# Binding of the glucocorticoid and estrogen receptors to the human H-*ras* oncogene sequences

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**Abstract.** There is evidence that hormone regulation of cellular oncogenes plays an essential role in human cancer. The c-H-*ras* gene is implicated through both mutation and abnormal gene expression in many types of human cancer. Computer scanning of this gene has revealed two putative hormone response motifs: A possible Glucocorticoid Response Element (GRE) at position 1261 of the first intron of the H-*ras*1 gene and a putative Estrogen Response Element (ERE), at position 3007 of the fourth intron of the gene. In DNA binding assays, using the HeLa and LATK$^-$ cell lines, we showed specific binding of the corresponding receptors at both putative H-*ras* glucocorticoid and estrogen response sequences, suggesting that hormones could be contributing to H-*ras* transcriptional regulation through interaction with their corresponding Hormone Response Elements (HREs).

## Introduction

Nuclear receptors, such as those for steroid/thyroid hormones, vitamin D, and retinoic acid, are known to act as gene regulators, controlling the transcription of target genes by binding to *cis*-acting DNA elements referred to as hormone responsive elements (HREs) (reviewed in ref. 1). Negative HREs, which repress expression of specific genes, have been characterized (2,3), however, the mechanism of their action is still poorly understood. Consensus steroid hormone REs correspond to palindromic sequences (4,5), however, either imperfect palindromic HREs (6,7) or their interference with other *cis*-acting regulatory elements (8,9) or factors (10), may also generate efficient HREs.

Glucocorticoids regulate transcription of a number of genes by the direct interaction of the hormone receptor complex with GREs. GREs contain an imperfect palindromic consensus sequence 5'-GGTACANNNTGTTCT-3', (1) with highly conserved TGTTCT oligonucleotide and quite variable upstream hexanucleotide (11,12). Interference between the glucocorticoid receptor and other transcription factors has also been shown (13,14). The hormone response elements in the conserved 5'-AGGTCANNNTGACCT-3' sequence is the ERE consensus oligonucleotide (5). Additionally, 5'-TGACCT-3' half palindromic oligonucleotides may act synergistically conferring estrogen response to a gene, even at a long distance from the gene promoter (15,16), possibly through estrogen receptor-associated proteins (17). GREs and EREs have been identified and characterized in a number of cellular genes (18-21), as well as in viral regulatory regions (22-25), but little is known about the presense of glucocorticoid and estrogen response elements in cellular oncogenes.

The H-*ras* gene, a member of the *ras* family of proto-oncogenes, is readily implicated in many types of human cancer both through mutation (26) and abnormal gene expression and is involved in multiple signal transduction pathways, under normal cellular growth as well (27). The fact that overexpression of *ras* genes may cause oncogenic transformation (28,29) and that expression of the normal H-*ras* gene can act as an onco-supressor (30), indicate the importance of understanding the way *ras* genes are regulated. Elevated expression of the *ras* genes has also been found in a variety of human tumors (for a review see ref. 31).

Considering the possible role of hormonal steroids as tumor promoters (32), we computer scanned the human c-H-*ras*1 sequence for potential hormone response elements. We observed a putative GRE motif with 100% similarity in the half palindrome consensus oligonucleotide at position 1261 (GGCCAGACCTGTTCT) of the conserved 3' end of the first intron (33) and a possible ERE motif, with 90% similarity, at position 3007 (GGGCCACCCTGACCT) of the fourth intron of the H-*ras* gene. Using gel retardation experiments with nuclear extracts from HeLa and LATK$^-$ cell lines, which possess hormone receptors, we showed that both glucocorticoid and estrogen receptors bind their cognate oligonucleotides of H-*ras* in a specific way, thus suggesting a role for glucocorticoids and estrogens in regulation of the H-*ras* gene expression.

