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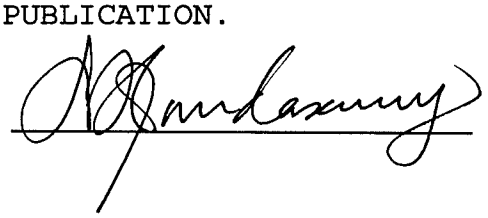

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Our previous studies in pubescent female rats suggested that nitrosomethylurea (NMU) mediated epigenetic effects on DNA conformation within the Ha-ras promoter contributes to mammary carcinogenesis. The DNA region affected by NMU includes a transcriptional 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. Despite the fact that HRE element present in the Has1 promoter resembles a binding site for ets transcription factors, we did not detect the binding of ets related proteins. 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 suggest that CBF-A plays an important role in regulation of Ha-ras promoter activity during mammary carcinogenesis.

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Introduction

Our previous finding suggested a new paradigm for non-mutagenic mammary carcinogenesis (1). We demonstrated that the activating *Hras1* mutations found in NMU-induced tumors arise as background mutations within cells of the developing gland, and that NMU enhances the phenotypic penetrance of these mutations by initiating alterations in DNA conformation. We subsequently identified a cell-type-specific, DNA structure or conformational switch (CS) within the *Hras1* promoter of normal rat mammary cells (RMCs) *in vivo* (2). 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. 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 are 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 was delayed somewhat because we were able to obtain a polyclonal antibody from other investigators but is now underway. Technical Objective 4 is essentially complete, with the exception of defining all post-translational modifications of CBF-A.

The Ras proteins are closely related set of genes encode membrane-associated proteins involved in cell proliferation and differentiation and. 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 (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). 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 (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 accord with the observations that mutant ras genes are frequently over-expressed in human tumors (5,6). 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* (7). 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 (8). 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 (9). The rat and human Ha-ras promoters have been cloned and a number of regulatory elements were identified (10,11). 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 (11). 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 (11). 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 (11), 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 (12). 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 suggest that CBF-A mediated transactivation may play an important role in Ha-ras deregulation during carcinogenesis in rodents and humans.

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 (13). Released nuclei were used for protein extraction according a previously described method (14). 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.

Electrophoretic Mobility Shift Assays (EMSA).

Oligonucleotides were purchased from Integrated DNA Technologies, Inc (IDT, Inc.,IA). For EMSA double strand probe was labeled with polynucleotide kinase (NE Biolab, MA) and [α^{32}P]-ATP (NEN Dupon, MA). Approximately 10 ug aliquots of protein from each nuclear extract was incubated for 30 min in the 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 double strand probe, 1ug of the nonspecific binding inhibitor, poly dIdC (Sigma, MO) in a total volume 15 ul. In competition experiments, a 40 or 50-fold molar excess of unlabeled, double strand oligonucleotides

were added to the reaction. The complexes formed were separated on the 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 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).

Northern blot.

Extracted RNA was dissolved in formamide. Twelve ug of RNA were loaded on a 1.1% agarose gel containing formaldehyde. Following electrophoretic separation, RNA was transferred to nylon membrane Hybond-N Plus (Amersham Life Science, Inc., IL) by electroblotting for 2 hours at 1mA in 25mM sodium phosphate buffer pH6.5 using a transblot apparatus (BioRad, CA). Membranes were hybridized to the Ha-ras cDNA probe labeled by random priming with [α^{32}P]-dCTP (NEN Dupon, MA) according to manufacturer's recommendations and 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 (15) 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 (16) 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^{0}C 1 hour. Proteinase K was inactivated 15 min at 94^{0}C. To measure the transfected plasmid copy numbers in nuclei of transfected cells (16), 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 94^0 C - 35 sec, 49^0 C- 35 sec and 72^0 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 43 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 (17,18). 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 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 (12) 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 (19) 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 (20), 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) (21). 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 (22). 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 (21), 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.

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 (23). 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. 5) 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 (24). 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). 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 a presence of L-mimosine were significantly lower compare to levels in cells stimulated with serum only (Fig.6B). As predicted, in the presence of L-mimosine cells were arrested in G1 phase resulting in 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 during cell cycle progression.

