated by YY1 and DPBF (33). Other studies showed that serum response factor (SRF) is required for muscle-specific transcriptional activation through CArG boxes (68) and that SRF competes with YY1 for binding to wild-type CArG elements (51). CBF-A has significant similarity with the group of heterogeneous nuclear ribonucleoproteins (hnRNPs) comprised of approximately 30 proteins (for review see (45). The main structural feature of hnRNPs are the presence of a RNA binding domain (RBD) and a glycin-rich motif, which may play an important role in the protein-protein interactions (11). Most hnRNPs genes also generate number of isoforms by alternative splicing and post-translational modification.

HnRNPs have been implicated in a variety of important cellular functions. In addition to splicing, transport and protection of the RNA, some hnRNPs are involved in the transcriptional regulation. For example, the interaction of hnRNP K with the CT-rich element within c-myc promoter correlates with transcriptional activation (56) . In another study (27), hnRNP K was found to be a transcriptional repressor of neuronal acetylcholine receptor β4 subunit gene, probably by interfering with SP1-mediated transactivation. Similarly, hnRNPs K inhibits transactivation of alpha-1 acid glycoprotein gene, possibly by interaction with the CCAAT/enhancer binding protein (C/EBP) β, preventing the formation of a Nopp-C/EBP β activator complex (55) .

Another notable function of the hnRNPs is their role in maintenance of telomere length. Telomeres in the erythroleukemic cells lacking hnRNP A1 are significantly shorter than in the same cells expressing exogenous hnRNP A1 (46). In addition, hnRNP D and E were found to interact *in vitro* with oligonucleotides containing telomere repeats ([Ishikawa, 1993 #63]).

Although CBF-A was initially identified as a transcriptional repressor (42) , our study showed that CBF-A can also function as a transcriptional activator of the Ha-ras gene (57) . This duplicity in transcriptional regulation is not surprising since the CArG regulatory element can interact with a number of other transcription factors, including ets related factors Elk-1 and SAP-1 (20, 38) ,E12, NF-IL-6 (54) and the HMG-I family of proteins (16). It is therefore plausible that CBF-A complexes with, or displaces other transcriptional factors depending on the context of the CArG box within a given promoter, and possibly in a cell type or cell cycle dependent manner. For example, functional antagonism between SRF and YY1 proteins at CArG elements has been described in chicken skeletal muscle cells(36).

Our previous study in a rat mammary carcinoma cell line demonstrated that CBF-A binding activity correlates with activation of the wild type Ha-ras promoter (57) . We also showed that CBF-A has a much higher affinity for the Ha-ras element (HRE) than it does for the CArG box originally identified as the consensus sequence for CBF-A binding. Although Western blot analyses detected two isoforms of CBF-A with estimated molecular weights of 42 and 44 Kda, only the 42 KDa isoform binds to the HRE (57). Similarly, Bemark et al. found that only the 4kDa isoforms binds with high affinity to the pd element of the globin gene promoter (6). We further observed that ectopic over-expression of the CBF-A failed to induce a significant increase in specific binding activity to target sequences or transcriptional activation of HRE reporter constructs. These results suggested that additional post-translational modifications are required for activation CBF-A binding activity *in vitro*.

To further define the role of CBF-A in mammary carcinogenesis, we next investigated the regulation of CBF-A expression and interaction with the HRE target sequence *in vitro* and *in vivo*. Inhibition of either PI3 kinase activity with Wortmannin or ERK phosphorylation with PD98059, both reduced CBF-A binding to HRE within 2 hours of treatment. Likewise, overexpression of dominant negative ras (ras N17) also resulted in reduced CBF-A interaction to the target DNA. By contrast, CBF-A binding activity was induced in Ha-ras, src, fos, neu and trk transformed fibroblasts. In the latter experiments, the levels of CBF-A protein expression remained constant, indicating that post-translational modification of CBF-A was required for the increased binding activity *in vitro*. We further demonstrated activation of CBF-A binding activity *in vivo* in chemically-induced rodent tumors harboring activated Ha-ras oncogenes. Rat mammary carcinomas induced by N-methyl-N-nitrosourea and mouse skin carcinomas induced by 9,10-Dimethyl-1,2-benzanthracene both showed elevated CBF-A binding activity. However, in contrast to the results of the *in vitro* experiments, the levels of CBF-A protein were increased in 22 out of 23 mammary tumors relative to the levels detected in adjacent normal mammary gland. Similarly, increased levels of CBF-A protein were detected in all mouse skin tumors. However in both cases, the level of CBF-A mRNA were only marginally increased, indicating that post-transcriptional regulation also plays a role in accumulation and binding activity of CBF-A protein *in vivo*. Together, our results suggested that activation Ha-ras signaling increases CBF-A binding activity *in vitro* and *in vivo*, and that CBF-A binding may play a role in cell transformation mediated via the Ha-ras pathway.

## Methods

**Cell culture, cell treatment and cell cycle analysis.**

The BICR rat mammary gland carcinoma cell line was grown in DMEM and 5% fetal calf serum (HyClone Laboratories, Inc, UT). Cell cultures were harvested during exponential growth or following appropriate treatment times. Following cell disruption cytoplasmic fractions were used for RNA extraction (17). Released nuclei were used for protein extraction according a previously described method (23) . In some experiments, cells were serum starved for 48 hours and stimulated with 5% FCS. At the indicated times after stimulation, cells were harvested for extraction of RNA and nuclear protein. Treatment with L-mimosine (Sigma, MO) was performed at a final concentration 200 ug/ml for 10 hours before as well as during serum stimulation. For cell cycle analysis, cells cultures were used at 70-80% confluence. After appropriate treatments, cultures were harvested by trypsinization, fixed in 35% ethanol, stained with propidium iodide, and analyzed using Becton Dickinson flow cytometer.

Rat-1 fibroblasts, Me12 cells (Rat-1 transformed with mutant Ha-ras) and E12 cells (site specific recombinants expressing mutant Ha-ras under control of it's own promoter) were provided by Dr. Finney (29) . Cells transformed with src and corresponding parental Rat-1 fibroblasts obtained from Dr. Jonathan Cooper (FHCRC). Rat-1 fibroblasts transformed with the neu, c-trk, or v-fos (1302) oncogenes, and the phenotypic revertant from v-fos induced transformation (EMS-1-19) were previously described (79). Cells were grown in DMEM supplemented with 10% fetal calf serum (HyClone Laboratories, Inc, UT). The BICR-M1Rk rat mammary gland carcinoma cell line was grown in DMEM and 10% bovine serum (Summit Biotechnology, Co). Cell cultures were harvested during exponential growth or following appropriate treatment times. In some experiments, cells were serum-starved for 48 hours and then stimulated with 5% bovine serum. Treatment with Wortmannin (Sigma, MO) was performed at a final concentration of 10 and 100 pM. Treatment with PD 98059 (New England Biolabs, MA) was performed at final concentration 50 uM for two hours. Inhibition of ERK phosphorylation following PD98059 treatment was verified on by Western blot analysis using an antibody against phospho-ERK (Santa Cruz, CA). Inhibition of PI3 kinase following Wortmannin treatment was verified by induction of apoptosis. In this assay, cells were irradiated with 20 mJoule of UV (254 nm) radiation. Twenty hours after exposure, 10pM of Wortmannin were added to the irradiated cultures. Following an additional 3 hours of incubation at 370C, floating cells were harvested and genomic DNA was extracted and loaded on 1.5% agarose gel to document DNA fragmentation in a nucleosome dependent manner.

**Cell transfection.**

Dominant negative rasN17 was a gift from Dr. Eugenio Santos (62). Purified plasmid was co-transfected into cell with selectable marker pMEXneo using Lipofectamine Plus reagent according to manufacture's protocol (Gibco BRL, MD). Individual transfectants were selected with G418. Inducible expression of ras17 was verified on the Western blot using antibody against Ha-ras (Santa Cruz, CA). Functional activity of ras17 expression was tested by plating cell in soft agar and by sensitivity to induction of apoptosis following 20 mJoule of UV. Apoptosis was scored 18 hours later by the presence of genomic DNA ladder extracted from cells. Soft agar was assessed by seeding 1 X $10^4$ cells in DMEM, with 10 % Bovine serum and 0.33% molten Difco noble agar. Plates were

incubated at 37° C in a 5% CO2 atmosphere and fed with 1.0 ml of 0.33% agar medium every 7-10 days.

**Electrophoretic Mobility Shift Assays (EMSA).**

Oligonucleotides were purchased from Integrated DNA Technologies, Inc (IDT, Inc., IA). For EMSA, a double stranded probe corresponding to the Ha-ras Response Element (HRE) sequence (5'- TAG GGG TTC CGG CGA ACT CTG T-3') was labeled with polynucleotide kinase (NE Biolab, MA) and [α32P]-ATP (NEN Dupon, MA). Aliquots of approximately 8-10 ug of protein from each nuclear extract were incubated for 30 min in binding buffer D (20mM HEPES, pH7.9, 20% Glycerol, 100mM KCl, 0.2mM EDTA, 1.0 mM PMSF, 1mM DTT), containing 0.5 nM of 5'end-labeled probe, and 1ug of the nonspecific binding inhibitor, poly dI•dC (Sigma, MO), in a total volume of 15 ul. The complexes formed were separated on 6-8% TBE polyacrylamide gels (Fisher Scientific, PA).

**Extraction of RNA, nuclear protein and total protein.**

After harvesting cell, nuclei were released and used for extraction of nuclear protein according to a previously described methods (23) . RNA was extracted from cytoplasmic fraction of the same cells. To extract nuclear proteins from rat normal tissues and mammary tumors, tissues were disrupted in liquid nitrogen using pestel and mortar with following nuclear protein extraction as described above. For extraction of total proteins, cell from disrupted tissues were lysed with RIPA buffer on ice for 30 min. Tissue lysates were then passed through a 21 guage needle. Before each analysis, nuclear and total proteins were centrifuged for 30 min at 30,000g. Total RNA extraction from rat tissues was performed with Trizol reagent (Gibco BRL, MD) according to manufacture recommendation.

**Northern blot analysis.**

Total RNA extracted from cells was dissolved in formamide. Twelve ug of each RNA sample was loaded on a 1.1% agarose gel containing formaldehyde. Following electrophoretic separation, RNA was transferred to GeneScreen Plus (NEN DuPon, MA) nylon membrane by electroblotting for 2 hours at 1mA in 25mM sodium phosphate buffer, pH6.5, using a transblot apparatus (BioRad, CA). The CBF-A cDNA probe was labeled by random priming with [$\alpha$-$^{32}$P]-dCTP (NEN Dupon, MA) and hybridized to the blotted membranes for 18 hours in 1M NaCl, 0.1 SDS, 1ug/ml of sheared calf thymus DNA (12). RNA loading was verified following blotting by photographing of ethidium bromide stained membrane. Membranes were hybridized to the Ha-ras cDNA probe labeled by random priming with [$\alpha$-$^{32}$P]-dCTP (NEN Dupon, MA) according to manufacturer's recommendations and subjected to autoradiography using Hyperfilm-MP (Amersham Life Science, Inc., IL).

**Western blot analysis.**

Nuclear proteins from cultured cells or total proteins from mammary tissues were separated on 12% SDS-PAGE gels and elecroblotted onto PVDF membranes (Millipore, MA). Membranes were probed with CBF-A antibody (6) using standard procedures. Blotted antigens were detected using chemoluminescent detection (NEN DuPont, Boston, MA). Protein molecular weight standards were from Bio-Rad (Hercules, CA). The quality of transfer and protein loading was assessed by staining of the membrane with Coomassie Brilliant Blue G (Sigma, St. Louis, MO) or by detection of actin with an appropriate antibody (Santa Cruz, CA).

**UV crosslinking.**

For UV crosslinking of oligonucleotide probes to specific binding proteins, EMSA reactions were subjected to UV irradiation for 30 min using transilluminator (Fotodyne, Inc., WI) Protein-DNA complexes were boiled in a sample buffer with 5% mercaptoethanol and separated in 10% SDS-PAGE. After electrophoresis, gels were dehydrated and were subjected to autoradiography using Hyperfilm-MP (Amersham Life Science, Inc., IL).

**Luciferase assay for Ha-ras promoter activity.**

Ha-ras promoter sequences were derived from the pNMU-1 plasmid (70) and inserted into SmaI site of pGL2 plasmid. Wild type promoter sequence at position -573 (CCGG) was replaced to GCGC using Sculptor Kit (Amersham Life Science, Inc., IL) according manufacturer's recommendation. The presence of the mutation within the promoter was verified by DNA sequencing of plasmids constructs. Transient transfection assays were performed using a modified methods developed in our laboratory (5) to normalize for possible differences in transfection efficiencies of different DNA's. BICR-M1Rk cells were transfected in serum free conditions with Lipofectamine Plus reagent (Gibco BRL, MD) according to manufacturer's protocol. Twenty four hours following addition of serum, cells were harvested, counted using a Coulter Counter (Coulter Electronics Ltd., UK) and lysed by three cycles of freezing and thawing in 25mM Tris pH 8.0. Cytoplasmic fractions were used for the luciferase assay using standard procedure. Released nuclei were lysed in lysis buffer (1X AmpliTaq buffer II (Perkin Elmer) containing 1mM MgCl2, 0.45% of Nonidet P-40 and 0.45% Tween 20) and digested with proteinase K (0.1 ug/ul) at 56°C 1 hour. Proteinase K was inactivated 15 min at 94°C. To measure the transfected plasmid copy numbers in nuclei of transfected cells [Bahramian, 1994 #62], a 2 ul aliquots of extracted DNA from each transfection was subjected to PCR amplification using 20 pM/ul luciferase gene of primers, pLZ1 (ATA CGC CCT GGT TC) and pLZ2 (CCC TGG TAA TCC GT). PCR reactions were carried out at 94° C - 35 sec, 49° C- 35 sec and 72° C 40 sec. Amplification was performed 8 cycles in termal cycler (Perkin Elmer Cetus) in a presence of 3 uCi of p32 dCTP (NEN Dupon, MA) per reaction in 25 ul of the total volume. Standards included DNA from untransfected cells and known number of plasmid copies. PCR products were separated on the 6% polyacrylamide gel (Fisher Scientific, PA) and quantitated by PhosphorImager (Molecular Dynamics, CA) analysis. Negative control reaction included water and

cytoplasmic fraction taken for luciferase assay. Activities of the promoter construct were plotted as luciferase values per plasmid copy number per cell number. Final results are presented as fold-activation of the wild type or mutant promoter construct divided by the expression detected with the control pGL2 plasmid. Transfection experiments were performed at least four times using two independent plasmid preparations.

**Protein purification**

Protein purification was performed starting with approximately from 30 ml of a BICR-M1Rk wet cell pellet (800xg). Nuclear extracts were prepared as described above using 5-6 ml of cell pellet at the time. The resulting nuclear extracts were clarified at 30, 000xg and dialyzed against buffer D (see above). Each batch of nuclear extract was tested for binding activity using EMSA under conditions described above. Biotinylated sense and unmodified antisense oligonucleotides (same as above) were obtained from Research Genetics (Huntsville, AL), annealed and attached to streptavidin-agarose (Pierce, IL.) for use in affinity chromatography. Annealing of oligonucleotides was performed in excess of anti-sense strand to ensure complete annealing of the biotinylated strand. Affinity columns were similarly prepared using mutant oligonucleotides.

The individual batches of nuclear protein extracts were first incubated with poly dI•dC at 20 ug/ml to titrate non-specific DNA binding proteins, and centrifuged at 30,000 x g to remove precipitates. To reduce amount of non-specific DNA binding activity, the extracts were first passed over an affinity chromatography column generated with the mutant binding site. Column eluates were then passed over a column of the wild-type binding site to capture specific binding proteins. To ensure complete binding, the nuclear extract was passed over the column repeatedly overnight at 4°C using a peristaltic pump. Proteins bound to the wild-type column were then eluted with a 0.1 M step gradient of 0.2-1.0 M KCl. Collected fractions were dialyzed against buffer D and tested for binding activity by EMSA. All fractions showing specific binding were pooled and loaded on the specific column and eluted again as above. Positive fractions were concentrated (Millipore, MA), loaded on the 10% SDS gel and stained with Coomasie Brilliant Blue R-250 (Bio-Rad laboratories, CA). Detected bands with estimated molecular weights of 42 and 43 kDa were excised and eluted from a gel. One fifth of the eluate from the most abundant protein band (42kDa) was used for in UV crosslinking reactions to verify the presence of specific binding activity. Elution of protein for UV crosslinking was performed by vigorous shaking of crushed polyacrylamide gel slice overnight in 50mM Tris (pH 7.5), 0.1M EDTA, 100 mM NaCl, 20% glycerol, 100 ug BSA per ml, 1% TritonX-100, 10 mM DTT. The remainder of the 42 kDa band was used for peptide identification by capillary HPLC - Mass Spectrometry described below. The 4 kDa band was forwarded to Harvard Microchemistry Laboratory directed by Dr. W. Lane where protein identification was performed by microcapillary reverse-phase HPLC electrospray tandem mass spectrometry.

**Protein identification by capillary HPLC - Mass Spectrometry.**

The protein band was excised from a one dimensional preparative SDS-PAGE and digested with 0.5 microgram of Trypsin (Promega, Madison, WI). The digested peptide mixture was extracted and analyzed by microcapillary LC system connected online to an electrospray ionization ion trap mass spectrometer (Finnigan-MAT, Model LCQ, San Jose, CA). Peptides were concentrated and

separated on a micro C18 column with an inner diameter of 50 micrometer. Separation was accomplished by applying a gradient of 5% to 65 % B over 20 minutes. The gradient was delivered by a Magic 2002 HPLC system (Michrom BioResource,Inc., Pleasanton CA) and the flow delivered over the column was adapted with a pre-column flow splitter to 200 microliter per min. Eluting peptides were introduced into the mass spectrometer by electrospray via a home built microESI ion source and analyzed by data dependent MS/MS (28, 37) . The collision induced dissociation spectra generated during the experiment were searched against protein as well as nucleotide databases using Sequest software to identify possible sequence matches.

**Animal care and tumor induction**

All rodent tumors used in this study were obtained from two ongoing studies funded by the NIH and the American Cancer Society (ACS). The samples used to leverage the present study represented excess tumor tissue generated by the other ongoing studies. Use of these tumor samples for analysis of CBF-A mRNA and protein expression levels in the present study did not require the treatment of additional animals, in no way compromise the other studies, and were made available at no cost to the present study.