## Materials and methods

*Cells and culture conditions.* Human cervical epithelial tumor cells (HeLa) and tumor mouse fibroblasts (LATK$^-$)

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were grown exponentially in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. The cells were collected by scraping and used for preparation of nuclear extracts.

*Nuclear extract preparation.* The cells were homogenized in hypotonic buffer (25 mM Tris HCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The nuclei were pelleted, washed with isotonic buffer (25 mM Tris HCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 0.2 mM sucrose) and lysed with an extraction buffer (25 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1% Triton, 0.5 mM DTT, 0.5 mM PMSF). Nuclear debris was removed by centrifugation at 25,000 rpm for 1 h at 4°C. The protein concentration of the supernatant was measured by the method of Bradford.

*Preparation of double stranded oligonucleotides and labelling.* The PAS GRE probe was obtained by annealing the following two oligonucleotides: 5'-AGCTTTCCGCCGTG GCCAGACCTGTTCTGGAGGACA-3'; 5'-AGCTTGTCC TCCAGAACAGGTCTGGCCACGGCGGAA-3'. The RAS ERE probe was generated by the oligonucleotides: 5'-AGCTTGGGCCCCGGGCCACCCTGACCTTTGAGGGGA-3'; 5'-AGCTTCCCCTCAAAGGTCAGGGTGGCCCGGGGC CCA-3'. RAS GRE and RAS ERE probes contain the corresponding hormone response elements of the H-*ras* gene. The hMTIIA GRE probe, which contains the GRE of the human metallothionein IIA gene promoter (18), was generated by the oligonucleotides: 5'-AGCTTGGTACACTGTG TCCTGAATTCA-3'; 5'-AGCTTGAATTCAGGACACA GTGTACCA 3'. The Vitellogenin ERE probe, which encompasses the ERE of the *Xenopus* Vitellogenin A2 gene (33) was generated by annealing of the oligonucleotides: 5'-AGCTTCAAAGTCAGGTCACAGTGACCTGATCAAAG A-3'; 5'-AGCTTCTTTGATCAGGTCACTGTGACCTGACTT TGA-3'. These probes contain a HindIII recognition site located at the end. Double stranded oligonucleotides were 5' end-labelled. Radioactive end-labelling was performed using T4 polynucleotide kinase (Boehringer) and [$\gamma$-$^{32}$P] ATP. For competition experiments, HIV-LTR AP-1, SP-1 and NF-1 unlabelled double stranded oligonucleotides were used as nonspecific competitors. HIV-LTR AP-1 is the 2ab element of the HIV-1 LTR which encompasses an AP-1 binding consensus site (35). SP-1 and NF-1 oligonucleotides contain binding sites for SP-1 and NF-1 factors respectively (35).

*Gel retardation assay.* DNA binding reactions were carried out in binding buffer (50 mM Hepes, pH 8.0, 500 mM NaCl, 0.5 mM PMSF, 0.5 mg/ml BSA, 20% glycerol, 1 mM EDTA) plus 1 mM DTT and 150 µg/ml poly(dI-dC). Protein components were incubated for 20 min at 0°C prior to the addition of 2000 cps of the radiolabelled probe. After incubation at 0°C for 30 min the complexes were electrophoresed on 6% polyacrylamide gels and the gels were dried and exposed to X-ray film at RM. For competition experiments a molar excess of unlabelled oligonucleotides was added before the addition of the radiolabelled probe. For band shift experiments, polyclonal (rabbit) anti-human GR antibody (PA1-510, Affinity Bioreagents) was included in the reaction mixture and incubated at 0°C for 15 min.

Table I. Possible GRE and ERE elements in the c-H-*ras*1 gene.[a]

| Consensus sequence | Putative HREs in c-H-*ras*1 |
|---|---|
| GRE: | GRE: |
| 5'-GGTACANNNTGTTCT-3' | 1252-GGCCAGACCTGTTCT-1266 |
| ERE: | ERE: |
| 5'-AGGTCANNNTGACCT-3' | 3007-GGGCCACCCTGACCT-3021 |

[a]The position of the elements in H-*ras* gene sequence and homology to GRE consensus and complementary consensus sequences are also shown.