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 (25) arose as background mutations within cells of the developing mammary gland prior carcinogen exposure (26). 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 (26). 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* (7). 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 (12) 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 (27-29), (30,31). Ets binding site were identified in several oncogene responsive promoters (29,30,32). 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 (30). For example, cooperation of activated Ha-ras and ets1 was shown stimulate activity of the rat prolactin promoter in pituitary cells (27). Expression of dominant negative ets inhibits Ha-ras induced transformation (33). 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 (21). The CArG box sequence was initially described in a number of genes showing muscle tissue specific expression (34-42). 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 (35,40,43). 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 (44). The RPN domain is common to heterogeneous nuclear ribonucleoproteins (hnRPN) A/C types involved in splicing, transport and protection of RNA (45). CBF-A was initially found to be transcriptional repressor (21). 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 (46,47), E12, NF-IL-6 (48), HMG-I family proteins (49). 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 (50). Likewise, studies have demonstrated that protein-protein interaction affect transcription from CArG box (49). For example, in the Arabedopsis APETALA3 gene, individual CArG boxes within a tandem array of three have opposite regulatory effects on the promoter activity (42). 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 (21). 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* (22). 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 (51) our results suggest that CBF-A stimulation of Ha-ras expression may elevate Ras to levels required to activate downstream pathways.

Figure legends:

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

Figure 2. Wild-type Ha-ras promoter is 3 fold more active compare to mutant promoter. BICR 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. Double mutation introduced in the Ha-ras promoter linked to luciferase gene is shown on the top.

Figure 3. Panel A: Coomasie Brilliant Blue stained gel following separation of DNA binding protein by affinity chromatography. Migration of molecular weight standards is shown on the left. Most abandon bands labeled as p42 and p43. Bands of lower intensity subjected to protein identification by micro-HPLC - mass spectrometry are shown with short arrows.
Panel B: Detection of DNA-protein complexes of the same molecular weight using UV crosslinking of p32 labeled rat HRE probe to the protein from whole nuclear extract (NE) and protein recovered from p42 band (p42). BSA - bovine serum albumin.

Figure 4. Panel A: Anti CBF-A antibody completely abrogated binding of the protein from nuclear extract to the rat HRE and human HRE (not shown) probes. EMSA was performed as described in

experimental procedure, 0.5 ul of anti CBF-A antibody or normal serum were added as indicated (+) and (-), respectively.
Panel B: Higher affinity binding of CBF-A to the rat HRE compare to CArG box. Competitor oligonucleotides rat HRE, CArG and EBS were added to the binding reaction in 50 fold molar excess. Specific DNA-protein complex shown with arrow.
Panel C: Comparison of rat HRE probe, EBS and CArG box oligonucleotide sequences. Conserved sequence is boxed.

Figure 5. HRE - CBF-A binding activity correlates with Ha-ras mRNA expression. Serum deprived cells were stimulated with 5% serum. RNA and nuclear proteins were extracted at indicated time points.
Panel A: Nuclear proteins were used in EMSA with AP-1, SP1 probes (top, only DNA-protein complexes are shown with arrows) or rat HRE probe (bottom).
Panel B: RNAs extracted from cells at the same time following serum stimulation were separated on the 1.1% agarose gel, blotted and probed with rat Ha-ras cDNA. Lower panel: membrane following RNA transfer
demonstrating equal RNA loading.

Figure 6. L-mimosine inhibits CBF-A binding to rat HRE and Ha-ras expression. Serum deprived cells were stimulated with serum or serum plus L-mimosine (200 ug/ml) and after 12 hours nuclear proteins and RNA were extracted. Additional plate of cell at each time point was taken for cell cycle analysis by Fluorescence Activated Cell Sorting.
Panel A: CBF-A binding to rat HRE probe on EMSA.
Panel B: Northern blot of RNA probed with Ha-ras cDNA.
Panel C: Cell cycle analysis shown as an average G1 to S ratio of three independent measurements. Error bars show standard deviations.

