Estrogen Inducibility of c-Ha-ras Transcription in Breast Cancer Cells

IDENTIFICATION OF FUNCTIONAL ESTROGEN-RESPONSIVE TRANSCRIPTIONAL REGULATORY ELEMENTS IN EXON 1/INTRON 1 OF THE c-Ha-ras GENE*

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Although mutation of ras gene is rare in human breast cancer, overexpression of normal c-Ha-ras gene is frequently observed. Using a mouse mammary metastasis model consisting of genetically related mammary tumor sublines with variant metastatic potential, we have previously (i) demonstrated a direct correlation between c-Ha-ras mRNA and protein levels and metastatic potential and (ii) identified a novel hormone-responsive transcriptional regulatory element in intron 1 of the mouse c-Ha-ras gene that contains the consensus half-site of a glucocorticoid response element and flanking consensus half-sites for estrogen response element. Here, we have examined the functionality of intron 1 sequence in context of upstream sequences by using transient transfection assays with plasmids expressing chloramphenicol acetyltransferase. Intron 1 sequence and sequences similar to intron 1 element located in exon 1 function as transcriptional regulatory elements that confer hormonal inducibility to chloramphenicol acetyltransferase gene expression both independently and in context of 5′-flanking sequences. Measurement of c-Ha-ras transcription rates and protein expression by nuclear run-on and metabolic labeling assays showed a 5–12-fold enhancement, respectively, following treatment with 17β-estradiol that was blunted by ICI 182,780 in the non-metastatic variant. In contrast, constitutive overexpression of c-Ha-ras transcripts and protein in the metastatic subline was unaffected by estrogen and ICI 182,780. Gel shift assays demonstrated specific interaction of c-Ha-ras exon 1 sequence with nuclear proteins of human breast cancer MCF-7 cells with formation of two complexes, one of which contains estrogen receptor. Our data demonstrate a direct (i) interaction of c-Ha-ras sequence with estrogen receptor and (ii) stimulatory effect of estrogen on c-Ha-ras gene transcription and suggest that alteration in transcriptional regulation of c-Ha-ras gene by estrogen may play an important role in progression of breast cancer.

Ras proteins are responsible for regulating the flow of information that is triggered by diverse extracellular signals that stimulate their respective cell surface receptors. The relay of their signals via ras proteins ultimately regulate the activities of nuclear transcription factors that control the expression of key genes that regulate cell growth and differentiation (1). Thus, regulation of transcription of members of the ras gene family plays an important role in controlling cell growth. The ras genes are classified as “housekeeping genes,” which are expressed at relatively constant levels in all tissues and stages of development. Alteration in structure and expression of the ras gene family has been found in human tumors (2) as well as in animal tumor model systems (3–5). Although mutation of ras genes is rare in human breast cancers, 50% of human breast carcinomas express elevated levels of normal Ha-ras protein (6–8). Experimental induction of ras levels has been shown to lead to transformation of certain recipient cell types (9). Tumors that lack activated Ha-ras genes frequently show overexpression of ras proteins, perhaps a result of transcriptional deregulation of the ras gene (10).

The 5′ region of the murine Ha-ras gene is highly homologous with the 5′ upstream region of both the rat (11) and human (12) c-Ha-ras genes. The Ha-ras promoter regions for mouse, rat, and human are all very G-C rich and contain numerous repeats of the core consensus sequences for binding Sp1 transcription factor, lack TATA box, and contain a consensus sequence for the CAAT box. The cloned mouse Ha-ras upstream region has been shown to possess powerful transcriptional machinery when tested in transient gene expression assays in primary cultures of mouse epidermal cells (13). This high level of expression in transfected cells is surprising in view of the low level of expression of the endogenous mouse Ha-ras gene in normal mouse epidermis (14, 15). This suggests that transcription of c-Ha-ras is tightly regulated in normal tissues, which when lost or deregulated could result in constitutive overexpression of Ha-ras that is frequently observed in tumor tissues.

Estrogen plays a crucial role in cell growth and proliferation of reproductive tissues such as uterus and mammary gland (16, 17). Although the precise role of estrogen in the biology of breast carcinogenesis is not known, the effects of estrogen on proliferation of target breast cells are believed to be mediated through transactivation of specific genes that are recognized by estradiol-estrogen receptor (ER) complex. This process stimulates DNA synthesis, cell division, division, and proliferation.

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1 The abbreviations used are: E₂, estradiol; ER, estrogen receptor; ERE, estrogen response element; GRE, glucocorticoid response element; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PCR, polymerase chain reaction; bp, base pair(s); GAPDH, glyceraldehyde-phosphate dehydrogenase; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
and production of biologically active proteins, such as pS2, tumor growth factor-α, and epidermal growth factor (18), that influence cell growth and differentiation. Although a sequence motif, 5′-GGTCGA_TGACC-3′, having 92% homology to the vitellogenin estrogen response element (ERE) and differing from the consensus sequence by one base, is present in the Ha-ras gene at position −1420 (numbered according to Brown et al. (19)), no significant induction of transcription of reporter gene from the mouse Ha-ras promoter was observed in presence of estrogen in estrogen-dependent human breast cancer MCF-7 cells (13).