Rat mammary tissue from chemically treated rats were available from an NCI funded study entitled Mapping "Genetic Suppressors of Epigenetic Carcinogenesis" (RO1 CA7722, Helmut Zarbl, P.I.) The overall goal of the latter study is to use genetic linkage analysis to map this novel suppressor(s) of mammary carcinogenesis on the rat genome in preparation for positional cloning and application to studies of human breast cancer risk, early detection and prevention. In this study female N2 backcross progeny rats [Fisher 344x(Fisher 344xCopenhagen)F1] were treated with 50 mg/kg body weight of N-methyl-N-nitrosourea (NMU) by i.p. injection. All animals were fed a standard laboratory diet enriched with 7% fat (lard), given water *ad libitum* and housed under a 12h light/12-h dark cycle. Animals were monitored weekly for development of mammary tumor and were asphyxiated by $CO_2$ inhalation when tumors reached a diameter of 1-1.5 cm. Tumors, as well as all normal mammary glands were dissected and frozen in liquid nitrogen. Tissues were stored at -80°C until needed.

Tissues from chemically treated rats were available from an ACS funded study To Dr. C.J Kemp (FHCRC). Mouse skin carcinomas were provided as frozen tissue by J.Philipp and C.J. Kemp. Skin tumors were induced in NIH strain mice (Harlan Olac, UK) with single application of 7,12-Dimethylbenz(a)anthracene (Sigma) followed a week later by twice weekly application of tetradecanoil13-phorbiol acetate (TPA) (Sigma) for 15 weeks as described in (61) . The work was funded by ACS res. grant to CJ Kemp

## Results

**Identification of the rat Ha-ras Response element (HRE).**

We first tested the hypothesis that disruption of the *in vivo* DNA conformation present in the Ha-ras promoter of mammary cells could unmask positive transcriptional elements. The region of the promoter (around nucleotide –573) that harbors the mammary gland specific DNA conformation (41) was found to include the sequence nucleotide sequence GGAA. This sequence corresponds to the *ets* transcription factor core binding site, albeit in complimentary and invert orientation. To determine whether any transcription factors can bind specifically to this region of the promoter, we performed EMSA using synthetic, double-stranded oligonucleotides (Fig.1A) and nuclear extract from the BICR mammary carcinoma cell line. The results presented in Figure 1B provide evidence for specific binding of proteins from nuclear extracts to the HRE probe. The stable protein DNA complexes formed were the result of sequence specific DNA binding, since a forty-fold molar excess of dsDNA probes (Mutants 1, 2 and 4) mutated within the putative ets binding site (CCGGAA) failed to compete with wild-type probe (Fig.1B). However, a 40-fold excess of dsDNA probes with mutation outside of the consensus CCGGAA ets motif (Mutant 3) were as effective as the wild-type sequence in competition experiments. The human HRE element possessing same core sequence was also effective in competing for the binding activity. Unrelated promoter elements such as SP1, AP-1, and Stat5/6 binding sites failed to compete for binding to the Ha-ras HRE element (not shown). Both the human and rat HRE probes formed similar protein(s) complexes with nuclear extracts from rat, mouse and human cells, as judged by mobility in EMSA gels (not shown).

To determine if the proteins bound to the rat HRE were members of the ets transcription factor family, we performed competitive binding experiments with an oligonucleotide probe comprising the binding site for the Drosophila melanogaster E74 ets transcription factor. The E74 probe (EBS) forms two distinct EMSA complexes (C1 and C2) with mammary cell nuclear extracts (Fig.1C). The rat HRE probe was able to compete effectively with the E74 probe, although the rat HRE affinity for protein complex C1 was higher than for the complex C2 (Fig.1C, lane 3). In the inverse experiments (Fig.1C, lines 4 and 5), labeled rat HRE probe formed predominantly complex C1, while complex C2 was very weak or undetectable. An excess of unlabeled oligonucleotide corresponding to the E74 binding site efficiently abrogated rat HRE binding. The latter result is consistent with the notion that the proteins bound to the rat HRE could be members of the *ets* protein family, or at least compete for binding to the same DNA sequences.

To investigate the role of rat HRE in the context of the promoter, we performed transient transfection assay using wild type and mutant rat Ha-ras promoter linked to the luciferase reporter gene. A double mutation that disrupts the Msp1 site at position –573 was introduced into the HRE using the mutant oligonucleotide (Mutant 2; See Fig. 1A). In transient transfection experiments the wild type promoter showed a 3-fold increase in activity relative to the mutant promoters in BICR-M1Rk cells (Fig. 2). The activity of mutant promoter was only slightly higher compare to the activity of the empty vector. Thus, in context of the whole promoter, the HRE has significant positive effect on the Ha-ras promoter activity in mammary cells.

We next used UV irradiation to cross-link the specific binding proteins to the DNA probe in order to estimate the approximate molecular weight of the specific binding protein. Analyses of the cross-linked products by SDS polyacrylamide gel electrophoresis demonstrated that the protein bound to the DNA probe has an estimated molecular weight of approximately 51-52kDa. Assuming the probe bound to the protein was single-stranded, the latter result suggested that the protein alone is ~43 - 44kDa in weight (data not shown and see Fig. 3B).

Taken together, these experiments suggested that the HRE sequences from rat and human Ha-ras promoter was a specific binding site for an *ets* related transcription factor present in mammary cell lines. However, ETS-1 and –2 (75) antibodies or Ets 1/Ets 2 antibodies (Santa Cruz, CA) designed to recognize broad spectrum of ets related proteins failed to super-shift the complexes formed between the HRE oligonucleotide probe and the mammary cell nuclear extracts (not shown). These results suggested that the HRE binding proteins in mammary cells were either novel members of the *ets* transcription factor family or unrelated proteins that recognized the same DNA sequences as *ets* proteins. We therefore performed experiments to identify and clone the HRE binding proteins.

**Purification of the protein and protein identification.**

The HRE binding protein was extensively purified using sequence specific DNA affinity chromatography as described in the Material and Methods. Bound proteins were eluted from the column with a KCl step gradient and fractions assayed for HRE binding by EMSA. Most of the binding activity eluted in 0.7-1.0M KCl (not shown). Fractions with binding activity were pooled, concentrated and analyzed on 12% SDS gel. Two main bands with estimated molecular weights of 42 and 43 kDa were detected in the fractions with binding activity (Fig. 3A). To confirm that most abundant protein band contained the HRE binding protein, the 42 kDa band was excised from Coomassie Brilliant Blue stained gel, and protein was eluted from 1/5 of gel slice. Following renaturation (65), the eluted proteins were incubated with radiolabeled HRE probe and any resulting protein-DNA complexes were crosslinked with UV light. The protein eluted from 42 kDa bands formed stable complex with the HRE probe that was indistinguishable from the crosslinked complexes formed with the protein in total nuclear extract by SDS-PAGE. (Since the amount of the protein in 43 kDa band was significantly lower than in the former band, we did not to perform crosslinking experiments to conserve protein for further analysis.) In summary, crosslinking experiment above showed that the protein in the 42 kDa protein preparation contained an HRE binding protein, consistent with molecular weight estimated by UV crosslinking.

Independent protein analyses identified similar polypeptides present in both the 42 and 43 kDa proteins (summarized in Table 1). The polypeptides identified corresponded to sequences detected in the previously identified mouse CArG binding protein (CBF-A) (42). Together the polypeptides identified in our analysis encompassed almost 36% of the CBF-A amino acid sequence. Moreover, MS/MS analysis of these two and three additional bands with low intensity staining from the preparative protein gel (Fig.3A, showed with short arrows) failed to detect any *ets* related proteins (data not shown). Together these results suggested that the CBF-A or closely related protein was responsible for most of the HRE binding activity detected in mammary cells.

**CBF-A interacts with rat HRE with greater affinity compare to CArG-box.**

To verify that CArG binding protein interacts with rat and human HRE, we employed an anti-CBF-A antibody kindly provided by T. Leandersson (6) First, we verified that the polyclonal antibody against mouse CBF-A would cross- react with the rat protein. Rat CBF-A was cloned by PCR from BICR cells, *in vitro* translated and detected using Western blot (not shown). EMSAs performed demonstrated that the antibody completely and specifically abrogated the interaction of nuclear protein with the rat (Fig. 4A) or human (not shown) HRE probes. These results provided direct evidence that CBF-A was responsible for the HRE binding activity observed in mammary cells.

We next compared the relative affinities of CBF-A protein for rat HRE and the CArG-box originally described as its recognition sequence. A 50-fold molar excess of unlabeled, double-stranded oligonucleotides corresponding to CArG box (42), rat HRE or an EBS were used as competitors in binding experiments using labeled rat HRE probe and analyzed by EMSA. While the addition of 50-fold molar excess of cold rat HRE and EBS efficiently diminished DNA-protein binding, addition of same molar excess of CArG box oligomer demonstrated only partial competition with rat HRE (Fig.4B). The latter results suggested that CBF-A bind to the HRE elements present in the Ha-ras promoters of rats and humans with high affinity than the CArG box originally used to isolate the CBF-A. Similarly, CBF-A is able to bind ets binding site (EBS) with higher affinity than the CArG box. From these competition experiments (also see Fig. 1), we conclude that sequence CCGGAA is important for high affinity CBF-A binding to DNA.

In a previous study it was shown that antibody against CBF-A recognizes two protein species in mammary cell extracts by Western blot (6). However, the authors demonstrated that only the lower molecular weight protein was found to interact with the A-T rich region of the pd element. To determine if rat HRE interacts with one or both protein species, we probed a Western blot containing protein fractions eluted from affinity column (see Material and Methods for details) with the CBF-A antibody on the Western blot. Figure 5 demonstrates that the same two protein species found in the nuclear extract of mammary cells (p44 and p42) are detected in protein fractions eluted from the affinity column. However, protein ratio (p44/p42) is reduced compare to nuclear extract before loading on the column. In our hands, under normal growth conditions this ratio is 0.5-1.2. This experiment together with microcapillary reverse-phase HPLC electrospray tandem mass spectrometry provided the evidence that slower migrating protein (44KDa) is related to CBF-A.

**Establishing a correlation between CBF-A binding to HRE, Ha-ras mRNA expression, and G1 to S cell cycle progression.**

Previous studies have demonstrated that serum stimulation of cells arrested in G0 leads to induction of Ha-ras expression (43). To establish correlation between HRE binding activity and Ha-ras mRNA expression, BICR-M1Rk cells were serum deprived for 48 hours to induce growth arrest, and then stimulated with serum. To control for activities among different preparations of extracted nuclear proteins, we assayed each extract for serum responsive binding activity to the AP-1 recognition sequence and binding to the SP1 recognition sequence, known to be involved in transcriptional regulation of Ha-ras. At the specified time points cells were harvested for extraction of

RNA and nuclear proteins. AP-1 binding was observed in serum starved cells (Fig. 6) and was increased by 24 hours. Serum transiently stimulated binding to the HRE element binding between 4-12 hours, and gradually decreased by 24 hours. Interestingly, binding to the rHRE was maximal at 8 and 12 hours after treatment, at which time AP-1 and SP-1 binding activities were transiently reduced to somewhat lower levels. The latter results suggested that there is crosstalk among factors regulating Ha-ras promoter activity. As expected serum stimulated Ha-ras mRNA expression (Fig.4B), and the increase in RNA levels detected at 8 hours corresponded with the time of maximal CBF-A binding to the HRE. Thus in mammary carcinoma cells, there is a clear correlation between CBF-A binding to the HRE and increased expression of Ha-ras mRNA following serum stimulation. Reduced CBF-A - HRE binding at 24 hours time point suggested that Ha-ras expression at later time points following serum stimulation depend on other transcription factors.

Binding of CBF-A on the Ha-ras promoter was observed at very specific time following serum stimulation. To demonstrate that HRE binding activity and Ha-ras mRNA expression were linked to cell cycle progression, we stimulated cells with serum in the presence or absence of L-mimosine, a p53 independent inducer of the cyclin dependent kinase inhibitor, p21 waf-1 (1) . Induction of p21 waf-1 by L-mimosine was confirmed by Western blot (not shown). As expected from experiment above, treatment of cells with serum in the absence of L-mimosine, induced binding of CBF-A to the HRE (Fig. 6A), and resulted in elevated Ha-ras mRNA expression (Fig.6B) by 12 hours after serum stimulation. At the same time, we observed accumulation cells in the S phase of cell cycle, with a concomitant decrease G1/S ratio (Fig.6C).

To expand this observation we stimulated cells with serum in the presence or absence of L-mimosine, a p53 independent inducer of the cyclin dependent kinase inhibitor, p21 waf-1 (1). Induction of p21 waf-1 by L-mimosine was confirmed by Western blot (not shown). As expected from the experiment above, treatment of cells with serum in the absence of L-mimosine, induced binding of CBF-A to the HRE (Fig. 7A), and resulted in elevated Ha-ras mRNA expression (Fig.7B) by 12 hours after serum stimulation. At the same time, we observed accumulation of cells in the S phase of the cell cycle, with a concomitant decrease in the G1/S ratio (Fig.7C). L-mimosine treatment abrogated stimulation of CBF-A binding to the HRE sequence, and the levels of Ha-ras mRNA expression in cells stimulated with serum in the presence of L-mimosine were significantly lower compared to levels in cells stimulated with serum only (Fig.7B). As predicted, in the presence of L-mimosine, cells were arrested in G1 phase resulting in an increased G1/S ratio. Together, these results demonstrated a correlation between increased CBF-A binding to the rat HRE and stimulation of Ha-ras expression.

**Activation of CBF-A binding following serum stimulation requires post-translational modification of the p42 isoform.**

Our results indicated that the p42 isoform of CBF-A is the main HRE binding protein present in nuclear extracts from mammary cells, and that binding to the HRE was activated by serum stimulation of these cells (57) . To investigate the mechanisms regulating CBF-A binding activity, we

first determined if stimulation of CBF-A binding to HRE in response to serum was the result of protein accumulation or post-translational modification. We used EMSA and Western blot analyses to compare CBF-A binding activity and protein levels. To avoid possible variability among experiments, both assays were performed with the same nuclear extract. The results demonstrated that while serum stimulation activated CBF-A binding (Fig.8A) the level of p42 expression remained constant before and after serum stimulation (Fig.8B). These findings suggested that post-translational modifications of CBF-A in response to serum-stimulation were responsible for increased binding to the DNA target. By contrast, the level of expression of p44 isoform clearly increased following serum treatment. The effects of increased levels of the p44 isoform was not further investigated in the present study, since the latter shows very little HRE binding activity relative to the p42 isoform (57).

**Reduced CBF-A-HRE binding following inhibition of Ha-ras signaling pathways.**

Since serum-induced activation of CBF-A binding to the target DNA correlated with cell cycle progression (57) , it was reasonable to posit that activation of the ras pathway may be important for activation CBF-A binding to the Ha-ras HRE. To explore this possibility, we tested if inhibitors of MAP kinase and PI3 kinase, known downstream effectors of ras signaling (10), would block binding of CBF-A to the ras HRE. Exponentially growing BICR-M1Rk cells were treated with 50 uM of the MAP kinase inhibitor, PD98059 or left untreated. Inhibition of the ERK phosphorylation was verified on the Western blot with antiphospho-ERK antibody (Santa Cruz, CA)(data not shown). To inhibit PI3 kinase activity, cells were treated with either 10 or 100 uM of Wortmannin. We first tested if 10 uM Wortmannin was sufficient to block activity of PI3 kinase activity. Irradiation of mammary carcinoma cell line with UV and treatment cells with 10 uM of Wortmannin induced massive cell death by apoptosis, as documented by the generation of DNA ladder (not shown) (19). Both of the kinase inhibitors used in the experiment resulted in inhibition of CBF-A binding to the Ha-ras HRE within two hours of treatment (Fig.9A). The levels of CBF-A protein remain unchanged in the nuclear extract from treated versus untreated cells (Fig.9B). We concluded that MAPK and PI3 kinase pathways are involved in the regulation of CBF-A binding activity.

To confirm that the ras signaling pathway affects CBF-A binding to the HRE, we next transfected cells with a dominant negative mutant of Ha-ras (rasN17) under control of Dexamethasone (DEX) inducible promoter, and isolated clones for further analysis. Western blot analysis was used to confirm DEX-induced stimulation of dominant negative ras17 protein expression (not shown). Functional activity of rasN17 was verified by plating cells in the soft agar, and by assessing sensitivity to apoptosis following cell irradiation. Clones expressing rasN17 demonstrated significant inhibition of the anchorage independent growth (Fig. 10). Since BICR-M1Rk cells do not harbor an activated Ha-ras oncogene (unpublished observation), these findings suggested that their mechanism of transformation includes constitutive activation of the ras signaling pathway. Moreover, low doses of UV treatment produced efficient apoptosis response in cells expressing dominant negative ras (not shown), but not in the parental cells. Even the low level of expression from inducible rasN17 construct in the absence of DEX was sufficient to inhibit cell growth in the soft agar and to induce apoptosis following UV irradiation.

After verifying the efficient inhibition of Ha-ras pathway by rasN17, the two clones (B6 and B8) were selected for further analysis of CBF-A-HRE binding activity. The results of EMSA clearly demonstrated inhibition of CBF-A binding to the HRE following induction of dominant negative rasN17 with DEX (Fig.11A). No change in HRE binding activity was observed following DEX treatment of BICR-M1Rk cells transfected with empty vector. There was a low level of CBF-A binding to the target DNA before treatment with DEX. This low level of binding can be accounted for by the background expression from dominant negative rasN17 construct in the absence of DEX treatment. Significantly, DEX treatment failed to induce a detectable change in the levels of CBF-A protein, as assessed by Western blot analysis of the nuclear extracts used in the EMSA (Fig.11B). Taken together, our results provided convincing evidence that inhibition of ras pathway results in down regulation of the CBF-A - HRE binding activity. Moreover, both MAP kinase and PI3 kinase were implicated in regulation of the CBF-A-HRE interaction. From these results we concluded that regulation of CBF-A-HRE binding in the BICR-M1Rk mammary carcinoma cell line is primarily the result of post-translational modification of CBF-A.