*Computer scanning.* We used Cyborg/Pustell sequence analysis programs to search the nucleotide sequence of the human H-*ras*1 gene (36). For identifying possible GREs, we scanned the human c-H-*ras* sequence for the highly conserved hexanucleotide 5'-TGTTCT-3' and the complementary 5'-AGAACA-3' sequence. For EREs, the 5'-TGACCT-3' and the complementary 5'-AGGTCA-3' motifs were used.

## Results

*Computer scanning of the c-H ras1 gene.* Computer scanning of the human c-H-*ras*1 sequence for the highly conserved glucocorticoid response sequence, revealed several possible half palindrome GR motifs with 83% similarity and only one perfectly matching the conserved hexanucleotide. The sequence of this motif and its position in the H-*ras* gene and similarity with the consensus oligonucleotide is shown in Table I. The fact that the possible GRE at position 1261 of the conserved 3' end of the first intron perfectly matches, raised the question of it having a functional role in the H-*ras* gene regulation. Computer scanning of the H-*ras*1 gene for EREs revealed one putative ERE with 90% similarity at position 3016 of the fourth intron of the H-*ras* gene (Table I). The organization of the human c-H-*ras*1 gene and the positions of the putative GR and ER elements, as well as some putative regulatory elements possibly participating in hormone regulation of the gene, are shown in Fig. 1.

*DNA binding assays.* DNA binding assays were used to examine whether the hormone receptors bind their corresponding HRE motifs in a specific manner.

PAS GRE probe encompassing the H-*ras* GRE motif as well as the hMTIIA GRE, control probe containing the known GR DNA binding site of the human metallothionein-IIA gene, were $^{32}$P-labelled and used in gel retardation assays.

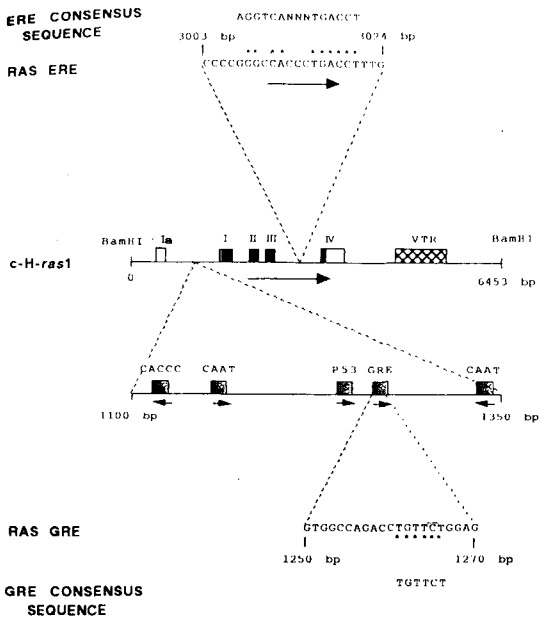


Figure 1. Organization of the human c-H-*ras*1 gene-sequences and positions of the H-*ras* glucocorticoid responce element (GRE) and estrogen response element (ERE). Some possible regulatory elements in the vicinity of the H-*ras* GRE are also shown. The coding sequences are represented by black boxes, the untranslated sequences by open boxes, the VTR by a crosshatched box and the regulatory elements by shadowed boxes. The direction of transcription is indicated by arrows. Asterisks indicate homology between hormone response elements and the corresponding consensus oligonucleotides.