We have previously reported identification of a novel dexamethasone-responsive transcriptional enhancer element in the intron 1 of the mouse c-Ha-ras gene (20). This regulatory sequence contains the consensus half-site of a glucocorticoid response element (GRE) that is point mutated in a metastatic mammary tumor line and has three 5′-flanking ERE half-sites in its immediate vicinity. To investigate the relevance of sequences similar to ras intron 1 element in the hormonal regulation of Ha-ras gene expression, we carried out functional analysis of intron 1 in the presence of intron 0, a region that is conserved in mouse, rat, and human c-Ha-ras genes and previously demonstrated to possess strong transcriptional regulatory activity (21). Our data show that sequences similar to the ras intron 1 element are present both in exon 1 and intron 1 and confer strong estrogen and dexamethasone inducibility to CAT reporter gene expression independently or in context of 5′-flanking sequences. The presence of the naturally occurring point mutation in the consensus GRE half-site of the ras intron 1 element selectively eliminates inducibility by dexamethasone. Pretranslational control of Ha-ras synthesis by estrogen or dexamethasone could be exerted at transcriptional and/or posttranscriptional levels. To investigate the molecular sites of action of estrogen, we estimated the relative rates of Ha-ras gene transcription following treatment with 17β-estradiol in two genetically related mouse mammary tumor sublines with variant metastatic capacities. Our data show that E2 enhances Ha-ras gene transcription in the otherwise low ras-expressing nonmetastatic variant and has no effect on constitutive overexpression observed in the metastatic subline. The E2-mediated enhancement of Ha-ras synthesis is regulated at the transcriptional level and is not dependent on de novo protein synthesis. Gel shift assays demonstrated specific interaction of Ha-ras exon 1 sequence with nuclear proteins of human breast cancer MCF-7 cells with formation of two specific complexes, one of which contains estrogen receptor. This is the first report demonstrating direct (i) interaction of Ha-ras sequence with estrogen receptor and (ii) stimulatory effect of estrogen on Ha-ras gene transcription and suggests that alteration in transcriptional regulation of Ha-ras gene by estrogen may play an important role in progression of breast cancer.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—In this study we have utilized MCF-7 human breast cancer cell line and two mouse mammary tumor sister sublines that are highly tumorigenic but differ in their ability to complete specific steps of the metastatic cascade (22). Line 168FAR is nonmetastatic because of a defect in its ability to extravasate, whereas the related 4T1 subline is highly metastatic and metastasizes spontaneously from the orthotopic site to lungs and liver (22). 168FAR and 4T1 sublines are grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10% fetal calf serum as discussed above. Cells were fed with fresh phenol red-free medium 3 h prior to transfection with 10 μg of plasmid DNA per dish. Cells were transfected by the calcium phosphate procedure (25). One day prior to transfection, 5 × 105 cells were seeded in 60-mm dishes in phenol red-free medium supplemented with 10% fetal bovine serum or fetal calf serum as discussed above. Cells were fed with fresh phenol red-free medium 3 h prior to transfection with 10 μg of the appropriate recombinant or empty plasmid DNAs. When β-galactosidase expression was used as an internal control, 5 μg of plasmid pSVβ-gal (Promega Corp., Madison, WI) was added. Following 6 h of transfection, medium was removed, and cells were shocked with 10% glycerol. After washes with phosphate buffered saline, cells were grown in phenol red-free media supplemented with E2 (0.001–10 nM; Sigma), dexamethasone (0.01–1000 nM; Sigma), or vehicle (ethanol to final concentration of 0.01%, v/v). The specificity of the hormone-induced effects were tested by using the inhibitors ICI 182,780 (a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, Cheshire, UK) or RU486 (Center de Recherche Roussel-Uclaf, Romainville, France) at 100-fold molarity excess of E2 or dexamethasone, respectively. Cells were harvested 48 h later for measurement of CAT activity.

CAT assays were done as described by Gorman et al. (26). β-Galactosidase activity was measured according to the manufacturer’s directions. The hydrolyzed and nonhydrolyzed products were scraped from the TLC plates, and radioactivity was measured by scintillation counting. Results are expressed as fold induction produced by E2 or dexamethasone relative to control based on acetylated chloramphenicol formed by per unit protein or per unit β-galactosidase activity. There was no significant difference in induction when radioactivity was normalized by protein or β-galactosidase activity.

Preparation of cDNA Probes—c-Ha-ras, β-actin, and glyceraldehydephosphate dehydrogenase (GAPDH) cDNAs were amplified by reverse transcriptase-PCR from 2 μg of RNA prepared from normal mouse liver in the presence of 1 μM each of dATP, dCTP, dTTP, and dGTP, 5 mM phosphate, by including the inhibitors, ICI 182,780 (a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, Cheshire, UK) or RU486 (Center de Recherche Roussel-Uclaf, Romainville, France) at 100-fold molar excess of E2 or dexamethasone, respectively. Cells were harvested 48 h later for measurement of CAT activity.
59 °C for 2 min, and 72 °C for 3 min. PCR products were further characterized by restriction mapping and cDNAs purified from gel following separation by electrophoresis. 32P-Labeled probes for Ha-ras and GAPDH cDNAs were random primed (Roche Molecular Biochemicals) using [α-32P]dCTP to a specific activity of 1 x 10^8 cpm/nmol.

Transcriptional Run-on Analysis—Ha-ras mRNA transcription rate was measured by nuclear run-on assay (27). 1 x 10^6 168FAR or 4T1 cells were seeded in 75-cm2 culture flasks in phenol red-free medium as described above. When cells reached ~60% confluence, fresh medium containing the appropriate ligands were added for the indicated times: 1 mM E2 (2, 6, 12, or 24 h), 1 mM E2 + 100-fold molar excess of ICI 182,780 (2, 6, 12, or 24 h), or vehicle (0.01% ethanol, v/v; 6 h). Cells were lysed on ice in 10 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 10 mM NaCl, 0.5% Nonidet P-40, and 1 mM dithiothreitol, and nuclei were collected by centrifugation for 5 min at 500 x g. Run-on transcription was carried out at 30 °C for 30 min in reaction mixture containing 1 x 107 nuclei, 5 mM each of ATP, CTP, and GTP, and 100 μCi of [α-32P]uridine triphosphate (specific activity, 3000 Ci/mmol; NEN Life Science Products) in a final volume of 200 μl. After RNase-free DNase I (75 units) and proteinase K (200 μg/ml) digestions, the labeled reactions were extracted with phenol chloroform (v/v) and precipitated with ethanol. The RNA pellets were resuspended in 10 mMTES, pH 7.4, 1 mM EDTA, 100 μg/ml yeast tRNA, and 100 μg/ml denatured salmon sperm DNA and then hybridized with 5 x 10^6 cpm of 32P-labeled RNAs at 65 °C for 3 days in hybridization buffer. Membranes were extensively washed with increasing stringency and subjected to autoradiography. Several different exposure times were chosen to obtain densitometric scans in the linear response range of the x-ray film. The autoradiograms were analyzed by measurement of band densities by scanner-densitometer (model 300A, Molecular Dynamics, Sunnyvale, CA). Ha-ras band densities were expressed as a ratio to β-actin.