The experiments outlined above suggested that activation of the ras pathway during cell transformation may lead to constitutive activation of CBF-A. To test this hypothesis we used cells transformed with a variety of oncogenes implicated in the ras signaling pathway. Rat-1 cells transformed with mutant ras (Me12) (29) had a 5-10 fold higher level of CBF-A binding when compared to parental fibroblasts or untransformed E12 cells harboring activated Ha-ras under control of its own promoter (Fig.12A). Similar activation of CBF-A binding was observed in src transformed fibroblasts compared to the parental cells (Fig.12B). v-fos-transformed Rat-1 fibroblasts also showed activated CBF-A-HRE interaction (Fig. 5C). Significantly, CBF-A binding comparable to that of the parental Rat-1 fibroblasts was present in non-tumorigenic revertants derived from v-fos transformants (EMS-1-19) (79) . It is important to note that enhanced CBF-A binding in v-fos transformants is not a direct result of elevated v-Fos levels, since EMS-1-19 cells express a functional v-fos oncogene at levels comparable to that in the v-fos-transformed. Despite the increased CBF-A binding observed in the transformed cell lines, CBF-A protein levels did not correlate well with CBF-A binding activity (compare Fig.12 D, E and F). These finding suggesting that as was the case in BICR-M1Rk cells, post-translational modification of the protein was responsible for modification of CBF-A binding activity in these transformed cells (Fig.12 D, E, F). Although we did we did not estimate the CBF-A protein levels, activation of CBF-A-HRE binding was also found in neu (not shown) and trk transformed fibroblasts (Fig.12C). In summary, our *in vitro* results suggested that transformation of fibroblasts with Ha-ras, or with oncogenes (src, fos, trk, neu) known to be activators of the ras signaling pathway, leads to constitutive activation of CBF-A binding to the ras HRE. *In vitro* experiments further suggested that activation of CBF-A binding during transformation is the result of post-translational modification.

**Activation of CBF-A binding and expression in mammary tumors.**

Activation of CBF-A binding in oncogene transformed cells prompted us to study CBF-A protein levels and HRE binding activity in chemically induced rodent tumors that frequently involve

activation of the Ha-ras oncogene. Previous studies in a numbe ro flabs including our own demonstrated that approximately 70-90% of these rat mammary tumors induced by NMU tumors harbor a Ha-ras oncogene with activating mutations in codon 12. Of the 23 mammary carcinomas tested , 22 showed CBF-A protein levels that were at least 10-20 fold higher than in normal tissue from the same tumor bearing animals (Fig.13A). By contrast, the levels of the p44 isoform were less correlated with well with the transformed phenotype, with many tumors showing the levels of the p44 isoform comparable to those in normal mammary glands (not all data shown). It is important to note that the abundance of the p42 isoform, which is responsible for most of the HRE binding activity (57), was especially low in normal mammary tissue. To investigate possible mechanisms responsible for CBF-A over-expression in tumors, we used Northern blot analysis to compare the levels of mRNA expression in the same samples (Fig.13B). We found that level of CBF-A mRNA expression in the tumors was at most about 2-3 fold higher than the to levels detected in normal mammary glands. This observation suggested that the high level of CBF-A protein in tumors was primarily the result of post-transcriptional regulation. To determine if CBF-A-HRE binding activity was constitutively activated in mammary carcinomas, we also performed EMSA with a subset of the samples from normal mammary gland and tumors. In all cases we found that increased CBF-A binding to HRE in mammary tumors relative to normal mammary cells (Fig. 13 C). Together, our finding suggested that activation of the Ha-ras pathway in MNU induced mammary carcinomas results in constitutively elevated levels CBF-A expression and HRE binding activity.

**Activation of CBF-A expression in mouse skin tumors.**

Another tumor model shown to frequently involve activation of ras oncogenes involves the induction of mouse skin tumors induced by 9,10-Dimethyl-1,2-benzanthracene and TPA. Approximately 90% of the resulting carcinomas show mutational activation of Ha-ras protooncogene at codon 61. J. Philipps, from the laboratory of C. Kemp (FHCRC) kindly provided skin carcinomas and normal skin samples were kindly provided by. Total proteins and RNA were extracted and respectively subjected to Western blot and Northern blot analyses. Skin carcinomas demonstrated a 10-15 fold increase in the level of CBF-A expression compared to normal skin (Fig.14). As in the case of mammary tumors, p42 isoform, accounted for most of the HRE binding activity, was very low in normal skin. Northern blots probed with CBF-A probe did not a reveal significant change in CBF-A mRNA levels between normal and tumor samples (not shown). Thus, DMBA-induced mouse skin tumors also demonstrated reproducible and persistent activation of CBF-A protein level due to post-transcriptional mechanism.

## Discussion

We previously demonstrated that the codon twelve GGA to GAA mutations frequently detected in the Ha-ras oncogene of N-nitroso-N-methylurea (NMU)-induced rat mammary tumors (80) arose as background mutations within cells of the developing mammary gland prior carcinogen exposure (14). Our results further demonstrated that the Ha-ras mutants were clustered within organ sectors, consistent with their origin as rare mutational events during an early stage of glandular development. Exposure of pubescent female rats to a single carcinogenic dose of NMU failed to

induce a detectable increase in the number of Ha-ras mutants, the fraction of organ sectors containing mutant cells, or the fraction of animals harboring mammary epithelial cells with Ha-ras gene mutations. Most importantly, we demonstrated that after exposure to NMU, tumors arose from within the clusters of pre-existing Ha-ras mutants. Thus, even though NMU is a strong mutagen, it may mediate its carcinogenic effects on the rat mammary epithelium via an epigenetic mechanism. Numerous *in vivo* and *in vitro* studies have shown that deregulated expression of Ha-ras may be required for expression of its transforming potential. More recent studies have demonstrated the crucial role of Ha-ras expression in tumor maintenance and progression *in vivo* (15) . We hypothesized that selective outgrowth of mammary cells harboring Ha-ras mutations could result from carcinogen-induced deregulation of gene expression, perhaps including the Ha-ras gene itself.

In order to test the latter hypothesis, we analyzed the methylation status of the Ha-ras gene promoter in mammary epithelial cells before and after a carcinogenic dose of NMU. While we failed to detect changes in DNA methylation, these studies led to the detection of a mammary cell-specific Ha-ras promoter response to NMU. In the present study we asked if the region of the Ha-ras promoter involved in this response (41) can bind specifically with any trans-acting transcription factor and if the binding of factors can regulate expression of the oncogene. We determined that double-stranded oligodeoxynucleotides corresponding to the sequences between positions -582 to -563 was able to bind specifically with transcription factors present in nuclear extracts from a rat mammary tumor cell line cells. Moreover, we found that the HRE element present in human Ha-ras promoter is able to bind same protein(s). Our result showed that the E74 oligonucleotide (which comprises a consensus ets binding site) efficiently competed for the binding with rat and human HRE probes, suggesting that the latter elements interact with the similar proteins, and that the binding factors could be members of *ets* transcription factor family. The HRE-protein(s) binding is appear to be highly conserved among different species since similar binding activities and EMSA profiles were detected in nuclear extracts from a variety of rat, mice and human cell lines using either the human or rat HRE elements as probe (not shown).

*Ets* phosphoproteins play important role in control of cell growth and development (8, 60, 66, 76). *Ets* binding site were identified in several oncogene responsive promoters (32, 66, 76). A number of studies have shown that *ets* related transcription factors may play important role in ras mediated signal transduction. Ets related proteins also appear to involved in regulation of number of genes downstream of Ras (76) . For example, cooperation of activated Ha-ras and *ets-1* was shown stimulate activity of the rat prolactin promoter in pituitary cells (8) . Expression of dominant negative *ets* inhibits Ha-ras induced transformation (77). It is thus reasonable to posit that *Ets* related proteins or proteins that compete with *Ets* proteins for specific binding sites could play an important role in Ha-ras mediated cell transformation.

To demonstrate a role for the HRE in Ha-ras promoter regulation, we used transient transfection assay of wild type and mutant Ha-ras promoter linked to the luciferase gene. We found that wild type construct showed 3-fold higher luciferase activity compare to mutant construct. Clearly, that rat HRE in mammary cells is relatively strong positive regulatory element. We concluded that despite the different relative positions of HRE within rat and human Ha-ras promoters, they functionally equivalent. Initially, competition experiment (see above) suggested that ets related

proteins with relative molecular weight 42-43 kDa interacted with the HRE. To further characterize the HRE binding proteins from mammary cells we employed affinity purification of the protein with following protein identification by micro HPLC-mass spectroscopy. Analysis of proteins eluted from two most abundant bands, with approximate molecular weight of 42 and 43 kDa, unexpectedly identified a set of overlapping peptide that corresponded to mouse CArG binding factor, CBF-A. We confirmed interaction of CBF-A with rat and human HRE in EMSA using CBF-A specific antibody. It remains unclear if lower intensity, 43 kDa band taken for protein analysis represents a gel artifact, a post-translationally modified form of the CBF-A protein or a CBF-A related protein with slightly higher molecular weight. Treatment of the eluted proteins with alkaline phosphatase failed to alter the mobility of the proteins, suggesting that the species did not represent differentially phosphorylated forms of CBF-A. Identified peptides encompassed almost 36% of the CBF-A amino acid sequence suggesting high level of homology between mouse and rat proteins. Indeed, comparison of cloned rat and mouse proteins showed that identified peptides localized within highly conserved central part of proteins (data not shown).

In attempt to detect any putative ets related protein, we performed protein analysis of additional bands of very low intensity detected on preparative Coomassie Blue stained gel. We failed to detect polypeptides corresponding to ets related proteins. Together these results suggested that *ets* related proteins are probably not involved in interaction with HRE site of the rat Ha-ras promoter in BICR-M1Rk mammary carcinoma cell line and that CBF-A is indeed the major binding HRE factor in these cells.

The CBF-A protein was discovered by screening an expression library with the CArG box DNA sequence as a probe (42). The CArG box sequence was initially described in a number of genes showing muscle tissue specific expression (13, 33, 49, 50, 59, 67, 68, 71, 82). For example, it was shown that serum response factor (SRF) can interact with CArG box and activate transcription of muscle-specific genes and immediate-early genes, such as fos (33, 51, 68). CBF-A is a protein with calculated molecular weight of 31 kDa and migrates with an apparent molecular weight of 42 kDa in the SDS PAGE gels. The protein has RPN domain that is thought to be involved in the binding to nucleic acid (34) . The RPN domain is common to heterogeneous nuclear ribonucleoproteins (hnRPN) A/C types involved in splicing, transport and protection of RNA (26). CBF-A was initially found to be transcriptional repressor (42). However, our study shows that CBF-A is transcriptional activator of Ha-ras in transient transfection assays. The discrepancy with published result is not surprising, since the CArG regulatory element can interact with a number of other transcription factors, including ets related factors Elk-1 and SAP-1 (20, 38), E12, NF-IL-6 (54), HMG-I family proteins (16). It is therefore plausible that CBF-A complexes with or replace other transcriptional factors, depending on the context of the CArG box. For example, functional antagonism between SRF and YY1 protein at CArG elements has been described (36). Likewise, studies have demonstrated that protein-protein interaction affect transcription from CArG box (16). For example, in the Arabedopsis APETALA3 gene, individual CArG boxes within a tandem array of three have opposite regulatory effects on the promoter activity (71). While the first two CArG boxes are positive regulatory elements, the third has a negative effect on the promoter activity. It was also noted previously that CBF-A is able to interact with single stranded DNA (42). However, in our experiment CBF-A we failed to detect any single stranded DNA binding activity (not shown). We speculate that CBF-A may demonstrate different

functional specificity, depending on affinity of interaction with target DNA and/or interactions with other factors. CBF-A modulation of transcription from CArG element may therefore be gene and cell type specific.

In our competition experiments, the affinities of CBF-A for rat HRE and ets binding site (E74) were clearly higher than its affinity for the interaction with CArG box. Comparison of human, rat HRE, E74 and CArG box sequences suggested that sequence CCGGAA is important for high affinity binding of CBF-A to DNA. Since this sequence is frequently present in a number of binding sites for ets proteins, we suggest a potential role of CBF-A in the regulation of *ets* responsive promoters. Our suggestion is supported by the fact that CBF-A is able to bind *ets* related proteins *in vitro* (6). Since a number of *ets* proteins are involved in the regulation of different genes, the role of CBF-A may be widespread.

The results of our transient transfection experiments clearly indicated that there is strong correlation between CBF-A-HRE binding, Ha-ras expression and cell cycle progression. Pharmacological arrest cells in G1 correlated with reduced CBF-A-HRE binding and Ha-ras expression. Since Ras protein is required throughout most of G1 following stimulation quiescent cells with growth factors (24) our results suggest that CBF-A stimulation of Ha-ras expression may elevate Ras to levels required for activation downstream pathways.

We identified CArG binding factor A as the main protein which interact HRE sequence present Ha-ras gene promoter (57). We, and others (6) , also showed that the p42 isform accounts for most of CBF-A binding to the HRE. The higher molecular weigh p44 isoform showed very little binding activity in our study. Interaction of p42 CBF-A with the target DNA correlated with proliferative activity in BICR-M1Rk cells stimulated with serum. Cells arrested in the G1 phase of the cell cycle showed dramatically reduced CBF-A binding activity. Since proliferative activity is associated with activation of Ha-ras pathway, we reasoned that modulation of Ha-ras activity during cell transformation and tumorigenesis may also activated CBF-A binding to HRE sequences. To test this hypothesis, we used inhibited the ras pathway using pharmacological inhibitors or dominant negative rasN17. In al cases reduction of ras signaling resulted in reduced interaction CBF-A with HRE sequences in a mammary carcinoma cell line. Also consistent with our hypothesis was the observation that transformation of cell with Ha-ras, or oncogenes that involve activation of Ha-ras signaling uniformly resulted in activation of CBF-A binding to the target DNA.

In preliminary experiments designed to demonstrate specific correlation with activation of ras signaling, we utilized non-tumorigenic revertant cell lines we previously isolated from the HeLa crevical carcinoma cell (4, 7). The transformed phenotype of the HeLa cells is known to be a result of inactivation of p53 and pRB by E6 and E7 proteins of HPV18. We compared CBF-A binding to HRE among these cell lines using EMSA. Nuclear extracts from Hela and two revertant cell lines (HA and HF) failed to reveal a dramatic difference in CBF-A-DNA binding activity (data not shown). Since ras signaling is not known to play a role in the transformation of HeLa cells, these results were consistent with the notion that activation of ras signaling during transformation and not cell transformation per se was responsible for constitutive activation of CBF-A-HRE binding. However, it

remains to be elucidated if cell transformation by other ras unrelated oncogenes affect CBF-A activity.

In summary, a variety of *in vitro* experiments yielded results to support the hypothesis that increased ras signaling leads to post-translational modification of CBF-A, which in turn lead to the increased affinity of CBF-A for the HRE sequence. To determine if ras signaling also activates CBF-A *in vivo*, we next analyzed examined HRE binding in rodent tumor cells shown to harbor Ha-ras oncogenes. Analysis of MNU induced mammary carcinomas and adjacent normal mammary tissues in rat, and DMBA induced skin carcinomas in mice provided an evidence for a positive correlation between CBF-A regulation and ras pathway activation during tumor growth *in vivo*. In both mammary and skin carcinomas we found elevated levels of CBF-A protein expression as compared tp adjacent normal tissue. However, elevated CBF-A protein in the tumors was not the result of enhancement of transcription, since levels of CBF-A transcript were only modestly increased in tumors. It is therefore likely that increased translation and/or post-translational modification (protein stabilization) of the CBF-A occur in tumors. Nonetheless, we did observe weak, but highly reproducible transcriptional activation of the CBF-A mRNA in mammary gland. CBF-A mRNA was previously found to be overexpressed in the ets transformed mouse fibroblasts in (63) and Ets proteins were shown to be targets of Ras-MAPK signaling pathway (76). However, it is not known if CBF-A binding to the target DNA is also increased in ets transformed fibroblast.

Recently, hnRNP protein AUF1 (hnRNP D) was found to be a major component in regulation of the Epstein-Barr Virus C promoter (30) . The authors also found that AUF1 binding activity is regulated by the cyclic AMP/Protein Kinase A signaling pathway and depends on post-translational modification. Together with our findings using kinases inhibitors, these results suggest a common mechanism in the activation of hnRNP binding to the target DNA. Treatment of cells with cAMP analogs results in the activation of AUF1 binding to and activation of the CD21 promoter (73) . There is recent evidence to suggest that the cAMP/PKA signaling pathway is linked to the Ha-ras pathway (2, 74). Since AUF1 and CBF-A are highly homologous proteins, it is plausible that they may under some conditions share similar pathways of activation.

Another important issue to be resolved is the posttranscriptional mechanisms mechanism for activation of CBF-A's sequence specific DNA binding activty. HnRNPs, including LR1 and hnRNP K, are known to be regulated by phosphorylation (22, 31, 40). Phosphorylation of hnRNP D affects its interaction with RNA, as well as protein-protein interaction (81). Presently the kinases that mediate phosphorylation of hnRNPs are unknown. Our data would seem to suggest to suggest that the pathway leading to activation of CBF-A binding to the target DNA involves kinases that are effector ras transformation.

Development of markers specific for activation of specific signaling is not only useful in dissecting the mechanisms of cell transformation, but may facilitate selection of targeted treatment strategies. For example, it was found that cell lines harboring ras mutations are more sensitive to 1-beta-D-arabinofuranosylcytosine (Ara-C) and 2,2'-O-cyclocytidine compared to the tumor lines with wild-type ras alleles (44). Activation of Ras oncogene has been found in approximately 30 % of human cancers, including malignancies of the lung, pancreas, colon, thyroid carcinomas, and myeloid

leukimia (64) . Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) was shown to be overexpressed in the human lung cancer (69, 72), esophagus and oral squamous cell carcinomas (35) (52). While lung carcinomas frequently harbor activated Ki-ras mutation (58), Ki-ras mutations are rare in esophageal cancer (3). Whether CBF-A protein levels and/or binding activity can be marker for activation of ras pathway in human tumors is an important question and remains to be resolved in additional studies.

In summary, we investigated the expression and DNA binding activity of the CArG binding factor A *in vivo* and *in vitro*. We demonstrated that *in vitro* inhibition and activation of ras signal pathway resulted in reciprocal modulation of CBF-A binding of the target DNA by post-translational mechanisms. Our data also suggested *in vivo* activation of CBF-A protein expression and/or binding in carcinogen induced tumors via post-transcriptional modification. These results suggested that CBF-A might be a marker for ras pathway activation.

**Figure legends:**

**Figure 1.** EMSA with rat HRE (HRE) probe and competition experiment with mutant HRE, human HRE (hHRE) and ets binding site (EBS). Competitor DNAs were added in 40-fold molar excess. NE-nuclear extract from BICR-M1Rk.
Panel A: Sequence of oligonucleotides used in the competition experiment. The conserved sequence CCGGAA found in the rat and human HRE (hHRE) and the ets binding site (EBS) is boxed. The summary of competition experiment results is on the right. Competition and absence of competition with the HRE probe are shown with "+" and "-", respectively.
Panel B: EMSA and competition experiment with human HRE and the different mutant oligonucleotides (from 1 to 4) listed on panel A. Specific DNA-protein complexes are shown with an arrow.
Panel C – EMSA competition experiment with EBS or rat HRE in the presence of excess rat HRE or EBS respectively. C1 and C2 – DNA-protein complexes are indicated. The rat HRE probe without nuclear extract added is not shown.