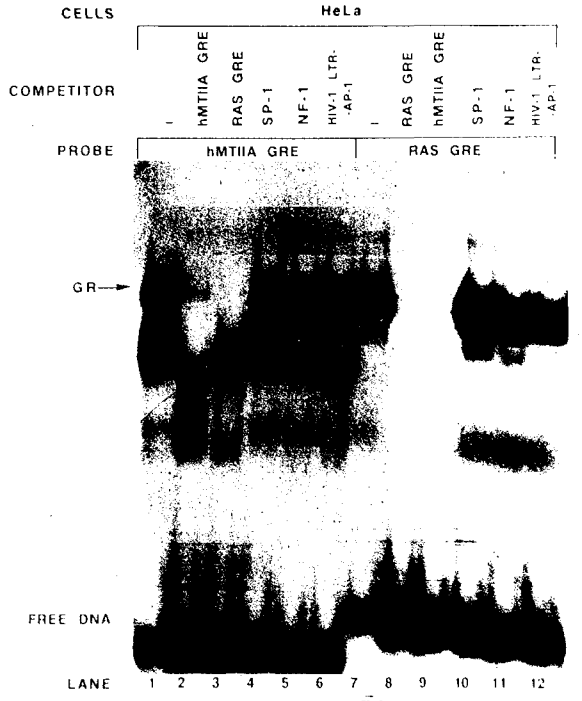


Figure 2. Sequence specific binding of GR to the H-*ras* GRE. Binding reaction mixtures contained 15 µg of HeLa total nuclear protein, 0.01 pmoles of $\gamma^{32}$P-end labelled probes hMTIIA GRE (lanes 1-7) and RAS GRE (lanes 8-12) and a 150 fold molar excess over the labelled probe of the unlabelled competitor oligonucleotide (lanes 2-6 and 8-12). Protein-DNA complexes were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. GR-RAS GRE complex is indicated by the arrow.

HeLa and LATK$^-$ nuclear extacts were used as a source of GR. Fig. 2 shows the interaction of GR and H-*ras* GRE. The labelled hMTIIA GRE and RAS GRE probes were incubated with HeLa nuclear extract in the absence (lanes 1,7) or presence of unlabelled oligonucleotide competitors. The labelled probes were competed for protein binding with a molar excess of unlabelled oligonucleotides: (lanes 2,8), with each other (lanes 3,9) and with the unrelated SP-1 (lanes 4,10), NF-1 (lanes 5,11) and HIV-1LTR AP-1 (lanes 6,12). A common retarded band was seen in lanes 1 and 7, indicating the presence of a GR-DNA complex. Binding of GR on RAS GRE probe was abolished in the presence of excess of GRE containing competitors, but was unaffected by the other competitors such as SP-1, NF-1 or HIV-1 LTR-AP-1. An equivalent picture resulted from the hMTIIA GRE probe. Fig. 2 showed a strong sequence specificity of a protein for the H-*ras* DNA target and suggested that this protein was the GR, as it was shown by the common band in lanes 1 and 7 and was displaced only by GRE-related competitors.

A further, more direct demonstration of the GR-H-*ras* GRE interaction is shown in Fig. 3. $^{32}$P-labelled RAS GRE probe was incubated with HeLa nuclear protein, in the absence (lane 3), or presence (lanes 4-6) of increasing amounts of polyclonal anti-human glucocorticoid receptor antibody. Control hMTIIA GRE probe was also incubated, in the absence (lane 1) or presence (lane 2) of polyclonal anti-human GR antibody. Inclusion of the antibody into the reaction mixture led to the disappearance of the specific retarded band, clearly indicating that this was due to GR-DNA interaction.

The hMTIIA GRE and RAS GRE radiolabelled probes were incubated with increasing amounts of GR-containing HeLa (lanes 6-11) and LATK$^-$ (lanes 1-5) extracts (Fig. 4). A retarded band was seen and a stronger GR-DNA complex was observed, in both cell lines, with increasing amount of nuclear extract.