Measurement of mRNA Stability—168FAR cells were grown in phenol red-free medium as described above. Cells were treated with vehicle (0.01% ethanol, v/v), 1 mM E2, or a combination of 1 mM E2 and 100-fold molar excess of pure antiestrogenICI 182,780, for 6 h. Media were then removed, rinsed, and replaced with fresh medium or medium containing actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) and incubated for another 6 h. Total RNA was isolated and analyzed by slot blot method. 10 μg of total RNA was denatured by heating at 65 °C in 2.2 mM formamide and 0.5% Denhardt’s solution, 0.5% SDS, 100 μg/ml yeast RNA, and 100 μg/ml denatured salmon sperm DNA and then hybridized with 5 x 10^6 cpm of 32P-labeled RNAs at 65 °C for 3 days in hybridization buffer. Membranes were extensively washed with increasing stringency and subjected to autoradiography. Different shear exposure times were chosen to obtain densitometric scans in the linear response range of the x-ray film. The autoradiograms were analyzed by measurement of band densities by scanner-densitometer (model 300A, Molecular Dynamics, Sunnyvale, CA). Ha-ras band densities were expressed as a ratio to β-actin.

RESULTS

Estrogen- and Dexamethasone-responsive Transcriptional Regulation Sequences Are Located in Exon 1 and Intron 1 of the Mouse c-Ha-ras Gene—Previous studies have identified the presence of functional GREs and a putative ERE in the distal promoter region of the murine Ha-ras gene at nucleotides −1506, −1655, and −1420, respectively (13). Although the GREs conferred responsiveness to dexamethasone in transient reporter gene expression assays, the putative ERE failed to confer estrogen inducibility to the Ha-ras promoter in estrogen-dependent MCF-7 human breast cancer cells (13). We have previously reported identification of a transcription enhancing palindromic sequence in the intron 1 of the mouse c-Ha-ras gene that confers dexamethasone responsiveness to CAT reporter gene (20). In this study, we have examined the functional relevance of this sequence in the regulation of c-Ha-ras gene expression in two genetically related mouse mammary tumor subpopulations that differ in metastatic potentials (22) and Ha-ras mRNA and protein expression levels (30). We constructed reporter plasmids containing the minimal 30-bp ras intron 1 element (+191/+220) with (pmutras) and without (pmutras) the naturally occurring point mutation (A912G at nucleotide 210). A reporter plasmid containing the entire intron 1 was also constructed (Fig. 1). All these sequences were ligated upstream of the −37 TK promoter, which in turn was linked to the bacterial CAT gene and the SV-40 poly(A) signal.

When pmutras was transfected into 168FAR cells, a 20-fold centrifugation and washed four times in lysis buffer. Bound proteins were solubilized in SDS buffer and subjected to SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels. After electrophoretic separation, gels were processed for fluorography and subjected to autoradiography at 70 °C. Amounts of c-Ha-ras protein bands were quantitated with a scanner-densitometer described above.

Gel Retardation Assay—Nuclear extracts were made by the procedure of Dignam et al. (28) from estrogen-dependent MCF-7 human breast cancer cells. Protein concentration was determined by the method of Bradford (Bradford protein assay; Bio-Rad). Three pairs of complimentary oligonucleotides were used for DNA binding assays: (i) 5′-ATCTAGTATGCTATCCAGGCTGAGG-3′ (wild type), (ii) 5′-ATCTAGTATGCTATCCAGGCTGAGG-3′, which contains the palindromic sequence (underlined region) between nucleotides 191 and 220 of the mouse c-Ha-ras gene; (iii) exon 1 sequence, 5′-GTCGCGTACCCTGCTGCAAGGACATCCTGACGACAGC-3′, which contains between nucleotides 1713 and 1742 of the human c-Ha-ras gene (23); and (iii) NF-1, 5′-TGTTTTCACTGCATGCTCAGGAGG-3′, a putative NF-1 binding element occurring in hepatitis B virus enhancer region (nucleotides 1290–1238; Ref. 29) to test specificity of binding. This oligonucleotide was chosen because the underlined sequence has 80% homology to the palindrome in the ras wild type intron-1. The oligonucleotides were annealed and end-labeled with [γ-32P]ATP using polynucleotide kinase. Binding reactions were carried out in 20 μl containing 20,000–25,000 cpm (0.5 ng) of 32P-labeled double-stranded oligonucleotides and 10–100 μg of nuclear matrix fraction (nuclei from wild type mouse Ha-ras intron 1 element or NF-1 sequence) were added together with the labeled human exon 1 probe. For supershift experiments, nuclear extracts were preincubated for 30 min in binding buffer with 0.5 μg of ER antibodies or equivalent amounts of rat or mouse preimmune IgG before the addition of the labeled double-stranded oligonucleotide. The following ER antibodies were used: rat monoclonal ER antibody H222 and D75 (a generous gift from Dr. Geoffrey Greer) and mouse monoclonal ER antibody clone 33 (Affinity BioReagents, Neshanic Station, NJ). The gels were dried and autoradiographed with intensifying screens at −80 °C.
induction of CAT activity over that of control transfected with the empty vector, p-37TKCAT was observed (Table I). Because the \textit{ras} intron 1 element contains the motif, TGATCC, which is identical to the half-site of GRE found in the chicken vitellogenin gene (31) and bears homology to the consensus ERE half-site, TGACC (32), we challenged \textit{pmutras}-transfected 168FAR cells with dexamethasone or E\textsubscript{2}. Treatment of \textit{pmutras}-transfected 168FAR cells with 0.1 or 1 \textmu{}m dexamethasone significantly induced CAT activity \textasciitilde{}3- and 4-fold, respectively, over cultures treated with vehicle (Table I). In contrast, cells transfected with \textit{pmutras} were not induced by similar concentrations of dexamethasone (Table I). These data suggest that the naturally occurring point mutation at base 210 that disrupts palindromic symmetry of the 10-bp sequence in \textit{ras} intron 1 selectively abolishes induction of reporter gene expression by dexamethasone (Table I and Fig. 1). When 168FAR cells transfected with \textit{pmutras} or \textit{pmutras} were challenged with E\textsubscript{2}, there was no induction of CAT activity, indicating that minimal \textit{ras} intron 1 element confers only dexamethasone inducibility (Table I). The observed induction of CAT activity was specific for dexamethasone because inclusion of 100-fold molar excess of RU486 blocks dexamethasone-induced responses (Table I).