**Figure 2.** Wild-type Ha-ras promoter is 3 fold more active compared to the mutant promoter. BICR-M1Rk cells were transfected with 1 ug of plasmid DNA and luciferase activity was measured after 24 hours. Normalization for transfection efficiency was performed as described in the experimental procedure. At the top is a schematic presentation of the constructs showing wild type and mutant sequences in the Ha-ras promoter linked to the luciferase gene.

**Figure 3.** Panel A: Coomasie Brilliant Blue stained gel following purification of the DNA binding protein by affinity chromatography. Migration of the molecular weight standards is shown on the left. The most abundant species are labeled p42 and p44. Bands of lower intensity which were also subjected to protein identification by micro-HPLC - mass spectrometry are shown with short arrows.
Panel B: Comparison of radiolabeled, UV crosslinked HRE-protein complexes from whole nuclear extract (NE) with the HRE-p42 complex. The p42 protein was recovered from gel shown on panel A. BSA - bovine serum albumin.

**Figure 4.** Panel A: Anti CBF-A antibody completely abrogated protein binding to the rat HRE and human HRE (not shown) probes. EMSA was performed as described in experimental procedure. Anti CBF-A antibody or normal serum were added as indicated (+) and (-), respectively. NE - nuclear extract from BICR-M1Rk cells.
Panel B: Comparison of the binding affinity of CBF-A to the rat HRE and CarG box. Competitor oligonucleotides, rat HRE, CArG and EBS (ets binding site), were added to the binding reaction in 50 fold molar excess. Specific HRE-protein complex are indicated with arrow.
Panel C: Comparison of rat HRE probe, EBS and CArG box oligonucleotide sequences. Conserved sequence is boxed.

**Figure 5.** Two protein species (p42 and p44) interact with rat HRE. The Western blot was performed with the CBF-A antibody against HRE binding proteins eluted from two sequential affinity columns.

Fifteen ul aliquots from each fraction were used. NE- nuclear extract before loading on the affinity column. The two protein bands, p42 and p44, are shown with arrows.

**Figure 6.** HRE - CBF-A binding activity correlates with Ha-ras mRNA expression. Serum deprived cells were stimulated with 5% calf serum. RNA and nuclear proteins were extracted at indicated time points.
Panel A: Nuclear proteins were used in EMSA with AP-1 (top, only DNA-protein complexes are shown with arrow) and rat HRE probes (bottom).
Panel B: RNAs extracted from cells at the same time points following serum stimulation were separated on the 1.1% agarose gel, blotted and probed with rat Ha-ras cDNA. Lower panel: Ethidium bromide staining of the membrane following RNA transfer demonstrates equal RNA loading. For every time point shown on the figure we also performed control experiments using nuclear extract (A) and RNA (B) from serum deprived cells harvested at a given time. The level of binding activity to the AP-1 and HRE probes and the level of Ha-ras expression did not differ from the zero time points in these samples. For simplicity these controls were removed from the final figure using image analysis software.

**Figure 7.** L-mimosine inhibits CBF-A binding to the rat HRE and expression of the Ha-ras mRNA. Serum deprived cells were stimulated with serum or serum plus L-mimosine (200 ug/ml) Twelve hours later, nuclear proteins and RNA were extracted from the same culture. At each time point, a duplicate cell culture was taken for cell cycle analysis by Fluorescence Activated Cell Sorting.
Panel A: EMSA of the CBF-A binding to rat HRE probe.
Panel B: Northern blot analysis of the RNA probed with Ha-ras cDNA. Equal loading was verified by ethidium bromide staining of RNA following transfer to the membrane (not shown).
Panel C: Cell cycle analysis shown the average G1 to S ratio from three independent measurements. Error bars represent standard deviations. Open and closed bars - cells untreated and treated with L-mimosine.

**Figure 8.** Stimulation of BICR-M1Rk cells with serum activates CBF-A-HRE binding but does not affect level of CBF-A protein expression. Serum starved cells (C) were stimulated with 5% bovine serum (S) for 8 hours. A. Extracted nuclear proteins were tested on EMSA with labeled HRE probe. Protein-DNA complex shown with arrow on the left. B. Same nuclear extracts were probed for total CBF-A protein on the Western blot using CBF-A antibody. P42 and p44 isoformes of CBF-A are shown with arrows on the left. Two independent experiments are shown.

**Figure 9.** Inhibition of PI3 kinase or MAP kinase reduces CBF-A-HRE binding but does not affect level of CBF-A expression. BICR-M1Rk were treated with 10 pM (w), 100 pM (w1) Wortmannin or 50 uM of PD98059 (P) for 2 hours. A. EMSA was performed using HRE probe B. Western blot were performed as for Figure 8.

**Figure 10.** Inhibition of soft agar growth of BICR-M1Rk cells transfected with dominant negative rasN17. BICR-M1Rk cells were transfected with rasN17 under control of Dexamethasone inducible promoter showing inducible expression of the rasN17 verified on the Western blot and induction of apoptosis following UV irradiation (not shown) were seeded on the soft agar in the

presence of Dexamethasone. Background expression of rasN17 (without Dexamethasone) was sufficient to inhibit soft agar growth (not shown for simplicity). Only clones B6 and B8 chosen for further analysis are shown. Large clones of cells growing on the soft agar were detected in control cells transfected with empty vector and shown with arrows.

**Figure 11.** Induction of the dominant negative ras expression inhibit CBF-A-HRE binding. A. BICR-M1Rk cells transfected with empty vector, B6 and B8 clones were left untreated (-) or were treated (+) with $5*10^{-5}$M of Dexamethasone (Dex) for 16 hours before harvest. EMSA was performed with HRE probe as described. P - probe only. B. Western blot was performed with the same nuclear extract obtained from Dexamethasone treated and untreated cells as described.

**Figure 12.** Activation of CBF-A-HRE binding in cells transformed with oncogenes. Level of CBF-A expression in the nuclear extracts does not correlate with the binding activity.
A, B, C. CBF-A -HRE binding activity in cells transformed with Ha-ras (Me12), src (Src9) and fos (1302) and c-trk oncogenes. Cells lacking transformed phenotype E12 (A) and 1-19 (C) show CBF-A binding activity comparable to parental Rat 1 cells. D, E, F Western blot analysis of the CBF-A level in the nuclear extracts in oncogene transformed fibroblasts. Nuclear extract from cells transformed with c-trk has not been tested for level of CBF-A expression.

**Figure 13.** High level of CBF-A protein expression in mammary tumors compare to normal mammary gland is not a result of transcriptional activation. A. Total proteins were extracted from normal mammary tissue (N), mammary tumors (T) and liver (L). Western blot was performed as described. B. Total RNA was extracted from mammary tissues (N) and from mammary tumors (T). Northern blot was probed with rat CBF-A cDNA. C. High level of CBF-A expression in mice skin carcinomas induced by DMBA and TPA. Total proteins were extracted from normal skin (N) and carcinomas (T) and Western blot was probed with anti CBF-A (upper panel) and actin antibody (lower panel).

**Figure 14.** Activation of the CBF-A HRE binding to the target DNA in rat mammary tumors (T) compared to normal mammary gland (N). Nuclear extracts from rat tissues were obtained as described in material and methods. EMSA was performed with HRE probe. P-probe only.

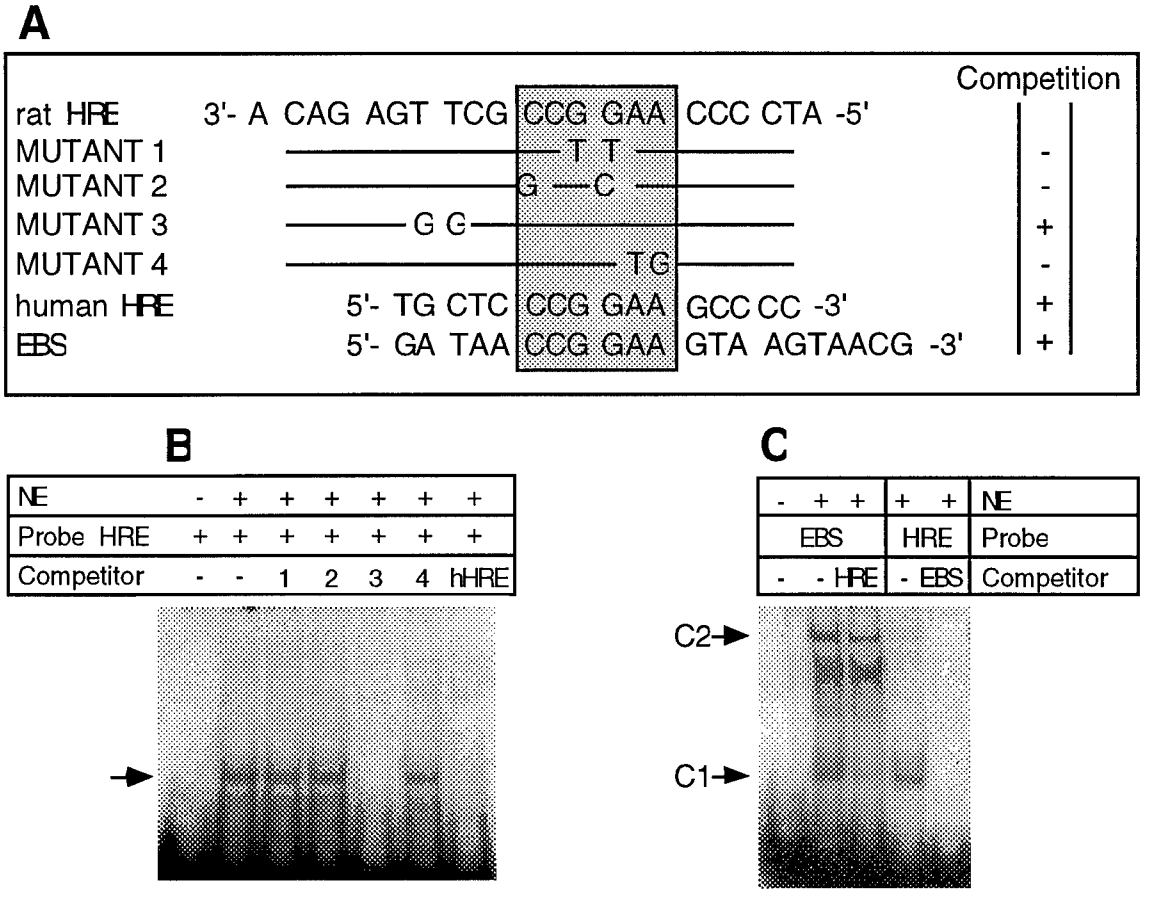
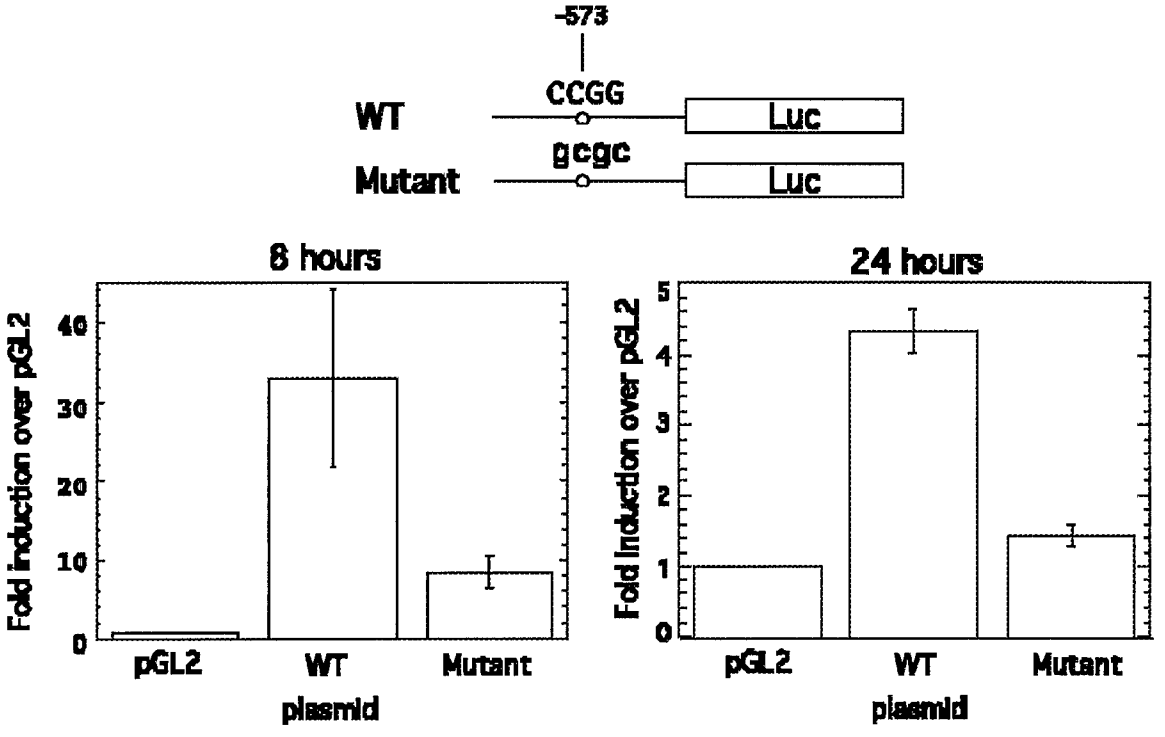

A
Competition
rat HRE 3'- A CAG AGT TCG CCG GAA CCC CTA -5'
MUTANT 1 T T -
MUTANT 2 G C -
MUTANT 3 G G +
MUTANT 4 T G -
human HRE 5'- TG CTC CCG GAA GCC CC -3' +
EBS 5'- GA TAA CCG GAA GTA AGTAACG -3' +
B
NE - + + + + + +
Probe HRE + + + + + + +
Competitor - - 1 2 3 4 hHRE
C
- + + + + NE
EBS HRE Probe
- - HRE - EBS Competitor
C2
C1

Figure 1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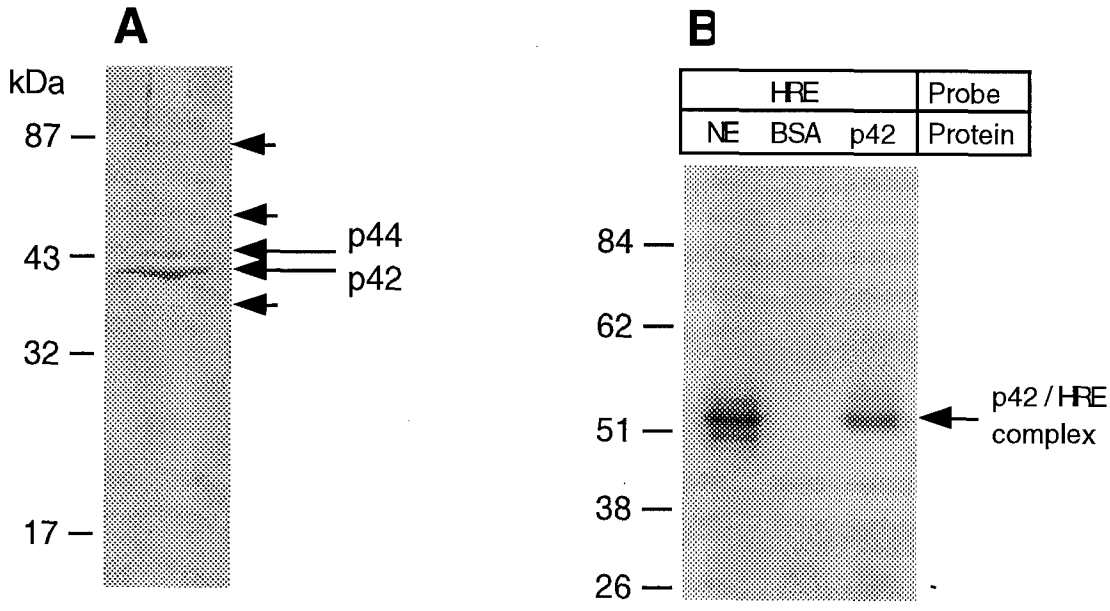
WT
Luc
gcgc
Mutant
Luc
8 hours
Fold induction over pGL2
40
30
20
10
0
pGL2
WT
Mutant
plasmid
24 hours
Fold induction over pGL2
5
4
3
2
1
0
pGL2
WT
Mutant
plasmid

Figure 2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kDa
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43
32
17
p44
p42
B
HRE
Probe
NE
BSA
p42
Protein
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62
51
38
26
p42 / HRE
complex