Figure 1

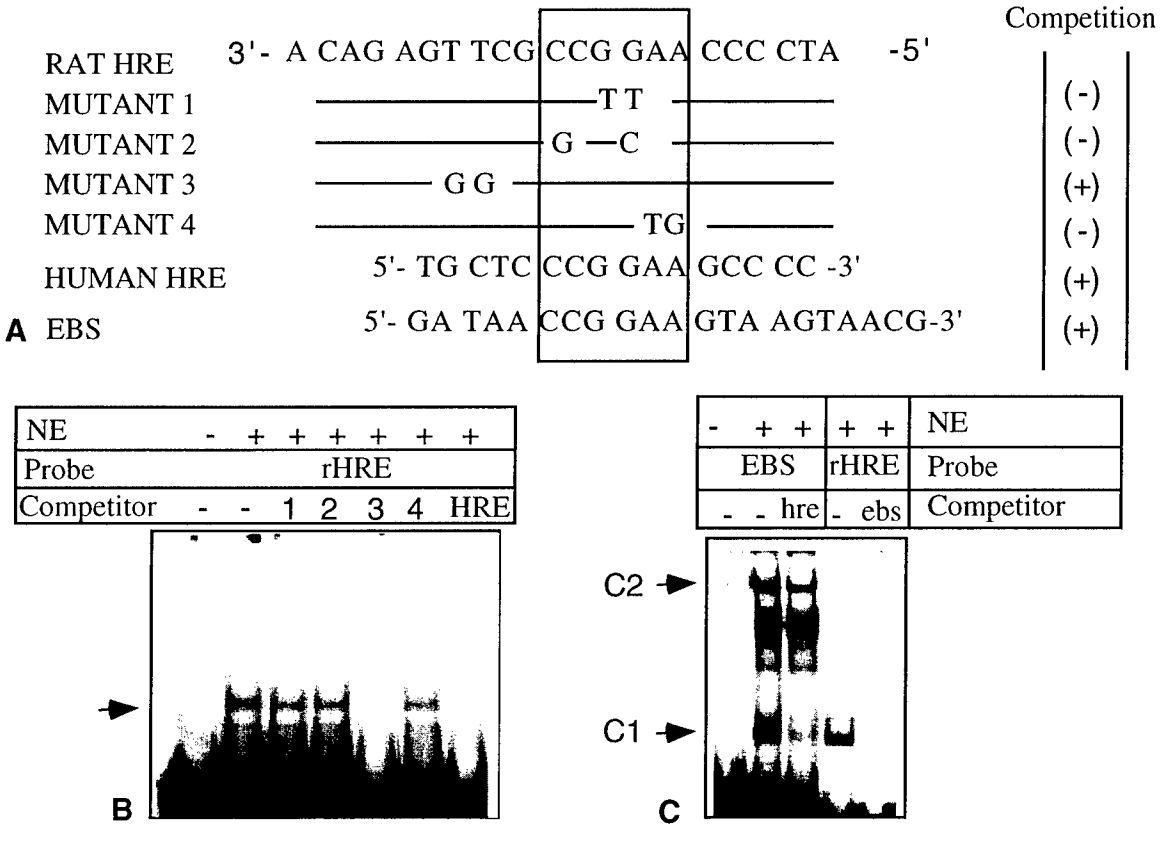

Competition
RAT HRE 3'- A CAG AGT TCG CCG GAA CCC CTA -5'
MUTANT 1 TT (-)
MUTANT 2 G C (-)
MUTANT 3 GG (+)
MUTANT 4 TG (-)
HUMAN HRE 5'- TG CTC CCG GAA GCC CC -3' (+)
A EBS 5'- GA TAA CCG GAA GTA AGTAACG-3' (+)
NE - + + + + + +
Probe rHRE
Competitor - - 1 2 3 4 HRE
B
- + + + + NE
EBS rHRE Probe
- - hre - ebs Competitor
C2
C1
C