Examination of exon 1 and intron 1 of the mouse c-Ha-	extit{ras} gene revealed the presence of one consensus ERE half-site, TGACC, at bases 55 in exon 1, and two consensus GRE half-sites at bases 150 (GTTGCA) and 163 (TGACC) in intron 1. The latter two ERE half-sites probably constitute a complete ERE sequence and differs from the consensus ERE (GTTGCANNTGACC) by having five extra bases in the spacer separating the two half-sites. Two GRE half-site motifs (TGATCC) by having five extra bases in the spacer separating the two ERE half-sites probably constitute a complete ERE sequence located at bases 150 (GGTCA) and 163 (TGACC) in intron 1. The latter revealed the presence of one consensus ERE half-site, TGACC (32), we challenged \textit{pmutras}-transfected 168FAR cells with dexamethasone caused a dose-dependent induction of CAT activity that was \textasciitilde{}4-fold higher with 1 \textmu{}m dexamethasone than vehicle-treated cells (Fig. 2B). However, similar exposure of mutant \textit{pQras}-transfected 168FAR cells to dexamethasone failed to stimulate an increase in CAT gene expression (Fig. 2B). The regulatory effects of E\textsubscript{2} and dexamethasone on CAT gene expression were completely abolished with the estrogen antagonist ICI 182,780 and glucocorticoid antagonist, RU486, respectively (Fig. 2).

**Estrogen- and Glucocorticoid-inducible Motifs Located in Exon 1 and Intron 1 of the Murine c-Ha-ras Gene Are Functional in Context with the 5′-Flanking Sequences**—Because the results of \textit{pQras} transfection showed the presence of estrogen and glucocorticoid-mediated transcriprional activation of the CAT reporter gene, it was of interest to determine whether the ERE and GRE half-sites identified in exon 1 and intron 1 of the Ha-ras gene can support hormone-induced transcription when placed in the context of 5′ upstream sequences. Reporter constructs (\textit{pQras}, –686/+355; \textit{pQras}, –545/+555; \textit{pQras}, –246/+355; Fig. 1) containing the entire intron 1 but lacking various lengths of proximal upstream sequences were transfected into 168FAR or 4T1 cells and treated with E\textsubscript{2},

**Table I**

| Construct | Conversion |
|-----------|------------|
| \textit{pQras} | 0.6 |
| \textit{mutras} | 11.1 |
| + 10\textsuperscript{−11} M E\textsubscript{2} | 13.8 |
| + 10\textsuperscript{−10} M E\textsubscript{2} | 14.8 |
| + 10\textsuperscript{−9} M E\textsubscript{2} | 15.1 |
| + 10\textsuperscript{−8} M E\textsubscript{2} | 12.8 |
| + 10\textsuperscript{−7} M DEX | 15.2 |
| + 10\textsuperscript{−6} M DEX | 19.7 |
| + 10\textsuperscript{−5} M DEX | 26.2 |
| + 10\textsuperscript{−4} M DEX | 37.1 |
| + 10\textsuperscript{−3} M DEX | 47.8 |
| \textit{mutras} | 5.1 |
| + 10\textsuperscript{−10} M E\textsubscript{2} | 7.0 |
| + 10\textsuperscript{−9} M E\textsubscript{2} | 5.9 |
| + 10\textsuperscript{−8} M E\textsubscript{2} | 6.8 |
| + 10\textsuperscript{−7} M DEX | 4.9 |
| + 10\textsuperscript{−6} M DEX | 6.3 |
| + 10\textsuperscript{−5} M DEX | 7.5 |
| + 10\textsuperscript{−4} M DEX | 5.2 |
| + 10\textsuperscript{−3} M DEX | 7.0 |

\textsuperscript{a} Significant increase in percentage of conversion over control cultures treated with vehicle (0.01% ethanol, v/v). Co-transfection with pSVβ-gal, which expresses β-galactosidase activity at a constant level regardless of E\textsubscript{2} or dexamethasone (DEX) concentration, confirmed that the results are not due to differences in transfection efficiency or cell viability (data not shown).

**Fig. 1.** Schematic representation of the 5′-flanking region, first coding exon, and intron 1 of the mouse c-Ha-ras gene. The numbering represents the distance in base pairs from the A of the initiation codon (19). The position of the various sequence motifs, SV40 core enhancer (○), AP1/ATF (□), ERE (●), and GRE (●) half-sites are indicated. Also shown is the sequence for the 30-bp ras intron 1 element containing the consensus GRE half-site (underlined) within the 10-bp palindromic. Presence of the naturally occurring point mutation resulting in A→G substitution at base 210 is indicated by *. Position of primers Y, Z, W, P, and Q that were used for amplification and construction of \textit{pQras}, \textit{pQras}, \textit{pQras}, \textit{pQras}, and \textit{pQras}-TK-CAT reporter plasmids are also shown.
porter construct and treated for 40 h with 17β-estradiol or dexamethasone (DEX) over a concentration range of 0.1–10 nM or 0.1 and 1 μM, respectively. Specificity of E2 or dexamethasone-mediated effects on CAT gene activation was tested by including 100-fold molar excess of ICI 182,780 or RU-486 (RU), respectively. Control cultures were exposed to vehicle (0.01% ethanol, v/v). Because no changes in CAT gene expression were observed in ~37TK-CAT-transfected cells exposed to steroid hormones as compared with untreated, the results are grouped together. Results obtained from three transfections are expressed as the means ± S.D. Co-transfection with pSVβ-gal that expresses β-galactosidase activity at a constant level regardless of E2 or DEX concentration confirmed that the results were not due to differences in transfection efficiency or cell viability (data not shown).