Figure 3

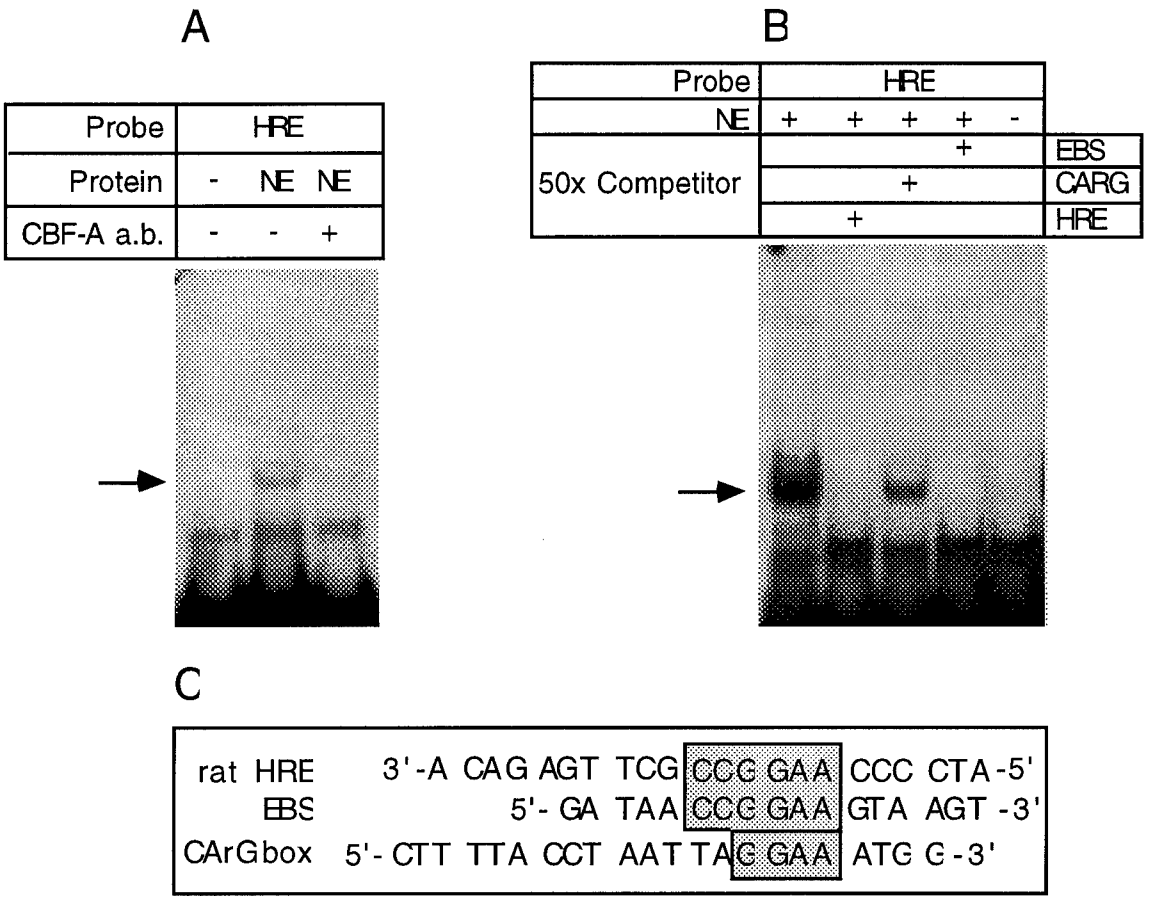
A
Probe
HRE
Protein
- NE NE
CBF-A a.b.
- - +
B
Probe
HRE
NE
+ + + + -
50x Competitor
+
EBS
+
CARG
+
HRE
C
rat HRE 3'-A CAG AGT TCG CCG GAA CCC CTA-5'
EBS 5'- GA TAA CCC GAA GTA AGT -3'
CArGbox 5'- CTT TTA CCT AAT TA G GAA ATG G-3'

Figure 4

Table 1. List of overlapping polypeptides identified by micro-HPLC-Mass Spectrometry of the 42 and 44 kDa proteins isolated by affinity chromatography.

| |
|---|
| MFVGGLSWDTSK |
| MFVGGLSWDTSKK |
| MFVGGLSWDTSKKDLKDYFTK |
| DLKDYFTK |
| |
| SRGFGFILFK |
| GFGFILFK |
| |
| IFVGGLNPEATEEK |
| IFVGGLNPEATEEKIR |
| GGLNPEATEEK |
| IREYFGQFGEIEAIELPIDPK |
| EYFGQFGEIEAIELPIDPK |
| |
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| GFVFITFKEEDPVK |
| |
| FHTVSGSK |
| |
| EVYQQQQYGSGGR |

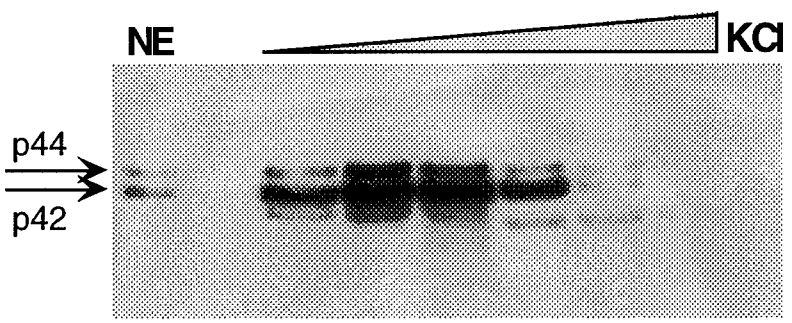



Figure 5

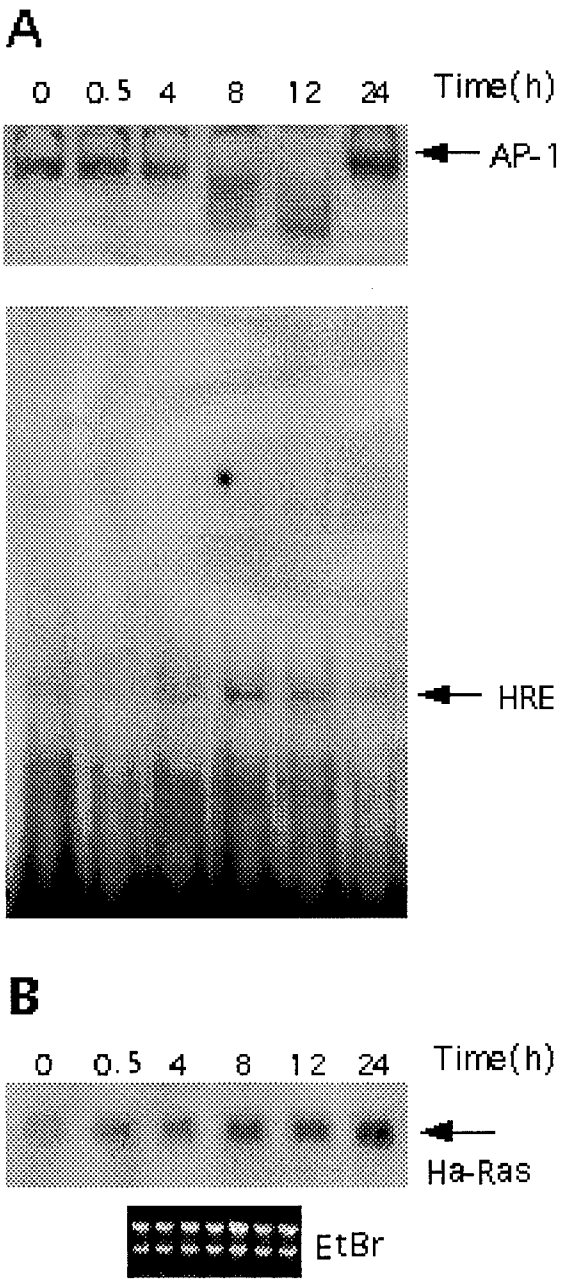
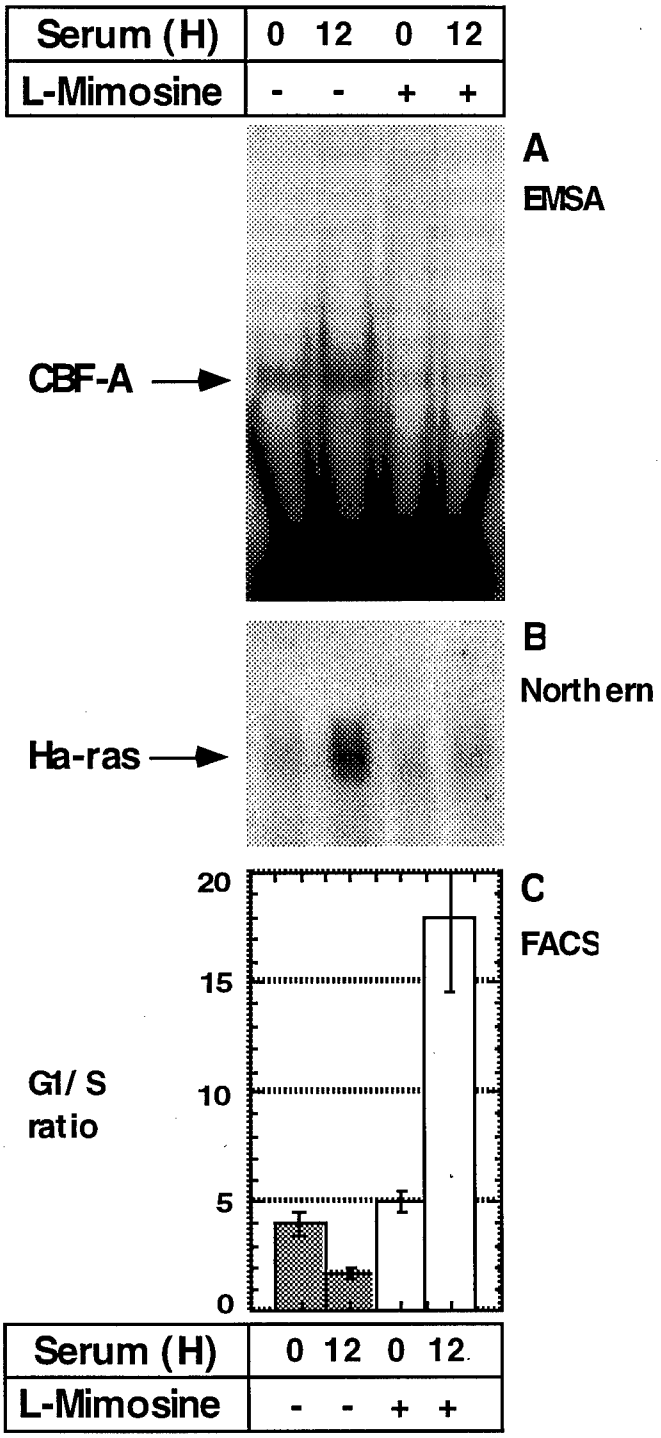



Figure 6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L-Mimosine - - + +
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EMSA
CBF-A
B
Northern
Ha-ras
C
FACS
G1/ S ratio
20
15
10
5
0
Serum (H) 0 12 0 12
L-Mimosine - - + +

Figure 7

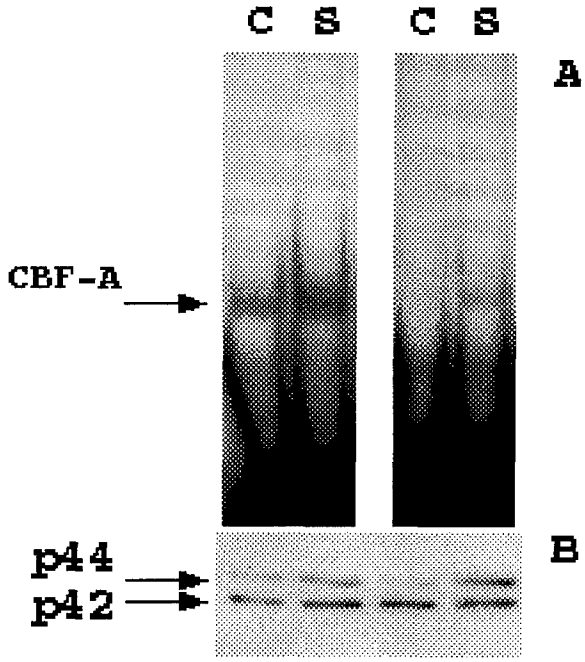


Figure 8

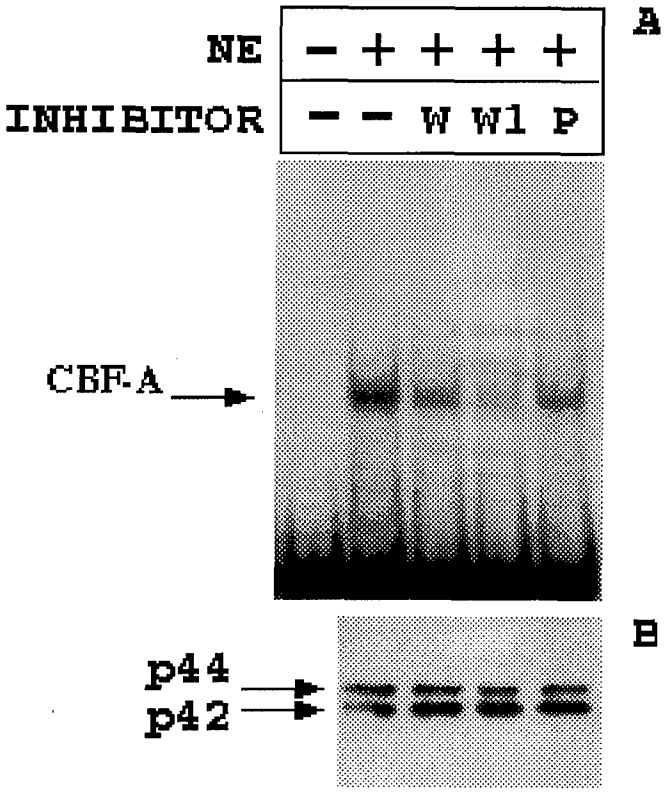


Figure 9

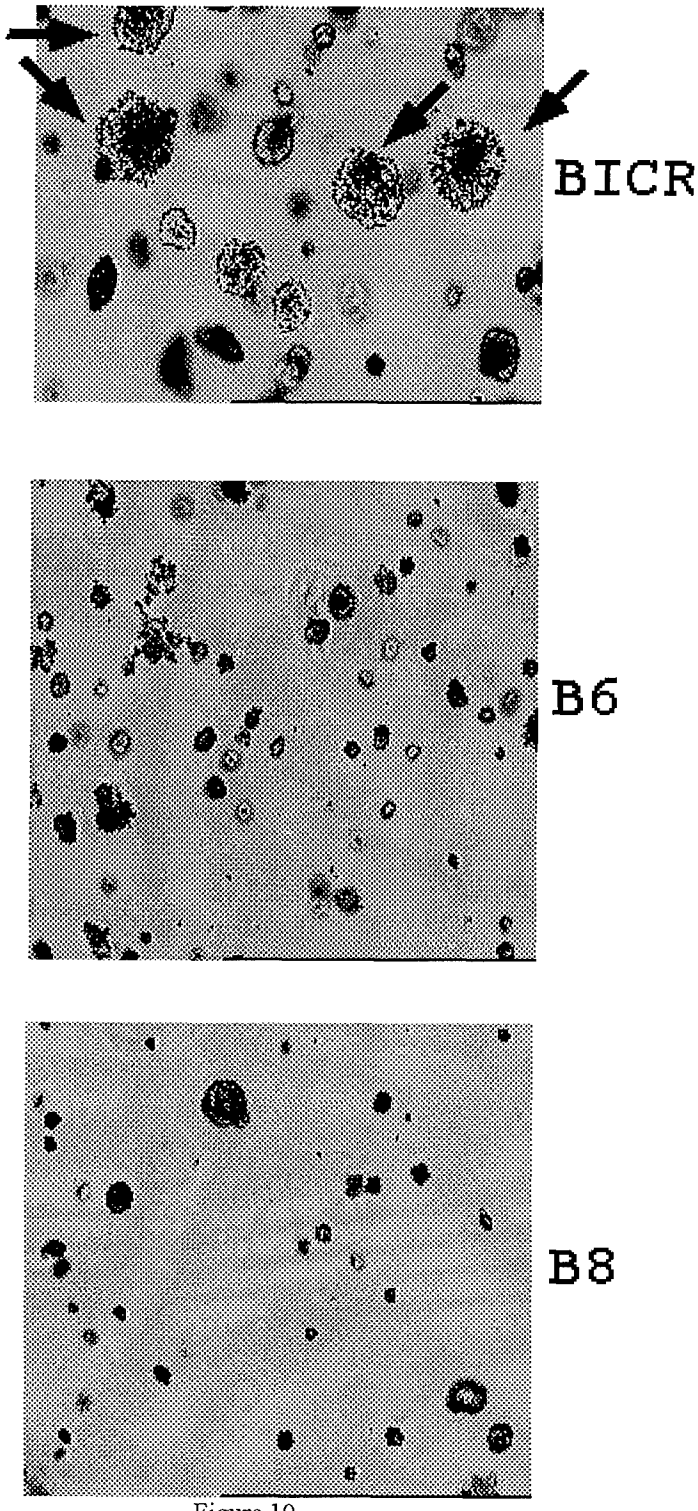


Figure 10

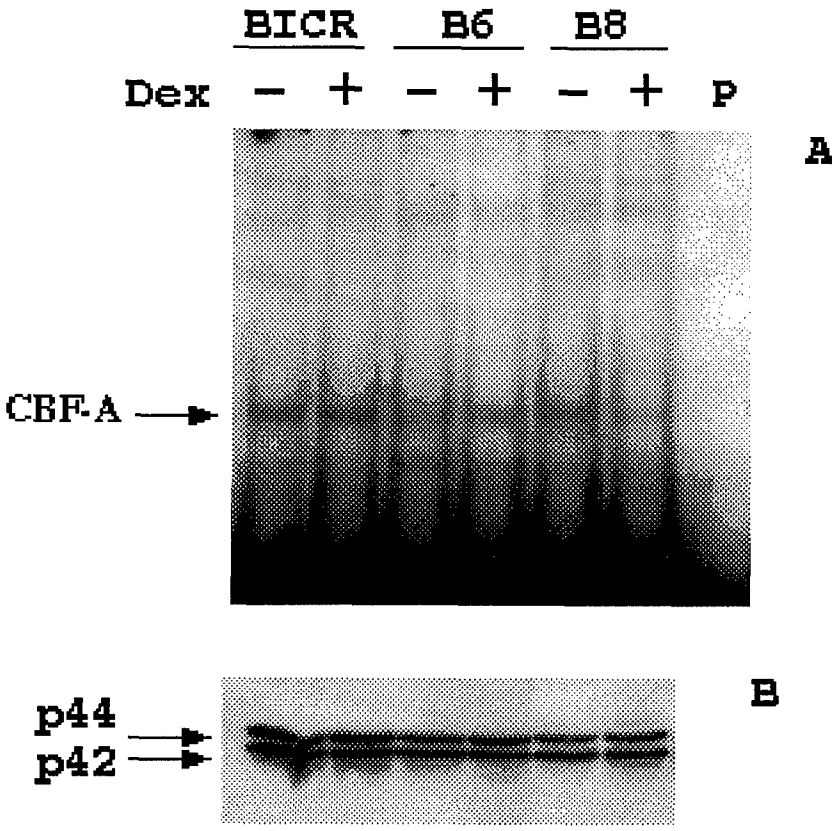


Figure 11

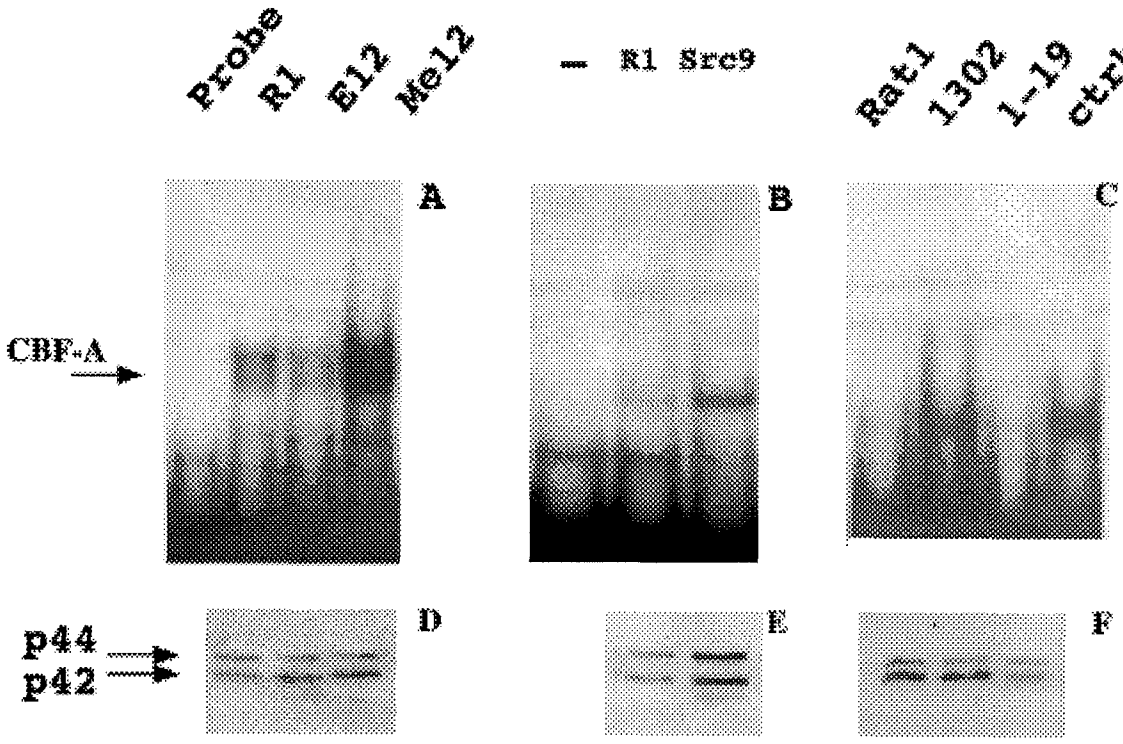
Probe
R1
E12
Me12
R1 Src9
Rat1
1302
1-19
ctrk
A
B
C
CBF-A
p44
p42
D
E
F