Figure 2

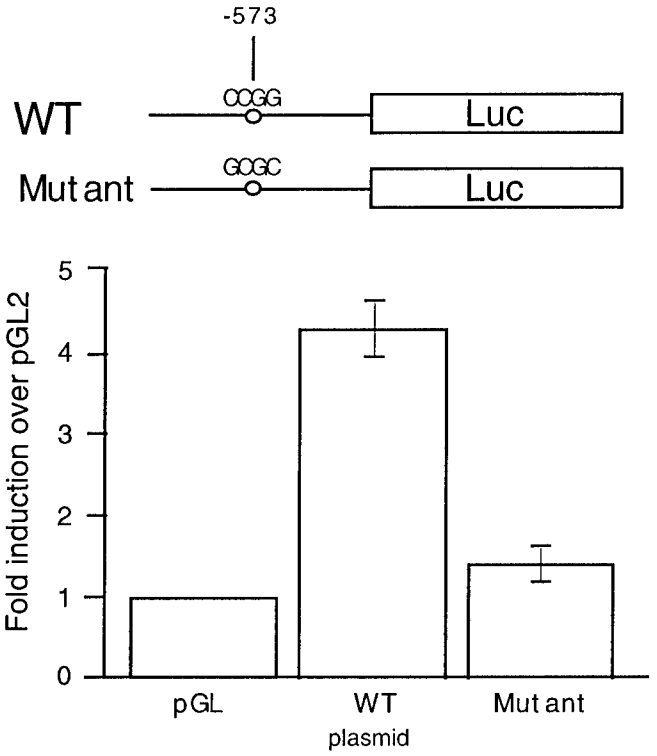

-573
WT
CCGG
Luc
Mutant
GCGC
Luc
Fold induction over pGL2
0
1
2
3
4
5
pGL
WT
Mutant
plasmid

Figure 3

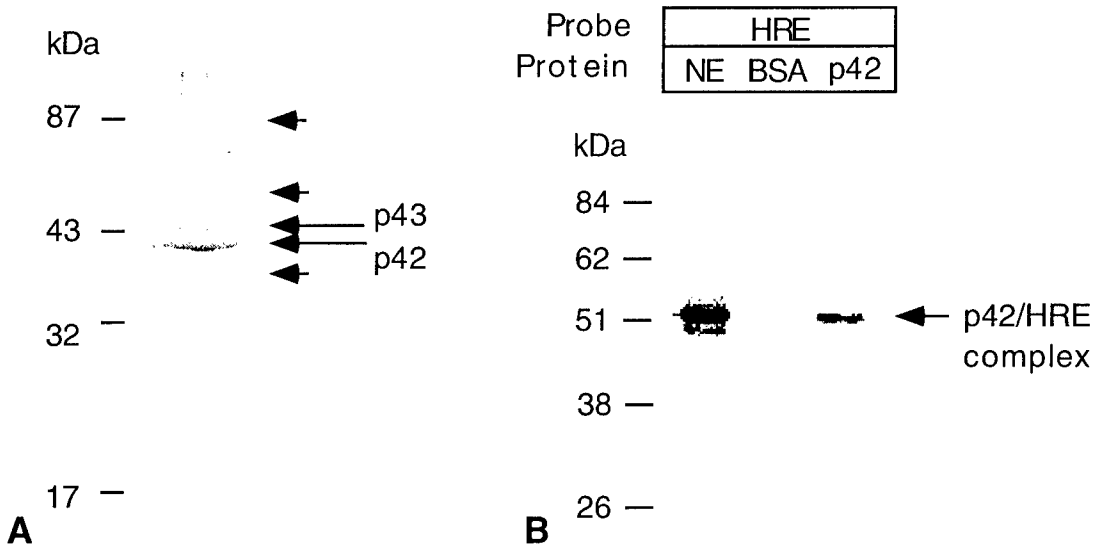
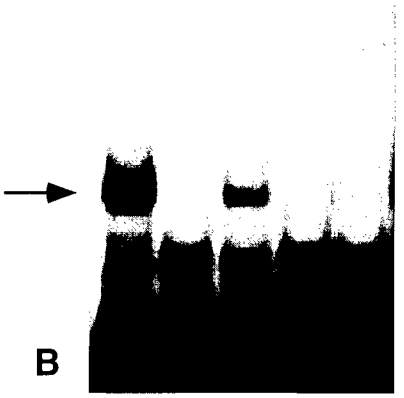

kDa
87
43
32
17
p43
p42
A
Probe
Protein
HRE
NE BSA p42
kDa
84
62
51
38
26
p42/HRE
complex
B