FIG. 3. Regulation of CAT gene expression by 17β-estradiol or dexamethasone in phrasexon1-TK-CAT-transfected human breast cancer MCF-7 cells. Cells grown in phenol red-free medium were transfected with the appropriate reporter construct and treated for 40 h with 17β-E2 or dexamethasone (DEX) over a concentration range of 0.1–10 nM or 0.1 and 1 μM, respectively. Specificity of E2 or dexamethasone-mediated effects on CAT gene expression and regulation by estrogen and dexamethasone in MCF-7 cells are similar to those observed in 168FAR cells and hence are not shown.

Sequences in Exon 1 of the Human c-Ha-ras Gene Bearing Homology to the Mouse Ha-ras Intron 1 Element Mediate Estragon and Glucocorticoid-responsive Transcriptional Enhancer Activity—To determine whether the sequence in exon 1 of the Ha-ras gene possesses transcriptional regulatory activity similar to the intron 1 element with which it bears 80% homology, we constructed reporter plasmid containing the 30-bp h-ras exon 1 sequence linked to the bacterial CAT gene and the SV-40 poly(A) signal.

When ph-rasexon1-TK-CAT was transfected into estrogen-dependent ER-positive human breast cancer MCF-7 cells, a 15-fold induction of CAT activity over that of control transfected with the empty vector, p-37TKCAT was observed (Fig. 3B). Because the ras exon 1 sequence contains the motifs, TGATCC (which is identical to the half-site of GRE found in the chicken vitellogenin gene; Ref. 31) and TGACC (which is identical to the half-site of GRE found in the chicken vitellogenin gene; Ref. 31), we exposed ph-rasexon1-TK-CAT-transfected MCF-7 cells with dexamethasone or E2. Treatment of ph-rasexon1-TK-CAT-transfected MCF-7 cells with 0.1 or 1 μM dexamethasone significantly induced CAT activity ~2- and 4-fold, respectively, over cultures treated with vehicle (Fig. 3). When MCF-7 cells transfected with ph-rasexon1-TK-CAT were challenged with E2, a 3–5-fold induction of CAT activity was observed with 1 and 10 nM E2, respectively (Fig. 3). The observed induction of CAT activity induced from pYQras, pZQras, or pWQras under the influence of E2 or dexamethasone is similar to that in cells transfected with the pPQras construct, these results indicate that activities of functional estrogen and glucocorticoid-responsive motifs in exon 1 and intron 1 of the murine c-Ha-ras gene are uninfluenced by 5′-flanking sequences. Results of CAT gene expression and regulation by estrogen and dexamethasone in 4T1 cells are similar to those observed in 168FAR cells and hence are not shown.
activity was specific for dexamethasone or E2 because inclusion of 100-fold molar excess of RU486 or ICI 182,780 blocked dexamethasone- or E2-induced responses, respectively (Fig. 3).

**Fig. 4. In vitro binding activity of MCF-7 nuclear proteins to human c-Ha-ras exon 1 sequence.** Double-stranded oligonucleotides corresponding to the human c-Ha-ras exon 1 sequence was 32P-labeled and incubated for 30 min with MCF-7 nuclear extract. Complexes were separated on 7% non-denaturing polyacrylamide gel. The positions of specific retardations are indicated as A, B, or C. NS indicates nonspecific complex. Panel A, MCF-7 nuclear proteins (2.5 μg) were incubated with labeled c-Ha-ras exon 1 sequence. 10-, 50-, or 100-fold excess of nonradioactive Ha-ras exon 1 or mouse ras intron 1 element were included as competitors with the labeled probe when indicated. 50-, 100-, or 250-fold excess of nonradioactive NF-1 were included with the probe to determine the specificity of complexes formed. Panel B, MCF-7 nuclear extracts containing 2.5 μg of proteins were incubated for 30 min at room temperature with 0.5 μg of ER antibodies, H222 (lane 5), D75 (lane 6), or clone 33 (lane 7), in the binding reaction mixture before addition of labeled c-Ha-ras exon 1 probe. Equivalent amounts of rat (lane 2) or mouse (lane 3) preimmune IgG were added as control. Lane 1 represents binding of c-Ha-ras exon 1 to MCF-7 nuclear proteins, and lane 4 represents nuclear extracts incubated with ER antibody H222 prior to the addition of labeled c-Ha-ras exon 1 and 50-fold excess of nonradioactive c-Ha-ras exon 1. A* shows the position of complex A retarded by the presence of H222 ER antibody (lane 5), and C indicates the position of the faster migrating band resulting from addition of ER antibodies D75 (lane 6) or clone 33 (lane 7). Note the concomitant disappearance of complex A and appearance of complex C in lanes 6 and 7 and supershifting of complex A (A*) in lane 5.

DNA-Protein Complexes Formed with Ha-ras Exon 1 Transcriptional Regulatory Sequence Contain Estrogen Receptor—Because the Ha-ras exon 1 sequence bears homology to the mouse ras intron 1 element and confers transcriptional enhancer activity to CAT gene expression in MCF-7 cells, we examined the ability of nuclear proteins from MCF-7 cells to bind the human Ha-ras exon 1 motif in electrophoretic mobility shift assays. Fig. 4A shows that when labeled human Ha-ras exon 1 motif was used as a probe, two DNA-protein complexes (indicated as complexes A and B) are observed that are specifically competed with 10–100 fold molar excess of unlabeled human Ha-ras exon 1 motif (third, fourth, and fifth lanes). Inclusion of unlabeled mouse ras intron 1 element caused efficient blocking of complex B formed with labeled human Ha-ras exon 1 motif, whereas a substantial amount of complex A remained even after adding 100-fold molar excess of the unlabeled ras intron 1 element (Fig. 4A, sixth, seventh, and eighth lanes). These results suggest that protein(s) involved in complex B formation have similar affinities for the exon 1 motif and intron 1 element. Both complexes are specific as neither of them are competed with 50–250-fold excess (Fig. 4A, ninth, tenth, and eleventh lanes) of NF-1 (putative NF-1 binding element occurring in the hepatitis B virus enhancer region; Ref. 29), although this sequence has 80% homology to the 10-bp palindrome in the ras intron 1 element.