Figure 12

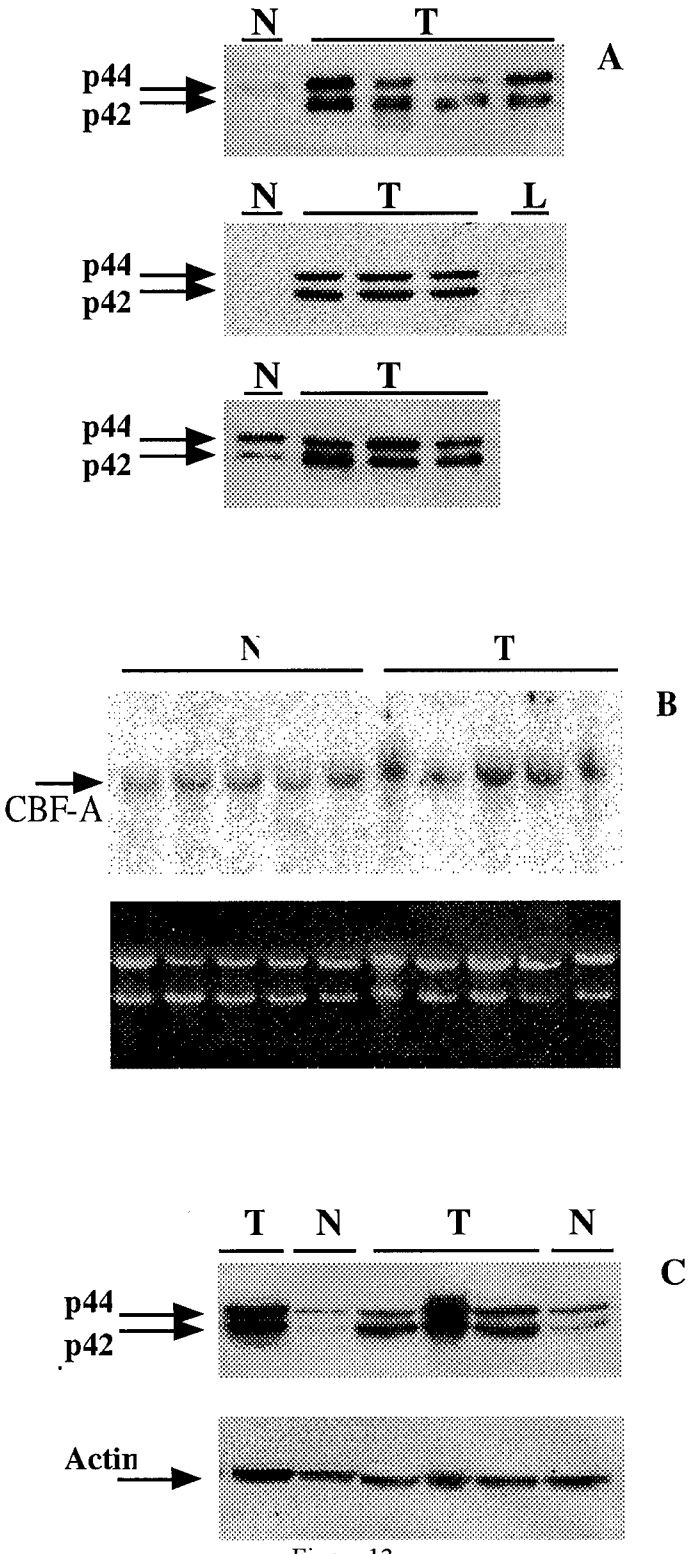


Figure 13

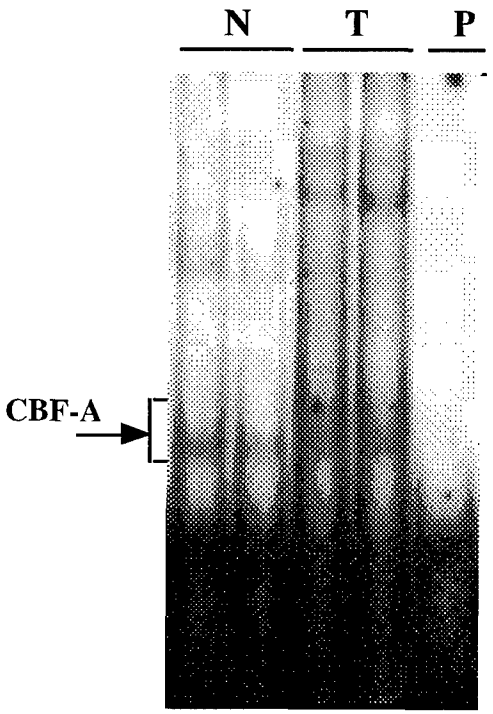

Figure 14

## Key Research Accomplishments

- We demonstrated the high affinity interaction of CArG binding factor (CBF-A) within Harvey Ras Element (HRE) present in the Ha-ras promoters in rodents and humans
- We demonstrated that the HRE functions as a positive regulatory element in Ha-ras promoter
- We showed CBF-A binds to HRE with higher affinity compare to CArG box initially identified as recognition sequence for CBF-A
- We did not find any *ets* related proteins capable of high affinity binding to the HRE in mammary cells
- We found a correlation of CBF-A binding to the HRE and Ha-ras mRNA expression, and cell cycle progression suggests
- Inhibition of either PI3 kinase activity with Wortmannin or ERK phosphorylation with PD98059, both reduced CBF-A binding to HRE within 2 hours of treatment
- Over-expression of dominant negative ras (ras N17) also resulted in reduced CBF-A interaction to the target DNA.
- CBF-A binding activity was induced in Ha-ras, src, fos, neu and trk transformed fibroblastswhile the levels of CBF-A protein expression remained constant, indicating that post-translational modification of CBF-A was required for the increased binding activity *in vitro*
- Rat mammary carcinomas induced by N-methyl-N-nitrosourea and mouse skin carcinomas induced by 9,10-Dimethyl-1,2-benzanthracene both showed elevated CBF-A binding activity
- In the rodent tumors, level of CBF-A mRNA were only marginally increased, indicating that post-transcriptional regulation also plays a role in accumulation and binding activity of CBF-A protein *in vivo*.

## Reportable Outcomes

- A publication entitled "CArG Binding Factor A (CBF-A) is Involved in Transcriptional Regulation of the Rat Ha-ras Promoter" in Nucleic Acids Research (Nucleic Acids Res. 28: 3762-3770, 2000) and authored by Mikheev, A. M., Mikheeva, S A., Zhang, Y., Aebersold, R., and Zarbl, H., describes the isolation, and characterization of CBF-A.

- A manuscript entitled "Signaling -Dependent Activation of the CBF-A Transcription Factor *in vitro* and *in vivo*" and authored by Mikheev, A.M., Mikheev, S.A., Lee, V., Leanderson, T. and Zarbl H., describing the regulation of CBF-A activity has been submitted to Cancer Research.

- Dr. Zarbl is a member and the Director of the Environmental Carcinogenesis Research Core within the University of Washington/ National Institiute for Environmental Health Sciences Center for Ecogenetics and Environmental Health (CEEH, David Eaton, P.I.). Based on the results obtained in this study, Dr. Zarbl has procured a pilot funding from the CEEH to generate CBF-A knockout mice within the CEEH facilities. The generation of these mice is in progress.

- An RO1 proposal to continue our studies on the role of CBF-A in chemically induced mammary carcinogenesis and human breast cancer will be submitted to the NCI-NIH on June 1, 2001.

## Conclusions

In summary our results demonstrated the high affinity interaction of CArG binding factor (CBF-A) within Harvey Ras Element (HRE) present in the Ha-ras promoters in rodents and humans. Furthermore, Harvey Ras Element is a positive regulatory element in Ha-ras. We also showed that CBF-A binds to HRE with higher affinity compare to CArG box initially identified as recognition sequence for CBF-A. Contrary to expectations, we did not find any *ets* related proteins capable of high affinity binding to the HRE in mammary cells. The correlation of CBF-A binding to the HRE and Ha-ras mRNA expression, and cell cycle progression suggests that CBF-A may be involved in control of cell cycle and carcinogenesis in mammary cells.

We next investigated the regulation of CBF-A expression and interaction with the HRE target sequence *in vitro* and *in vivo*. Inhibition of either PI3 kinase activity with Wortmannin or ERK phosphorylation with PD98059, both reduced CBF-A binding to HRE within 2 hours of treatment. Likewise, overexpression of dominant negative ras (ras N17) also resulted in reduced CBF-A interaction to the target DNA. By contrast, CBF-A binding activity was induced in Ha-ras, src, fos, neu and trk transformed fibroblasts. In the latter experiments, the levels of CBF-A protein expression remained constant, indicating that post-translational modification of CBF-A was required for the increased binding activity *in vitro*. We further demonstrated activation of CBF-A binding activity *in vivo* in chemically-induced rodent tumors harboring activated Ha-ras oncogenes. Rat mammary carcinomas induced by N-methyl-N-nitrosourea and mouse skin carcinomas induced by 9,10-Dimethyl-1,2-benzanthracene both showed elevated CBF-A binding activity. However, in contrast to the results of the *in vitro* experiments, the levels of CBF-A protein were increased in 22 out of 23 mammary tumors relative to the levels detected in adjacent normal mammary gland. Similarly, increased levels of CBF-A protein were detected in all mouse skin tumors. However in both cases, the level of CBF-A mRNA were only marginally increased, indicating that post-transcriptional regulation also plays a role in accumulation and binding activity of CBF-A protein *in vivo*. Together, our results suggested that activation Ha-ras signaling increases CBF-A binding activity *in vitro* and *in vivo*, and that CBF-A binding may play a role in cell transformation mediated via the Ha-ras pathway.

While *ras* oncogene mutations are not frequently detected in human breast cancers, carcinogen-induced disruption of the CS is unlikely to be an anomaly of the rat. Several lines of evidence suggest that carcinogen-induced CS disruption also play an important role in human breast cancer. Our preliminary data had indicated the presence of an analogous structure within a conserved sequence of the human *Hras* promoter. Other studies have also shown that the *Hras* gene is frequently overexpressed in human breast cancer, suggesting that disruption of the CS in human breast cells could also contribute to the pathogenesis of human breast cancer. There is also no reason to posit that these hormonally regulated DNA structures are restricted to the promoters of *Hras1* genes in mammary cells. Our preliminary studies indicated that the sequences comprising the *Hras1*

CS include an *ets*-like responsive element in both the human and rat promoter. The results obtained so far suggest that this promoter element is not an *ets* binding site in mammary cells. Rather, the proteins that bind to this element are likely to be members of a family of proteins that bind to CarG box elements. These proteins may bind to alternative DNA conformations such as bent or single-stranded sequences. Our previous studies indicated that the *Hras1* promoter and other genes can adopt alternative, tissue specific conformations *in vivo*. The presence of these structural variants and proteins that recognize them could therefore play a role in tissue specific regulation of growth related genes such as *Hras*. It is plausible that carcinogen-induced alterations of DNA conformations permit binding of transcription factors such as CBF-A and lead to deregulation of these genes. Further characterization of these binding proteins and their role in regulation of tissue specific gene expression should provide further insight into their contributions to epigenetic carcinogenesis in both rats and humans.

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# Appendix

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# CArG binding factor A (CBF-A) is involved in transcriptional regulation of the rat Ha-*ras* promoter

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## ABSTRACT

**In the present study we identified a positive transcriptional element within the rat Ha-*ras* promoter previously known as Ha-*ras* response element (HRE) and identified a *trans*-acting factor that binds HRE sequences in rat mammary cells. To identify the binding protein we employed sequence specific DNA affinity chromatography. Amino acid sequence analysis of the affinity-purified proteins was performed by tandem mass spectroscopy. The results unexpectedly demonstrated that in rat mammary cells CArG box-binding factor A (CBF-A) is the major protein species that bind specifically to the rat and human HRE sequences with high affinity. The affinity of CBF-A binding to HRE was significantly higher than to the CArG box described as a recognition sequence for CBF-A protein. Transient transfection assays using reporter plasmids verified that mutations within the HRE that disrupt binding of CBF-A also reduced the activity of the rat Ha-*ras* promoter. Despite the fact that the HRE within the Ha-*ras* promoter resembles a binding site for Ets transcription factors, we did not detect the binding of Ets-related proteins to the rat HRE in BICR-M1Rk cells. We further demonstrated a correlation between the presence of HRE binding activity and induction of Ha-*ras* mRNA expression following serum stimulation in the mammary carcinoma cell line. Taken together, our results suggest that CBF-A may play an important role in transcriptional regulation of Ha-*ras* promoter activity during normal mammary cell growth and carcinogenesis.**

## INTRODUCTION

The Ras proteins are a closely related set of genes that encode membrane-associated proteins involved in cell proliferation and differentiation. The Ras proteins belong to the family of small GDP/GTP-binding proteins that transduce signals from activated cell surface tyrosine kinase receptors to the nucleus by activating a cascade of secondary messengers within the cytoplasm (1,2).

Activated Ras proteins are able to transform a number of immortalized cell lines *in vitro*, and decrease tumor latency and increase tumor frequencies in transgenic animals. However, transformation in these experimental models is usually associated with expression of activated Ha-*ras* alleles at levels that exceed those observed in most cancers (3).

When introduced into Rat 1 fibroblasts under the control of its own promoter, activated Ha-*ras* failed to transform immortalized Rat-1 fibroblasts (4). Furthermore, transformed clones arising during passage of these transfected cell populations invariably over-expressed the mutant allele as a result of either gene amplification or transcriptional deregulation. The latter studies are in accordance with the observations that mutant *ras* genes are frequently over-expressed in human tumors (5–7). A recent study of transgenic animals harboring an inducible Ha-*ras* transgene demonstrated that continued expression of the oncogene is necessary for the genesis and maintenance of solid tumors *in vivo* (8). A variety of *in vitro* transformation experiments have demonstrated that even wild-type Ras proteins have transforming potential when expressed above normal levels. An *in vivo* correlate of this observation is the finding that deregulation of Ha-*ras* pathways is frequently detected in human breast cancers, although *ras* gene mutations are rare (~5%) in these tumors (9). Taken together, these studies support the hypothesis that deregulated expression of the mutant or wild-type Ras may be important for cancer development and maintenance *in vivo*. Understanding the mechanisms underlying ras deregulation therefore has implications for diagnosis and therapeutic intervention.

The Ha-*ras* proto-oncogene is constitutively expressed in all cell types and can be induced in response to a number of mitogenic stimuli (10). The rat and human Ha-*ras* promoters have been cloned and a number of regulatory elements identified (11,12). The Ha-*ras* promoter in both species is G+C rich and lacks a TATA box, features characteristic of constitutively expressed 'housekeeping' genes. Six GC boxes, two NF-1 binding sites and two potential AP-2 sites were identified within the upstream regulatory region of human Ha-*ras*. In

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addition, two copies of Ha-*ras* conserved sequence (HRC) and an Ha-*ras* element I (HRE-I) were identified in the human promoter (12). The individual GC boxes appear to have different effects on the promoter activity: only GC II, which binds SpI, shows a positive effect on Ha-*ras* promoter activity. The NF-I elements themselves have weak effects on the promoter activity. Deletion of the NF-I binding site along with the HRE and GC-II site decreases transcription by 2.5-fold in the context of the whole promoter (12). Overall, the rat and human Ha-*ras* promoters are highly conserved, sharing similar regulatory elements located in similar positions within the promoter relative to the start site. Only the Ha-*ras* element (HRE) site present in the human promoter, which is thought to be responsive to the Ets family of transcription factors (12), was not previously reported to have a counterpart in the rat promoter.

Our previous studies of carcinogen induced mammary tumors suggested carcinogen mediated effects on the Ha-*ras* promoter *in vivo* (13). Here we demonstrate that the region of the promoter sensitive to carcinogen treatment includes a positive transcriptional element identical to the HRE found in the human Ha-*ras* promoter, albeit in the inverted and complementary orientation. We demonstrate that the CArG Binding Factor-A (CBF-A) protein, originally defined by its ability to interact with CArG box, binds to both the human and rat HREs. CBF-A binds to the rat and human HRE with higher affinity than the CArG box, originally described as the recognition site for this protein. Furthermore, we failed to detect any Ets transcription factor binding to the rat HRE. These results indicated that in mammary cells, CBF-A is the major protein that binds to recognition sequences commonly accepted as Ets binding sites. CBF-A binding was correlated with increased Ha-*ras* promoter activity in mammary cells and there was a direct correlation between the presence of the HRE binding activity and induction of Ha-*ras* mRNA expression. Taken together, our results suggest that CBF-A mediated transactivation may play an important role in Ha-*ras* deregulation during carcinogenesis in rodents and humans.