RAS ERE probe, encompassing the H-*ras* ERE sequence and Vitellogenin ERE probe, encompassing the estrogen response sequence from *Xenopus* Vitellogenin A2 gene were used for ER binding experiments. The labelled Vitellogenin ERE and RAS ERE probes were incubated with HeLa nuclear extract in the absence (lanes 1,5) or presence of an excess of unlabelled oligonucleotide competitors (Fig. 5). The labelled probes were competed for protein binding with related (lanes 2,6) and unrelated (lanes 3,4,7,8) unlabelled oligos. A common retarded band was seen in lanes 1 and 5, indicating the presence of an ER-DNA complex. Binding of ER on the RAS ERE probe was abolished only in the presence of excess of ERE related competitors, indicating specific binding of estrogen receptor on the H-*ras* ERE.

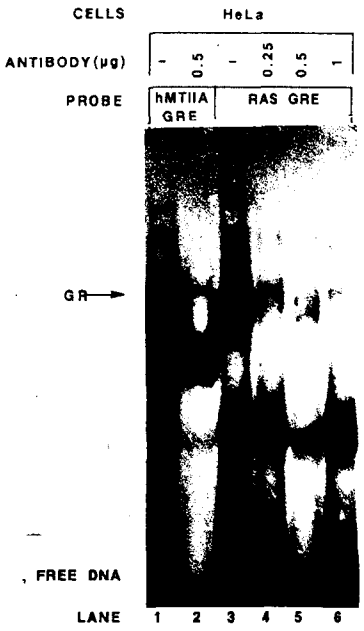


Figure 3. Identification of GR-H-*ras* GRE complexes by specific antibody. Binding reaction mixtures contained 13 μg of HeLa total nuclear protein, 0.01 pmoles of $^{32}$P labelled hMTIIA GRE and RAS GRE probes, polyclonal anti-human glucocorticoid receptor antibody in amounts indicated above each lane. Protein-DNA complexes were separated as in Fig. 2.

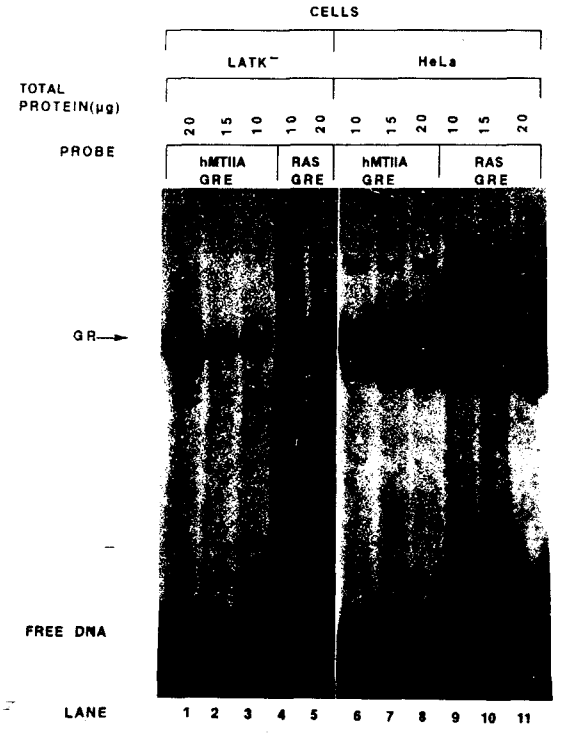


Figure 4. GR binding to GRE derived from two different cell lines. Binding reaction mixtures contained 0.01 pmoles of hMTIIA GRE (lanes 1-3 and 6-8) or RAS GRE (lanes 4,5 and 9-11) radiolabelled probe and 10,15, or 20 μg of nuclear protein, as indicated above the lanes. LATK⁻ (lanes 1-5) or HeLa (lanes 6-11) nuclear extracts were used. Protein-DNA complexes were separated as in Fig. 2.