Because the human Ha-ras exon 1 motif conferred estrogen responsiveness to CAT gene expression in estrogen-dependent MCF-7 cells, we examined whether the complexes formed with Ha-ras exon 1 motif contained estrogen receptor. As shown in Fig. 4B, incubation with monoclonal ER antibodies, clone 33 (Affinity BioReagents) or D75 with epitopes directed to the DNA-binding domain of ER, selectively inhibited complex A formation with resultant appearance of a faster migrating band (complex C) that displayed enhanced DNA binding activity (lanes 6 and 7). Incubation of binding reaction with the ER antibody H222 with epitope directed to the hormone binding domain of ER caused selective supershifting of complex A (Fig. 4B, lane 5), whereas inclusion of rat or mouse preimmune IgG had no influence on both complexes (Fig. 4B, lanes 2 and 3, respectively). These results indicate that the complex A formed by the human Ha-ras exon 1-motif contains ER. It is interesting to note that competition assays demonstrated similar affinities of exon 1 motif and intron 1 element for protein(s) in complex B and markedly reduced affinity of ER and/or other protein(s) constituting complex A for the ras intron 1 element. These data are consistent with the observation that transcriptional enhancer activity mediated by potras construct that contains the minimal 30-bp ras intron 1 element is unaffected by estradiol (Table 1).

**Estrogen Regulates Transcription of c-Ha-ras Gene in Nonmetastatic Mammary Tumor Subline.—**Because our data from Fig. 2 demonstrate the presence of sequences in exon 1 and/or intron 1 of the murine Ha-ras gene that are capable of conferring strong estrogen inducibility to the CAT reporter gene, we tested whether estrogen can directly influence transcription of the murine c-Ha-ras gene in metastatic and nonmetastatic mammary tumor variants. In vitro transcript elongation or nuclear run-on assays were performed using purified nuclei prepared from 168FAR and 4T1 cells treated with vehicle (0.01% ethanol, v/v) or E2. When the amount of 32P-labeled nascent RNA complementary to Ha-ras cDNA was compared with that hybridizing with β-actin cDNA, the transcription rate of c-Ha-ras gene in 168FAR cells was more than doubled (relative to control) by 2 h after treatment with 1 nM E2 (Fig. 5). Transcriptional activity increased to about five times control (vehicle-treated cells) by 6 h (Fig. 5). Levels of Ha-ras transcription decreased gradually but remained elevated at 12 h and returned to control levels by 24 h of treatment with E2 (Fig. 5). Inclusion of ICI 182,780 significantly blocked the E2-induced increase in c-Ha-ras gene transcription confirming that E2-mediated induction of Ha-ras transcription in nonmetastatic 168FAR cells resulted via the E2-ER pathway. The Ha-ras increase was not associated with changes in transcription rates of β-actin. A similar analysis of Ha-ras transcription rates in metastatic 4T1 cells showed the presence of ~20-fold higher basal levels of Ha-ras transcripts in control 4T1 cells as compared with control nonmetastatic 168FAR cells (Fig. 5). However, unlike 168FAR cells where E2 exerts a stimulatory effect on the kinetics of Ha-ras transcription, estrogen failed to significantly influence the high basal levels of Ha-ras transcription observed in 4T1 cells (Fig. 5). Addition of the pure antiestrogen ICI 182,780 had no effect on Ha-ras transcription.
confirming the absence of E₂/ER-mediated effects on Ha-ras transcription in 4T1 cells (Fig. 5). These data imply major differences in transcriptional regulation of the Ha-ras gene in nonmetastatic 168FAR and metastatic 4T1 mammary tumor cells; nuclear transcript elongation (run-on) assay. Cells grown in phenol red-free medium were incubated with medium in the presence of vehicle (0.01% ethanol, v/v), 1 nM E₂, or a combination of 1 nM E₂ plus 100-fold molar excess of estrogen antagonist ICI 182,780. Treatment with E₂ was carried out for 2, 6, 12, or 24 h. Cultures were exposed to vehicle (control) or inhibitor for 6 h. Nuclei were isolated at the indicated time points, and nuclear run-on reactions were performed as described under “Experimental Procedures.” γ²P-Labeled nascent RNAs isolated from run-on reactions were hybridized to immobilized probes on the membrane. c-Ha-ras band densities were quantified by densitometry and expressed as a ratio to β-actin; the relative transcriptional rates of c-Ha-ras gene in 168FAR (filled bar) and 4T1 (shaded bar) cells are shown graphically in panel B.

**FIG. 5. Effect of 17β-estradiol on c-Ha-ras gene transcription in nonmetastatic 168FAR and metastatic 4T1 mammary tumor cells; nuclear transcript elongation (run-on) assay.** Cells grown in phenol red-free medium were incubated with medium in the presence of vehicle (0.01% ethanol, v/v), 1 nM E₂, or a combination of 1 nM E₂ plus 100-fold molar excess of ICI 182,780 (lanes 2 and 3), or addition of estrogen antagonist effectively blocked the estrogen-mediated increase in Ha-ras protein levels (Fig. 6, lane 4). In contrast to estrogen-inducible expression of Ha-ras protein in 168FAR cells, similar analysis of Ha-ras protein in the metastatic 4T1 subline revealed ~7-fold higher levels of Ha-ras protein relative to control 168FAR cells and demonstrated a lack of modulation by E₂ or ICI 182,780 (Fig. 6, lanes 2’–4’).