## MATERIALS AND METHODS

### Cell culture, cell treatment and cell cycle analysis

The BICR-M1Rk rat mammary gland carcinoma cell line was grown in DMEM and 5% fetal calf serum (FCS; HyClone Laboratories, Inc., UT). Cell cultures were harvested during exponential growth or following appropriate treatment times. Following cell disruption, cytoplasmic fractions were used for RNA extraction (14). Released nuclei were used for protein extraction according to a previously described method (15). In some experiments, cells were serum starved for 48 h and stimulated with 5% FCS. At the indicated times after stimulation, cells were harvested for extraction of RNA and nuclear protein. Treatment with L-mimosine (Sigma, MO) was performed at a final concentration of 200 μg/ml for 10 h before as well as during serum stimulation. For cell cycle analysis, cell cultures were used at 70–80% confluence. After appropriate treatments, cultures were harvested by trypsinization, fixed in 35% ethanol, stained with propidium iodide and analyzed using a Becton Dickinson flow cytometer.

### Electrophoretic Mobility Shift Assays (EMSA)

Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Inc., IA). For EMSA, double-stranded probe was labeled with polynucleotide kinase (NE Biolab, MA) and [α-$^{32}$P]ATP (NEN Dupon, MA). Aliquots (~10 μg) of protein from each nuclear extract were incubated for 30 min in binding buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1.0 mM PMSF, 1 mM DTT) containing 0.5 nM of 5′-end-labeled probe, 1 μg of the non-specific binding inhibitor poly dI•dC (Sigma, MO) in a total volume 15 μl. In competition experiments, a 40- or 50-fold molar excess of unlabeled, double-stranded oligonucleotides were added to the reaction. The complexes formed were separated on 6–8% TBE polyacrylamide gels (Fisher Scientific, PA).

### UV crosslinking

For UV crosslinking of oligonucleotide probes to specific binding proteins, EMSA reactions were subjected to UV irradiation for 30 min using a transilluminator (Fotodyne, Inc., WI). Protein–DNA complexes were boiled in sample buffer with 5% mercaptoethanol and separated by 10% SDS–PAGE. After electrophoresis, gels were dehydrated and subjected to autoradiography using Hyperfilm-MP (Amersham Life Science, Inc., IL).

### Northern blot

Extracted RNA was dissolved in formamide. RNA (12 μg) was loaded on a 1.1% agarose gel containing formaldehyde. Following electrophoretic separation, RNA was transferred to the nylon membrane Hybond-N Plus (Amersham Life Science, Inc., IL) by electroblotting for 2 h at 1 mA in 25 mM sodium phosphate buffer, pH 6.5, using a transblot apparatus (BioRad, CA). The Ha-*ras* cDNA probe was labeled by random priming with [α-$^{32}$P]dCTP (NEN Dupon, MA) and hybridized to the blotted membranes according to manufacturer's recommendations (Amersham Life Science, Inc., IL).

### Luciferase assay for Ha-*ras* promoter activity

Ha-*ras* promoter sequences were derived from the pNMU-1 plasmid (16) and inserted into the *Sma*I site of pGL2 plasmid. The wild-type promoter sequence at position –573 (CCGG) was replaced with GCGC using the Sculptor Kit (Amersham Life Science, Inc., IL) according to the manufacturer's recommendation. The presence of the mutation within the promoter was verified by DNA sequencing. Transient transfection assays were performed using a modified method developed in our laboratory (17) to normalize for possible differences in transfection efficiencies of different DNAs. BICR-M1Rk cells were transfected in six-well plates with Lipofectamine Plus (Gibco BRL, MD) according to the manufacturer's protocol. 8 and 24 h following addition of serum, cells were harvested, counted using a Coulter Counter (Coulter Electronics Ltd, UK) and lysed by three cycles of freezing and thawing in 25 mM Tris pH 8.0. Cytoplasmic fractions were used for the luciferase assay using standard procedure. Released nuclei were lysed in lysis buffer [1× AmplyTaq buffer II (Perkin Elmer) containing 1 mM $MgCl_2$, 0.45% of Nonidet P-40 and 0.45% Tween 20] and digested with proteinase K (0.1 μg/μl) at 56°C for 1 h. Proteinase K was inactivated for 15 min at 94°C. To measure the transfected plasmid copy numbers in nuclei of transfected

cells (17), a 2 µl aliquot of extracted DNA from each transfection was subjected to PCR amplification using 20 pM/µl of luciferase gene primers, pLZ1 (ATA CGC CCT GGT TC) and pLZ2 (CCC TGG TAA TCC GT). PCR reactions were carried out at 94°C for 35 s, 49°C for 35 s and 72°C for 40 s. Amplification was performed for eight cycles in a thermal cycler (Perkin Elmer Cetus) in the presence of 3 µCi of p32 dCTP (NEN Dupon, MA) per reaction in 25 µl total volume. Standards included DNA from untransfected cells and known copy number of the plasmid DNA. PCR products were separated on a 6% acrylamide gel (Fisher Scientific, PA) and quantitated by using a PhosphorImager (Molecular Dynamics, CA) analysis. Negative control reactions included water and cytoplasmic fraction from transfected cells. Activities of the promoter construct were plotted as luciferase values per plasmid copy number per cell number. Final results are presented as fold-activation of the wild-type or mutant promoter construct divided by the expression detected with the control pGL2 plasmid. Transfection experiments were performed at least four times using two independent plasmid preparations.

### Protein purification

Protein purification was performed starting with ~30 ml of a BICR-M1Rk wet cell pellet (800 *g*). Nuclear extracts were prepared as described above using 5–6 ml of cell pellet per preparation. The resulting nuclear extracts were clarified at 30 000 *g* and dialyzed against buffer D (see above). Each batch of nuclear extract was tested for binding activity using EMSA under the conditions described above. Biotinylated sense and unmodified antisense oligonucleotides (same as above) were obtained from Research Genetics (Huntsville, AL), annealed and attached to streptavidin-agarose (Pierce, IL) for use in affinity chromatography. Annealing of oligonucleotides was performed in excess of anti-sense strand to ensure complete annealing of the biotinylated strand. Affinity columns were similarly prepared using mutant oligonucleotides.

The individual batches of nuclear protein extracts were first incubated with poly dI•dC at 20 µg/ml to titrate non-specific DNA binding proteins, and centrifuged at 30 000 *g* to remove precipitates. To reduce the amount of non-specific DNA binding activity, the extracts were first passed over an affinity chromatography column generated with the mutant binding site. Column eluates were then passed over a column of the wild-type binding site to capture specific binding proteins. To ensure complete binding, the nuclear extract was passed over the column repeatedly overnight at 4°C using a peristaltic pump. Proteins bound to the wild-type column were then eluted with a 0.1 M step gradient of 0.2–1.0 M KCl. Collected fractions were dialyzed against buffer D and tested for binding activity by EMSA. All fractions showing specific binding were pooled and loaded on the specific column and eluted again as above. Positive fractions were concentrated (Millipore, MA), loaded on a 10% SDS gel and stained with Coomassie Brilliant Blue R-250 (BioRad laboratories, CA). Detected bands with estimated molecular weights of 42 and 44 kDa were excised. Protein from one-fifth of the most abundant band (42 kDa) was eluted and renatured (18). Resulting protein was used in UV crosslinking reactions to verify the presence of specific binding activity. The remainder of the 42 kDa band was used for peptide identification by capillary HPLC–mass spectrometry as described below. The 44 kDa band was forwarded to Harvard Microchemistry Laboratory directed by Dr W. Lane where protein identification was performed by microcapillary reverse-phase HPLC electrospray tandem mass spectrometry.

### Protein identification by capillary HPLC–mass spectrometry

The protein band was excised from a one-dimensional preparative SDS–polyacrylamide gel and digested with 0.5 µg of Trypsin (Promega, Madison, WI). The digested peptide mixture was extracted and analyzed by a microcapillary LC system connected online to an electrospray ionization ion trap mass spectrometer (Finnigan-MAT, Model LCQ, San Jose, CA). Peptides were concentrated and separated on a micro C18 column with an inner diameter of 50 µm. Separation was accomplished by applying a gradient of 5–65% B over 20 min. The gradient was delivered by a Magic 2002 HPLC system (Michrom BioResource, Inc., Pleasanton, CA) and the flow delivered over the column was adapted with a pre-column flow splitter to 200 µl/min. Eluting peptides were introduced into the mass spectrometer by electrospray via a home built microESI ion source and analyzed by data dependent MS/MS (19,20). The collision induced dissociation spectra generated during the experiment were searched against protein as well as nucleotide databases using Sequest software to identify possible sequence matches.

## RESULTS

### Identification of the rat HRE

We first tested the hypothesis that the region of Ha-*ras* promoter around nucleotide –573, involved in the response to carcinogen treatment (13), is able to interact with proteins *in vitro*. This region of the Ha-*ras* promoter was found to include the nucleotide sequence GGAA. This sequence corresponds to the Ets transcription factor core binding site, albeit in the complimentary and inverse orientation. To determine whether any transcription factors can bind specifically to this region of the promoter, we performed EMSA using synthetic, double-stranded oligonucleotides (Fig. 1A) and nuclear extract from the BICR-M1Rk mammary carcinoma cell line. The results presented in Figure 1B provide evidence for specific binding of proteins from nuclear extracts to the HRE probe. The stable protein–DNA complexes formed were the result of sequence specific DNA binding, since a 40-fold molar excess of dsDNA probes (Mutants 1, 2 and 4) mutated within the putative Ets binding site (CCGGAA) failed to compete with wild-type probe (Fig. 1B). However, a 40-fold excess of dsDNA probes with mutation outside of the consensus CCGGAA Ets motif (Mutant 3) were as effective as the wild-type sequence in competition experiments. The human HRE possessing the same core sequence was also effective in competing for the binding activity. Unrelated promoter elements such as SP1, AP-1 and Stat5/6 binding sites failed to compete for binding to the HRE (not shown). Both the human and rat HRE probes formed similar protein(s) complexes with nuclear extracts from rat, mouse and human cells, as judged by mobility in EMSA gels (not shown).

To determine if the proteins bound to the rat HRE were members of the Ets transcription factor family, we performed competitive binding experiments with an oligonucleotide probe comprising the binding site for the *Drosophila*

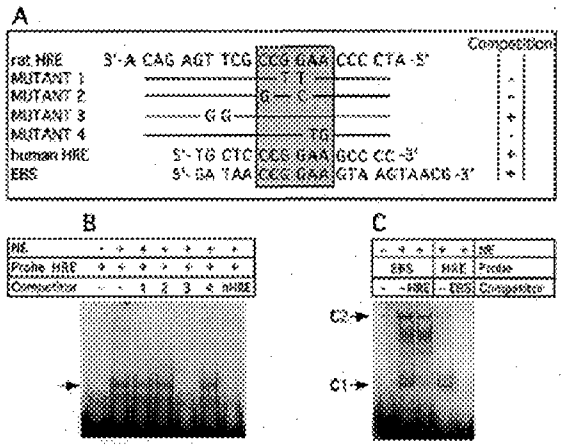


**Figure 1.** EMSA with rat HRE probe and competition experiment with mutant HRE, human HRE (hHRE) and EBS. Competitor DNAs were added in 40-fold molar excess. NE, nuclear extract from BICR-M1Rk. (**A**) Sequence of oligonucleotides used in the competition experiment. The conserved sequence CCGGAA found in the rat HRE and hHRE and the EBS is boxed. The summary of competition experiment results is on the right. Competition and absence of competition with the HRE probe are indicated by + and –, respectively. (**B**) EMSA and competition experiment with hHRE and the different mutant oligonucleotides (from 1 to 4) listed on (A). Specific DNA–protein complexes are shown with an arrow. (**C**) EMSA competition experiment with EBS or rat HRE in the presence of excess rat HRE or EBS respectively. C1 and C2 DNA–protein complexes are indicated. The rat HRE probe without nuclear extract added is not shown.

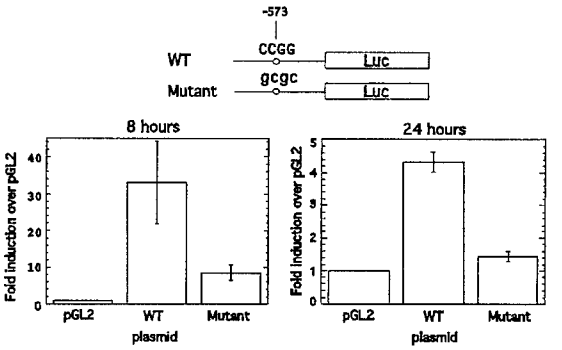


**Figure 2.** Wild-type Ha-*ras* promoter is 3–3.9-fold more active compared to the mutant promoter. BICR-M1Rk cells were transfected with 1 μg of plasmid DNA in serum free conditions and luciferase activity was measured after 8 and 24 h following serum stimulation. Normalization for transfection efficiency was performed as described in Materials and Methods. At the top of the figure is a schematic presentation of the constructs showing wild-type and mutant sequences in the Ha-*ras* promoter linked to the luciferase gene. Error bars represent standard deviations.

*melanogaster* E74 Ets transcription factor. The E74 probe (EBS) forms two distinct EMSA complexes (C1 and C2) with mammary cell nuclear extracts (Fig. 1C). The rat HRE probe was able to compete effectively with the E74 probe, although the rat HRE affinity for protein complex C1 was higher than for the complex C2 (Fig. 1C). In the inverse experiments (Fig. 1C), labeled rat HRE probe formed predominantly complex C1, while complex C2 was very weak or undetectable. An excess of unlabeled oligonucleotide corresponding to the E74 binding site efficiently abrogated rat HRE binding. The latter result is consistent with the notion that the proteins bound to the rat HRE could be members of the Ets protein family, or at least compete for binding to the same DNA sequences.

To investigate the role of rat HRE in the context of the promoter *in vivo*, we performed a transient transfection assay using wild-type and mutant rat Ha-*ras* promoter linked to the luciferase reporter gene. A double mutation that disrupts the Msp1 site at position –573 was introduced into the HRE using the mutant oligonucleotide (Mutant 2; see Fig. 1A). In transient transfection experiments in BICR-M1Rk cells the wild-type promoter showed strong (33-fold) activation 8 h following serum stimulation relative empty vector, and a 3.9-fold increase in activity relative to the mutant promoter (Fig. 2). At 24 h after serum stimulation the activity of the wild-type promoter was lower compared to an 8 h time point, but it remained 3-fold more active compared to mutant promoter. The activity of the mutant promoter at 24 h was only slightly higher than the activity of the empty vector. Thus, in the context of the whole promoter, the HRE has a strong positive effect on the rat Ha-*ras* promoter activity in mammary cells and correlates with increased HRE binding activity following serum stimulation (see Fig. 6).

We next used UV irradiation to cross-link the specific binding proteins to the DNA probe in order to estimate the approximate molecular weight of the specific binding protein. Analyses of the cross-linked products by SDS–polyacrylamide gel electrophoresis demonstrated that the protein bound to the DNA probe has an estimated molecular weight of ~51–52 kDa. Assuming the probe bound to the protein was single-stranded, the latter result suggested that the protein alone is ~43–44 kDa in size (data not shown and see Fig. 3B).

Taken together, these experiments suggested that the HRE from rat and human Ha-*ras* promoter is a specific binding site for an Ets related transcription factor present in mammary cell lines. However, Ets-1 and -2 antibodies (21) or Ets 1/Ets 2 antibodies (Santa Cruz, CA) designed to recognize a broad spectrum of Ets related proteins failed to super-shift the complexes formed between the HRE oligonucleotide probe and the mammary cell nuclear extracts (not shown). These results suggested that the HRE binding proteins in mammary cells were either novel members of the Ets transcription factor family or unrelated proteins that recognize the same DNA sequences as Ets proteins. We therefore performed experiments to identify and clone the HRE binding proteins.

### Purification of the protein and protein identification

The HRE binding protein was extensively purified using sequence specific DNA affinity chromatography as described in Materials and Methods. Bound proteins were eluted from the column with a KCl step gradient and fractions assayed for HRE binding by EMSA. Most of the binding activity eluted in 0.7–1.0 M KCl (not shown). Fractions with binding activity were pooled, concentrated and analyzed on a 12% SDS gel. Two main bands with estimated molecular weights of 42 and 44 kDa were detected in the fractions with binding activity (Fig. 3A). To confirm that the most abundant protein band

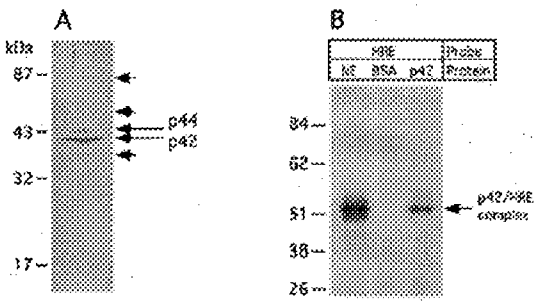


**Figure 3.** (**A**) Coomassie Brilliant Blue stained gel following purification of the DNA binding protein by affinity chromatography. Migration of the molecular weight standards is shown on the left. The most abundant species are labeled p42 and p44. Bands of lower intensity which were also subjected to protein identification by micro-HPLC–mass spectrometry are indicated by short arrows. (**B**) Comparison of radiolabeled, UV crosslinked HRE–protein complexes from whole nuclear extract (NE) with the HRE–p42 complex. The p42 protein was recovered from gel shown on (A). BSA, bovine serum albumin.