Figure 4

Probe	HRE		
Protein	-	NE	NE
CBF-A a.b.	-	-	+

Probe	HRE				
NE	+	+	+	+	-
50x Competitor	-	HRE	CArG	EBS	-

A



B

C

rat HRE	3'- A CAG AGT TCG	CCG GAA CCC CTA -5'
EBS	5'- GA TAA	CCG GAA GTA AGT -3'
CArG box	5'- CTT TTA CCT AAT	TAG GAA ATG G -3'

Figure 5

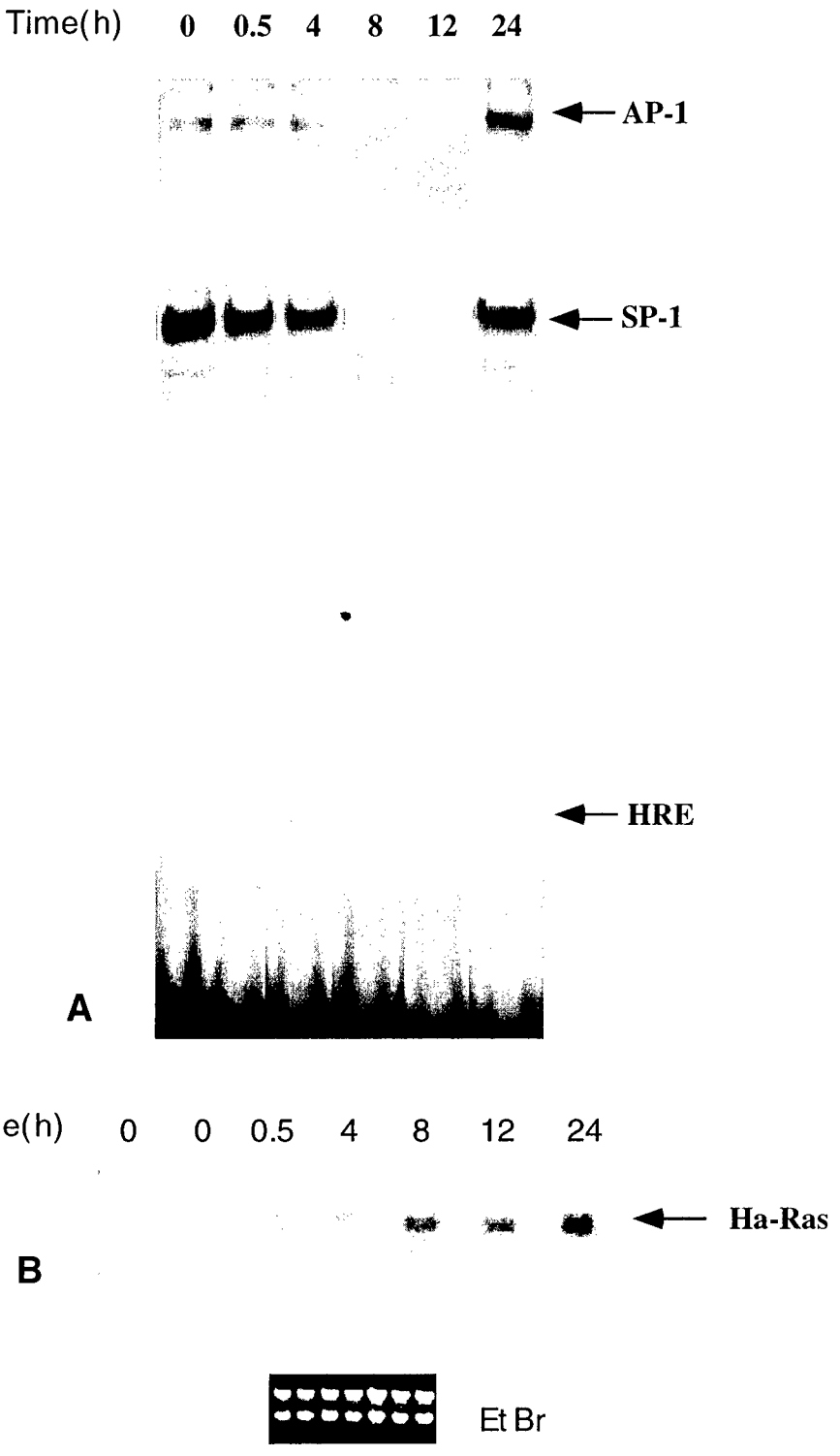
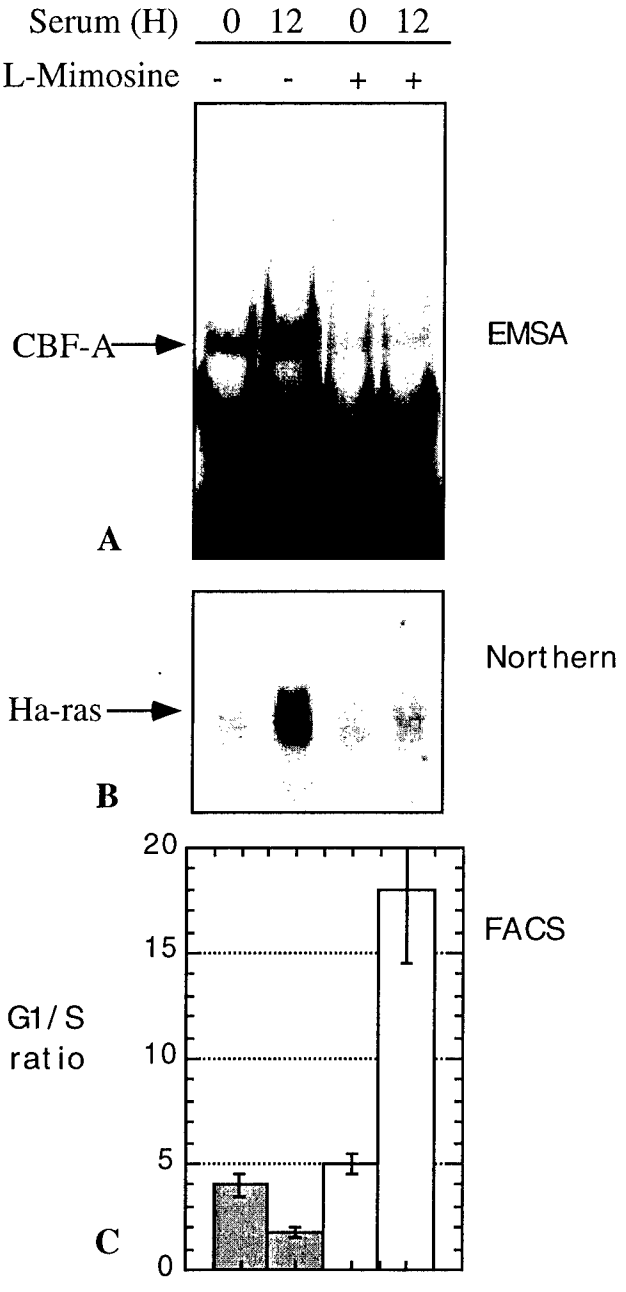


Figure 6


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

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

Reportable Outcomes

- A manuscript of the work presented in this report was submitted to the Journal of Biological Chemistry, and is prently being revised for resubmission

- Dr. Zarbl is a member of, 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 funds from the CEEH to generate CBF-A knockout mice within the CEEH facilities (see attached letter in appendix).

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.

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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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 5 Mar 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

DISTRIBUTION TO BE CHANGED TO UNLIMITED,
APPROVED FOR PUBLIC RELEASE

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