**Up-regulation of c-Ha-ras Gene Expression by Estrogen and Modulation by Actinomycin D—Results of Fig. 5 show that E₂ directly influences the rate of Ha-ras transcription in nonmetastatic 168FAR cells. To further characterize the effects of estrogen on Ha-ras mRNA stability, 168FAR cells were treated with vehicle or 1 nM E₂ for 6 h, and Ha-ras mRNA levels were measured 6 h later following exposure to actinomycin D or cycloheximide. Although, E₂ elicited only a 1.5-fold increase in steady-state level of Ha-ras mRNA over vehicle-treated control cultures (Fig. 7, second and third lanes), this difference was greatly enhanced to ~7-fold upon addition of a 100-fold molar excess of estrogen antagonist ICI 182,780 (Fig. 7, compare second and seventh lanes). Because addition of ICI 182,780 reduced Ha-ras mRNA levels to ~75% of that observed in vehicle-treated cells (Fig. 7, first and seventh lanes), these data imply that a portion of Ha-ras mRNA expressed in control cultures may be a result of stimulation by contaminating estrogen in the culture medium.

To determine whether the estrogen effect on Ha-ras expression was at the transcriptional level, E₂-treated cells were cultured with the transcriptional inhibitor actinomycin D at a concentration of 5 μg/ml. Levels of Ha-ras mRNA dropped ~8-fold in cultures exposed to actinomycin D when compared with corresponding E₂-treated cultures (Fig. 7, second and third lanes). Similarly, actinomycin D repressed the basal expression of Ha-ras mRNA (Fig. 7, fifth lane). These data suggest that Ha-ras mRNA expression by E₂ is transcriptionally regulated. We next tested the effect of cycloheximide on the maintenance of E₂-induced increase in Ha-ras mRNA levels in 168FAR cells. When 168FAR cells were cultured in the presence of cycloheximide (10 μg/ml) after exposure to E₂, no difference in levels of Ha-ras mRNA was observed when ratios of Ha-ras to GAPDH mRNAs were compared between cultures exposed to E₂ alone or in the presence of cycloheximide (Fig. 7, second and fourth lane). Similarly, cycloheximide alone had no...
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Effect on base-line Ha-ras mRNA levels (Fig. 7, compare first and sixth lanes), suggesting that de novo protein synthesis is not required for E2-dependent Ha-ras mRNA induction.

DISCUSSION

Ha-ras genes are rendered oncogenic either by mutation (23, 34–36) or by overexpression (37). Using a mouse mammary tumor model consisting of genetically related sister sublines with variant metastatic capacities, we have previously shown a direct correlation between metastatic behavior and expression levels of normal Ha-ras mRNA and protein (30). Although ras mutations are infrequent, occurring only in about 5% of human breast cancers, there is considerable evidence to suggest that the pathways regulated by ras are deregulated in breast cancer cells (38). Elevated levels of normal Ha-ras have been shown to play a crucial role in tumorigenesis. 50% of human breast carcinomas express elevated levels of Ha-ras (6–8). Thus, it is possible that the aberrant function of ras or ras related proteins may contribute to breast cancer development and/or progression. Overexpression of Ha-ras gene has been postulated to result from transcriptional deregulation (10). This is the first report that demonstrates existence of estrogen-mediated regulation of Ha-ras transcription in mammary tumor cells.

We have previously identified the presence of a glucocorticoid-responsive transcription enhancing element in intron 1 of the mouse Ha-ras gene. This transcriptional regulatory element is a palindromic sequence that contains the consensus GRE half-site (20). Identification of this intron 1 regulatory element has been facilitated by presence of a point mutation at the 3' end of the palindrome in the Ha-ras gene of the metastatic 4T1 subline (20). Although we do not yet know the exact role of this point mutation in regulation of Ha-ras gene expression, our data from transient reporter assays utilizing constructs containing only the ras intron 1 element or entire intron 1 show that the presence of this point mutation selectively abolishes transcriptional regulation by dexamethasone.

Previous functional analyses of murine and human Ha-ras genes have been limited to the promoter and intron 0 regions. The 5' region of the Ha-ras gene is highly conserved in the mouse, rat, and human (11, 12, 19). In the mouse c-Ha-ras gene, the intron 0 has been shown to contain a positive regulatory element (21). Although oncogenic ras enhancer sequences were detected within the intron 0 of the mouse Ha-ras gene, a 12-bp motif closely matching the SV40 enhancer core element was found at position -620 (19). Within this region several copies of a sequence motif that is similar to the elements that bind AP1 and ATF are found (19). These features imply that intron 0 of the mouse Ha-ras gene may play an important part in transcriptional regulation of Ha-ras. However, our results from reporter expression assays indicate that sequences in intron 0 are not responsible for hormone-mediated transcriptional enhancer activity, because the reporter plasmid pPQras does not contain this region, yet demonstrates similar levels of E2- and dexamethasone-induced CAT activities as do pYQras, pQras, or pWQras reporter plasmids that do contain intron 0. Our data show that intron 1 may play a significant role in hormone-mediated transcriptional regulation of Ha-ras gene because it can function independently or in the context of 5' upstream sequences. The importance of ras intron 1 element in regulation of Ha-ras gene expression is further strengthened by the fact that similar sequences are found in the mouse, rat, and human Ha-ras genes. Sequences bearing 100% (TGATCC-TGATCCATCA, +373/+388) and 80% (TGATCCATGC, +1535/+1544 and +1599/+1608) identity to the ras intron 1 element are located in introns 1 and 3, respectively, of the rat Ha-ras gene (39). Two motifs bearing 80% identity to the 10-bp palindrome of ras intron 1 element are located in exon 1 of the mouse, rat, and human c-Ha-ras genes. Motif 1 (TGACCATC/TGATCCATCA, 1719/1727, Ref. 23) contains a putative ERE half-site at bases 1719–1723, and motif 2 (TGATCCAAAG, 1731/1740; Ref. 23) contains a GRE half-site at bases 1731–1736 (19). Although the functional significance of this conserved sequence in regulation of human Ha-ras gene expression is yet not established, our data indicate that this sequence not only has transcriptional enhancer activity but is also capable of conferring estrogen responsiveness to CAT reporter gene expression. This is supported by demonstration of its ability to physically interact with ER in nuclear extracts of MCF-7 human breast cancer cells by gel retardation assays. Because the pPQras reporter plasmid includes the portion of exon 1 containing this sequence, estrogen-inducible transcriptional enhancer activity resulting from pPQras plasmid may be contributed at least in part by these hormonal response half-sites. Also, because the presence of a point mutation in intron 1 element selectively abolishes responsiveness to dexamethasone while maintaining estrogen regulation, sequences in ras intron 1 element, and not in exon 1, are required for dexamethasone-mediated stimulation of CAT activity.