## Discussion

The steroid hormone receptors are one of the most extensively studied group of the known regulatory factors, however, little is still known about hormonal regulation of cellular oncogenes (32,37,38). Using gel retardation assays we observed specific binding of hormone receptors in the H-*ras* gene, at positions which were previously predicted through computer scanning analysis. We showed that glucocorticoid receptor binds a consensus motif at position 1261 of the conserved 3' end of the first intron of the H-*ras* and that estrogen receptor binds a sequence at position 3007, at the fourth intron of the gene. These results provide data for hormone regulation of the human H-*ras*1 oncogene. Furthermore, potentially active HREs are found in sequences of the H-*ras* intron, suggesting a regulatory role for introns in the H-*ras* gene expression. Moreover, the end of the first intron is evolutionarily well conserved (33), suggesting a functional role for the H-*ras* GRE in human and other organisms as well. The existence of other possible regulatory elements in the vicinity of the H-*ras* GRE as shown in Fig. 1 (39-41), also implies a functional role for the H-*ras* GRE.

Previous data for regulation of the murine H-*ras* gene by glucocorticoids has been reported (42). The cooperation between glucocorticoid hormones and the T24 H-*ras* oncogene in cell transformation (43) suggests the involvement of the H-*ras* gene in hormonal promotion of breast tumors through abnormal hormonal regulation. In addition, development of breast carcinomas in rats has been shown to be initiated by mutations in the H-*ras* and K-*ras* genes and to depend on promotion by estrogens (44). The latency of *ras* oncogenes to induce cell transformation in the absence of hormonal stimulation (44) might be due to the inability of *ras* oncogenes to induce proliferation of the mammary precursor cells. Our experimental results suggest a more direct implication of H-*ras* in hormonal tumor promotion by proposing hormonal regulation of the H-*ras* gene (normal or mutated), also providing an explanation for the specificity of tumor development in systems involving *ras* genes and sexual development.

Using *ras* p21 as a possible marker for the disease may be of prognostic significance. Depending on the way the H-*ras* gene is regulated by hormones, an explanation of indications correlating the levels of hormone receptors and prognosis of the disease may also be provided (45-47).

In conclusion, more data on the hormone regulation of the H-*ras* gene through specific receptor binding could be provided by combining *in vitro* CAT assay experiments and

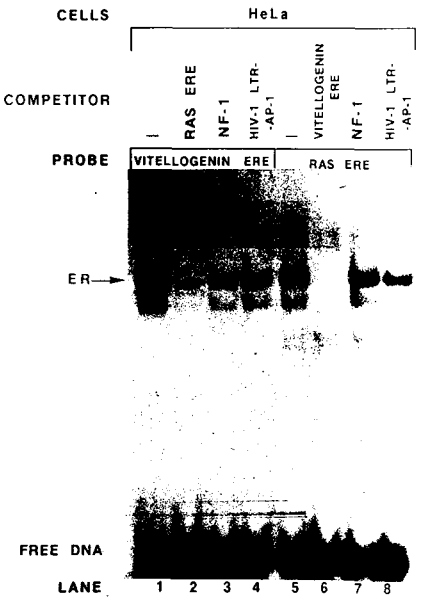


Figure 5. Binding of the ER to H-*ras* ERE. Binding reaction mixtures contained 0.01 pmoles of Vitellogenin ERE (lanes 1-4) or RAS ERE radiolabelled probe and 20 μg of nuclear protein. Unlabelled RAS ERE (lane 2) and Vitellogenin ERE (lane 6) probes, as well as the unrelated probes NF-1 (lanes 3,7) and HIV-1 LTR-AP-1 (lanes 4,8) were used in competition experiments. Protein-DNA complexes were separated as indicated in the Fig. 2 legend. ER-H-*ras* ERE complex is indicated by the arrow.

*in vivo* determination of the *ras* p21 levels in stable transfectants, when adding the corresponding hormone. Moreover, comparing the levels of the receptor binding in corresponding HREs in human tumors with the adjacent normal tissues, through gel retardation assays, may give additional evidence for the role of hormonal regulation of the H-*ras* gene in human tumor progression.

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