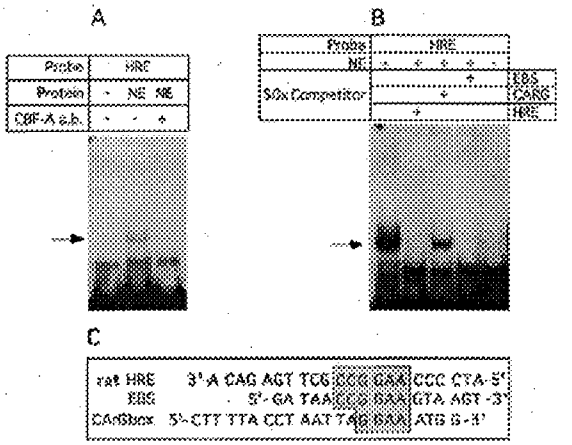


**Figure 4.** (**A**) Anti CBF-A antibody completely abrogated protein binding to the rat HRE and human HRE (not shown) probes. EMSA was performed as described in Materials and Methods. Anti CBF-A antibody or normal serum were added as indicated, + and –, respectively. NE, nuclear extract from BICR-M1Rk cells. (**B**) Comparison of the binding affinity of CBF-A to the rat HRE and CArG box. Competitor oligonucleotides, rat HRE, CArG and EBS were added to the binding reaction in 50-fold molar excess. Specific HRE–protein complex are indicated with arrow. (**C**) Comparison of rat HRE probe, EBS and CArG box oligonucleotide sequences. Conserved sequence is boxed.

contained the HRE binding protein, the 42 kDa band was excised from a Coomassie Brilliant Blue stained gel and protein was eluted from one-fifth of the gel slice. Following renaturation, the eluted proteins were incubated with radiolabeled HRE probe and any resulting protein–DNA complexes were crosslinked with UV light. The protein eluted from 42 kDa bands formed a stable complex with the HRE probe that was indistinguishable from the crosslinked complexes formed with the protein in total nuclear extract as shown by SDS–PAGE (Fig. 3B). Since the amount of the protein in the 44 kDa band was significantly lower than in the former band, we did not perform crosslinking experiments to conserve protein for further analysis. In summary, the crosslinking experiment above showed that the protein in the 42 kDa protein preparation contained a HRE binding protein whose molecular weight is consistent with that estimated by UV crosslinking.

Independent protein analyses identified similar polypeptides present in both the 42 and 44 kDa proteins (summarized in Table 1). The polypeptides identified corresponded to sequences detected in the previously identified mouse CBF-A (22). Together the polypeptides identified in our analysis encompassed almost 36% of the CBF-A amino acid sequence. Moreover, MS/MS analysis of these two and three additional bands with low intensity staining from the preparative protein gel (Fig. 3A, short arrows) failed to detect any Ets related proteins (data not shown). Together these results suggested that the CBF-A or a closely related protein was responsible for most of the HRE binding activity detected in mammary cells.

### CBF-A interacts with rat HRE with greater affinity as compared to CArG-box

To verify that CArG binding protein interacts with rat and human HRE, we employed an anti-CBF-A antibody kindly provided by T. Leandersson (Lund University, Sweden: 23). First, we verified that the polyclonal antibody against mouse CBF-A would cross-react with the rat protein. Rat CBF-A was cloned by PCR from BICR-M1Rk cells, *in vitro* translated and detected by western blot (not shown). EMSA demonstrated that the antibody completely and specifically abrogated the interaction of nuclear protein with the rat (Fig. 4A) or human (not shown) HRE probes. These results provided direct evidence that CBF-A was responsible for the HRE binding activity observed in mammary cells.

**Table 1.** List of the overlapping polypeptides identified by micro HPLC–mass spectrometry of the 42 and 44 kDa proteins isolated by affinity chromatography

| |
|---|
| MFVGGLSWDTSK |
| MFVGGLSWDTSKK |
| MFVGGLSWDTSKKDLKDYFTK |
| DLKDYFTK |
| SRGFGFILFK |
| GFGFILFK |
| IFVGGLNPEATEEK |
| IFVGGLNPEATEEKIR |
| GGLNPEATEEK |
| IREYFGQFGEIEAIELPIDPK |
| EYFGQFGEIEAIELPIDPK |
| GFVFITFKEEDPVKK |
| GFVFITFKEEDPVK |
| FHTVSGSK |
| EVYQQQQYGSGGR |

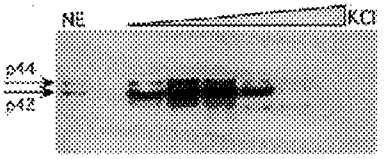


**Figure 5.** Two protein species (p42 and p44) interact with rat HRE. The western blot was performed with the CBF-A antibody against HRE binding proteins eluted from two sequential affinity columns with 0.1 M KCl step gradient. Aliquots (15 µl) from each dialyzed fraction were used. NE, nuclear extract before loading on the affinity column. The two protein bands, p42 and p44, are indicated by arrows.

We next compared the relative affinities of CBF-A protein for rat HRE and the CArG-box originally described as its recognition sequence. A 50-fold molar excess of unlabeled, double-stranded oligonucleotides corresponding to CArG box (22), rat HRE or an Ets binding site (EBS) were used as competitors in binding experiments using labeled rat HRE probe and analyzed by EMSA. While the addition of 50-fold molar excess of cold rat HRE and EBS efficiently diminished DNA–protein binding, addition of same molar excess of the CArG box oligomer demonstrated only partial competition with the rat HRE (Fig. 4B). The latter results suggested that CBF-A binds to the HRE with higher affinity than the CArG box originally used to isolate the CBF-A. Similarly, CBF-A is able to bind the EBS with higher affinity than the CArG box. From these competition experiments (also see Fig. 1), we suggest that sequence CCGGAA is important for high affinity CBF-A binding to DNA.

In a previous study it was shown that antibody against CBF-A recognizes two protein species in cell extracts by western blot (23). However, the authors demonstrated that only the lower molecular weight protein was found to interact with the A-T rich region of the pd element within the SP6 *k* promoter. To determine if rat HRE interacts with one or both protein species, we probed a western blot containing protein fractions eluted from affinity column (see Materials and Methods for details) with the CBF-A antibody on the western blot. Figure 5 demonstrates that the same two protein species found in the nuclear extract of mammary cells (p44 and p42) are detected in protein fractions eluted from the affinity column. However, protein ratio (p44/p42) is reduced compared to nuclear extract before loading on the column. In our hands, under normal growth conditions this ratio is 0.5–1.2. This experiment together with microcapillary reverse-phase HPLC electrospray tandem mass spectrometry provided the evidence that slower migrating protein (44 kDa) is related to CBF-A.

### Establishing a correlation between CBF-A binding to HRE and Ha-*ras* mRNA expression

Previous studies have demonstrated that serum stimulation of cells arrested in $G_0$ leads to induction of Ha-*ras* expression (24). To establish a correlation between HRE binding activity and Ha-*ras* mRNA expression, BICR-M1Rk cells were serum deprived for 48 h to induce growth arrest, and then stimulated with serum. To control for activities among different preparations of extracted nuclear proteins, we assayed each extract for

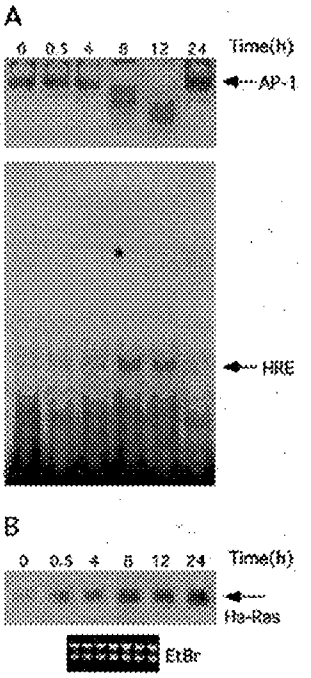


**Figure 6.** HRE–CBF-A binding activity correlates with Ha-*ras* mRNA expression. Serum deprived cells were stimulated with 5% calf serum. RNA and nuclear proteins were extracted at indicated time points from the same culture (see Materials and Methods for details). (**A**) Nuclear proteins were used in EMSA with AP-1 (top, only DNA–protein complexes are shown with arrow) and rat HRE probes (bottom). (**B**) RNAs extracted from cells at the same time points following serum stimulation were separated on the 1.1% agarose gel, blotted and probed with rat Ha-*ras* cDNA. Bottom, ethidium bromide staining of the membrane following RNA transfer demonstrates equal RNA loading. For every time point shown on the figure we also performed controls using nuclear extract (A) and RNA (B) from serum deprived cells harvested at a given time. The level of binding activity to the AP-1 and HRE probes and the level of Ha-*ras* expression did not differ from the zero time points in these samples. For simplicity these controls were removed from the final figure using image analysis software.

serum responsive binding activity to the AP-1 recognition sequence. At the specified time points following serum induction, cells were harvested for extraction of RNA and nuclear proteins. The AP-1 binding was observed in serum starved cells (Fig. 6) and was increased by 24 h post induction. Serum transiently stimulated binding to the HRE between 4 and 12 h, and decreased by 24 h. Interestingly, binding to the rat HRE was maximal at 8 and 12 h after treatment, at which time AP-1 binding activities were transiently reduced to somewhat lower levels. Reasons for modification of the AP-1 binding remain unknown and beyond the scope of this paper. As expected, serum stimulated Ha-*ras* mRNA expression (Fig. 6B), and the increase in RNA levels detected at 8 h, corresponded with the time of maximal CBF-A binding to the HRE. Reduced CBF-A–HRE binding at the 24 h time point suggested that Ha-*ras* expression at later time points following serum stimulation depend on other transcription factors and/or RNA stability.

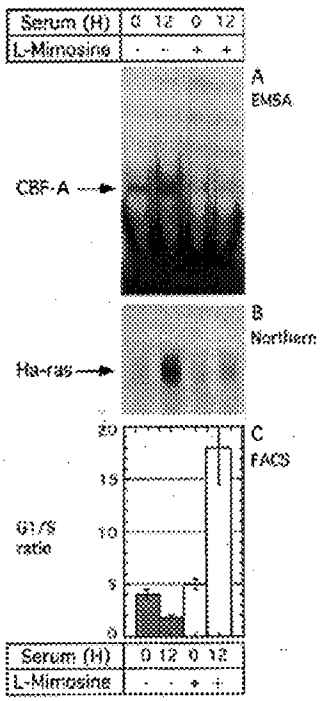


**Figure 7.** L-mimosine inhibits CBF-A binding to the rat HRE and expression of the Ha-*ras* mRNA. Serum deprived cells were stimulated with serum or serum plus L-mimosine (200 μg/ml). 12 h later, nuclear proteins and RNA were extracted from the same culture. At each time point, a duplicate cell culture was taken for cell cycle analysis by fluorescence activated cell sorting. (**A**) EMSA of the CBF-A binding to rat HRE probe. (**B**) Northern blot analysis of the RNA probed with Ha-*ras* cDNA. Equal loading was verified by ethidium bromide staining of RNA following transfer to the membrane (not shown). (**C**) Cell cycle analysis shown the average $G_1$/S ratio from three independent measurements. Error bars represent standard deviations. Open and closed bars, cells untreated and treated with L-mimosine, respectively.

To expand this observation we stimulated cells with serum in the presence or absence of L-mimosine, a p53 independent inducer of the cyclin dependent kinase inhibitor, p21 waf-1 (25). Induction of p21 waf-1 by L-mimosine was confirmed by western blot (not shown). As expected from the experiment above, treatment of cells with serum in the absence of L-mimosine, induced binding of CBF-A to the HRE (Fig. 7A), and resulted in elevated Ha-*ras* mRNA expression (Fig. 7B) by 12 h after serum stimulation. At the same time, we observed accumulation of cells in the S phase of the cell cycle, with a concomitant decrease in the $G_1$/S ratio (Fig. 7C). L-mimosine treatment abrogated stimulation of CBF-A binding to the HRE sequence, and the levels of Ha-*ras* mRNA expression in cells stimulated with serum in the presence of L-mimosine were significantly lower compared to levels in cells stimulated with serum only (Fig. 7B). As predicted, in the presence of L-mimosine, cells were arrested in $G_1$ phase resulting in an increased $G_1$/S ratio. Together, these results demonstrated a correlation between increased CBF-A binding to the rat HRE and stimulation of Ha-*ras* expression.

## DISCUSSION

In our previous study we found that the rat Ha-*ras* promoter between positions –582 and –563 is involved in response to specific carcinogen treatment (13). In the present study we asked if this region of the Ha-*ras* promoter can bind specifically with any *trans*-acting transcription factor and if the binding of factors can regulate expression of the oncogene *in vitro*. We determined that double-stranded oligodeoxynucleotides corresponding to the rat HRE were able to bind specifically with transcription factors present in nuclear extracts from a rat mammary tumor cell line. Our result suggested that rat and human HRE interact with members of the Ets transcription factor family. HRE–protein binding appears to be highly conserved among different species since similar binding activities were detected in nuclear extracts from a variety of rat, mice and human cell lines using either the human or rat HRE elements as probe (not shown).

Ets phosphoproteins play an important role in the control of cell growth and development (26–30). Ets binding sites have been identified in several oncogene responsive promoters (28,29,31). A number of studies have shown that Ets related transcription factors may play an important role in ras mediated signal transduction and involved in regulation of a number of genes downstream of Ras (28). It is thus reasonable to posit that Ets related proteins, or proteins that compete with Ets proteins for specific binding sites, could play an important role in Ha-*ras* mediated cell transformation.

In transient transfection assay we found that at 8 h after serum stimulation wild-type promoter construct showed strong 33-fold higher luciferase activity compared to construct lacking the HRE. The activity of the wild-type promoter in transient transfection assay correlated well with binding activity of the CBF-A to the target sequence (Figs 2 and 6). We concluded that despite the different relative positions of HRE within rat and human Ha-*ras* promoters, they are functionally equivalent. Our results are consistent with others showing that deletion of HRE from human Ha-*ras* promoter results in 2-fold drop in the promoter activity following transfection in HeLa cells (12). To further characterize the HRE binding proteins from mammary cells, we employed affinity purification of the protein followed by protein identification by micro HPLC–mass spectroscopy. Sequence analysis of the two most abundant proteins, with approximate molecular weights of 42 and 44 kDa, unexpectedly matched sequences corresponding to mouse CBF-A. We confirmed interaction of CBF-A with rat and human HRE in EMSA using CBF-A specific antibody. Our results also provided evidence that the slower migrating protein species (p44) detected on the western blot is a CBF-A related protein. The p44 is able to interact with the DNA target, although with lower affinity compared to the p42. We support the suggestion that the slower migrating protein is a post-translationally modified form of the CBF-A protein or an RNA splice form of slightly higher molecular weight (23). Identified peptides encompassed almost 36% of the CBF-A amino acid sequence suggesting a high level of homology between mouse and rat proteins. Indeed, rat and mouse cDNA for CBF-A are very conserved (S.Kamada and T.Miwa, EMBL accession no. D90151; A.Mikheev, L.Jing and H.Zarbl, EMBL accession no. AF216753). In an attempt to detect any putative Ets related protein, we performed sequence analysis of additional bands of

very low intensity detected on preparative Coomassie Blue stained gel. We failed to detect polypeptides corresponding to Ets related proteins. Together these results suggest that Ets related proteins are probably not involved in interaction with HRE site of the rat Ha-*ras* promoter in BICR-M1Rk mammary carcinoma cell line and that CBF-A is indeed the major binding HRE factor in these cells.

The CBF-A was discovered by screening an expression library with the CArG box DNA fragment as a probe (22). The CArG box sequence was initially described in a number of genes showing muscle tissue specific expression (32–40). For example, it was shown that the serum response factor (SRF) can interact with the CArG box and activate transcription of muscle-specific genes and immediate-early genes, such as fos (33,38,41). CBF-A is a protein with a calculated molecular weight of 31 kDa and migrates with an apparent molecular weight of 42 kDa in SDS–PAGE. The protein has an RPN domain that is thought to be involved in the binding to nucleic acid (42). The RPN domain is common to heterogeneous nuclear ribonucleoproteins (hnRPN) A/C types involved in splicing, transport and protection of RNA (43). CBF-A was initially found to be a transcriptional repressor (22). However, our study shows that CBF-A is a transcriptional activator of Ha-*ras* in transient transfection assays. The discrepancy with published results is not surprising since the CArG regulatory element can interact with a number of other transcription factors, including Ets related factors Elk-1 and SAP-1 (44,45), E12, NF-IL-6 (46) and HMG-I family proteins (47). It is therefore plausible that CBF-A complexes with, or replaces, other transcriptional factors depending on the context of the CArG box. For example, functional antagonism between SRF and YY1 proteins at CArG elements has been described (48). Likewise, studies have demonstrated that protein–protein interactions affect transcription from CArG box (47). For example, in the Arabidopsis APETALA3 gene, individual CArG boxes within a tandem array of three, have opposite regulatory effects on the promoter activity (40). While the first two CArG boxes are positive regulatory elements, the third has a negative effect on the promoter activity.

It was also noted previously that CBF-A is able to interact with single-stranded DNA (22). Binding of CBF-A to the single-stranded form of the A-T rich pd element is stronger compared to the double-stranded form (23). In our experiment we failed to detect any single-stranded DNA binding activity (not shown). We speculate that CBF-A may demonstrate different functional specificity, depending on the affinity of the interaction with target DNA and/or interactions with other factors. CBF-A modulation of transcription from the CarG, as well as other elements, may therefore be gene and cell type specific.

In our competition experiments, the affinities of CBF-A for the rat HRE and Ets binding sites (E74) were clearly higher than its affinity for the interaction with the CArG box, $CC(AT)_6GG$. Comparison of human and rat HRE, E74 and CArG box sequences suggests that the sequence CCGGAA is important for high affinity binding of CBF-A to DNA. Since this sequence is frequently present in a number of binding sites for Ets proteins, we suggest a potential role of CBF-A in the regulation of Ets responsive promoters. Our suggestion is supported by the fact that CBF-A is able to bind Ets related proteins *in vitro* (23). The functional significance of CBF-A and Ets protein interaction is not clear. Since a number of Ets proteins are involved in the regulation of different genes, the role of CBF-A may be widespread. CArG binding factor A was found to be overexpressed in NIH 3T3 cells transformed with *ets*-1 and *ets*-2 genes (49). This result suggests possible co-operation of Ets proteins and CBF-A in cell transformation. Our preliminary results suggested that the CBF-A protein undergoes post-translational modification which is required for binding activity. Ectopic overexpression of the CBF-A is not sufficient to induce efficient interaction to the target DNA and fails to induce Ha-*ras* expression.

In summary our results demonstrated the high affinity interaction of the CArG binding factor (CBF-A) with the HRE present in the Ha-*ras* promoters of both rodents and humans. Furthermore, the HRE is a strong positive regulatory element in the Ha-*ras* gene. Contrary to expectations, we did not find any Ets related proteins capable of high affinity binding to the HRE in mammary cells. The correlation of CBF-A binding to the HRE and Ha-*ras* mRNA expression suggests that CBF-A may be involved in control of cell cycle and carcinogenesis in mammary cells.

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