Estrogen is known to be a key requirement for the normal development of the mammary gland. Although some reports have shown estrogen to affect Ha-ras expression (40–46), there are reports contradicting such effects (47). Our data clearly indicate major differences in estrogen-mediated regulation of Ha-ras gene transcription in two genetically related mammary tumor sublines; thus the demonstration of a direct effect or lack thereof appears to be influenced by cell type. Regression of MCF-7 tumors in nude mice following estrogen ablation has been shown to be accompanied by a decrease in expression of c-Ha-ras, c-fos, and pS2 (48). Similarly, acquisition of an activated Ha-ras gene has been shown to confer hormone autonomy on the previously estrogen-dependent tumorigenicity of MCF-7 cells and causes up-regulation in secretion of growth factors in amounts that are comparable with estradiol stimulation (49). Kumar et al. (50) have shown the presence of Ha-ras oncogenes (H and K) in normal mammary glands of pubescent animals 2 weeks after nitrosoarine thylurea treatment, where they remain latent until exposure to estrogen occurs during sexual maturation. These studies demonstrate that the presence of ras oncogenes in the mammary gland of young animals is not sufficient to trigger neoplastic development but rather that the ras oncogenes need to cooperate with physiological processes required for sexual maturation to exert their neoplastic properties (50).

The effects of estrogen on Ha-ras gene expression have not been examined in detail. Sequence motifs that have 92 and 50%
homology to the vitellogenin ERE have been identified in the promoters of Ha-ras gene of the mouse (13) and human (12), respectively; however, this putative ERE failed to confer estrogen inducibility to the Ha-ras promoter (13). Our results from transient CAT gene expression assays clearly show that ERE half-sites (+58/+62, +150/+154, and +163/+167) present in pQras and ph-ras exon 1 constructs are functional and respond to physiological concentrations of E_2 via ER as these responses are blocked by the pure antiestrogen, ICI 182,780. Although these sequences do not contain a complete ERE, ERE half-sites separated from each other by more than 100 bp have been shown to confer estrogen inducibility by acting synergistically on the proximal ovalbumin gene promoter or heterologous promoters (33). It is interesting to note that both introns 1 and 3 of the rat Ha-ras gene also contain ERE half-sites in the immediate vicinity of the sequence resembling the mouse ras intron 1 element.

To determine the primary site at which E_2 acts to control expression of Ha-ras gene, we measured transcription rates by nuclear run-on assays. A comparative analysis of Ha-ras transcription in nonmetastatic and metastatic mammary tumor sublines revealed fundamental differences not only in basal transcription rates but also in their ability to be modulated by estrogen. Estrogen enhances Ha-ras gene expression in nonmetastatic 168FAR cells at transcriptional levels, which is not dependent on new protein synthesis. Although both sublines express ER, expression of Ha-ras mRNA and protein is unaffected by estrogen or its antagonist in the metastatic subline. In the treatment of ER-positive breast cancers, intrinsic or secondary hormone resistance is a major clinical problem (51). This is reflected both in treatment of advanced disease, where the development of resistance to tamoxifen and other endocrine agents is inevitable, and in the adjuvant setting, where tamoxifen prevents some but not all relapses. A molecular understanding of this phenomenon is important to help identify those tumors that despite being ER+ are intrinsically endocrine-resistant. The fundamental role of Ha-ras in signal transduction pathways argues that processes that cause deregulation of Ha-ras gene expression may play an important role in breast cancer development and progression. Alteration(s) in transcriptional regulation of Ha-ras gene resulting in constitutive overexpression of Ha-ras may represent one such mechanism of antiestrogenic resistance. Overexpression of ras proteins has been associated with resistance to chemotherapeutic agents and radiation (52–54). NIH3T3 cells transfected with normal or mutant c-Ha-ras oncogene are significantly more resistant to chemotherapeutic agents than normal cells (52). Although the detailed mechanisms responsible for these resistance phenenoma are not entirely clear, accumulating evidence indicate that ras-induced transactivation of AP-1 proteins, c-jun, c-fos, and Fra-1 (55, 56) may play a role. Experiments are in progress to correlate Ha-ras gene transcription with ER status, AP-1 levels, activity, composition, and regulation by estrogen and antiestrogens in human breast tumors.

Our data from transient in vitro assays have revealed the presence of estrogen-responsive elements in exon 1/intron 1 of the Ha-ras gene. However, definition of the exact role of exon 1/intron 1 motifs in in vivo regulation of the endogenous gene by estrogen requires a complete mapping of the Ha-ras gene by DNase I hypersensitivity or genome footprinting assays. Because sequences bearing similarity to the exon 1/intron 1 motifs are present elsewhere in the gene, it is possible that these regulatory elements may function independently or in concert with those identified in exon 1/intron 1. However, it is important to note that in vivo regulation of Ha-ras gene expression by estrogen may be more complex and may involve other regulatory elements and/or transcription activating factors. In conclusion, our data suggest that alterations in transcriptional regulation of the Ha-ras gene by estrogen may play an important role in progression of breast cancer.
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