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Glucocorticoid and Estrogen Receptors Have Elevated Activity in Human Endometrial and Ovarian Tumors as Compared to the Adjacent Normal Tissues and Recognize Sequence Elements of the H-*ras* Proto-oncogene

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We examined the level of receptor binding in H-*ras* elements, using nuclear extracts derived from human endometrial and ovarian lesions and from adjacent normal tissue in gel retardation assays. We found increased binding of the glucocorticoid receptor (GR) to the H-*ras* GR element in more than 90% of endometrial tumors and in all ovarian tumors tested, as compared to the corresponding adjacent normal tissue. Additionally, we found elevated binding of the estrogen receptor (ER) in H-*ras* ER element in all pairs of ovarian tumor/normal tissue tested, whereas in ER-negative control breast tumor/normal tissue pairs, no differences in ER DNA-binding levels were observed. These results suggest that steroid hormone receptor binding could directly activate the H-*ras* oncogenic potency in human endometrial and ovarian lesions, providing additional evidence for the role of H-*ras* expression in hormonally responsive human cancers.

Key words: Glucocorticoid receptor — Estrogen receptor — Endometrial tumor — Ovarian tumor — H-*ras* proto-oncogene

Steroid hormone receptors differentially control gene expression during embryonic development and adult homeostasis as hormone-activated transcriptional regulators. Upon activation, they specifically bind to palindromic DNA sequences, called hormone response elements (HREs), as homodimers and stimulate gene activity,[1,2] or sometimes repress transcription, by interacting with components of the transcription initiation complex.[3,4]

Steroids are considered to be tumor promoters.[5] They create a milieu which generally stimulates cell division and growth of the target organs during normal development and early neoplastic changes as well.[6] The influence of steroid levels on cure rates and survival of patients with genital tumors has been examined[7–9] and women who receive long-term unopposed estrogen substitution have a high risk for developing endometrial and ovarian cancer.[10–12] The molecular mechanisms by which steroids act as tumor promoters are not well defined. However, transcriptional activation of cellular proto-oncogenes, which are known to play a central role in cellular communication systems, appears to be one of them. Regulation of c-*fos*, c-*jun*, c-*myc* and cyclin D1 by steroids in breast cancer cells provides a paradigm for further understanding of cell cycle control by steroids.[13–15]

The *ras* family of cellular oncogenes is one of the most frequently detected families of transformation-inducing genes in human solid tumors[16,17] and is involved in multiple signal transduction pathways during normal cellular growth as well.[18] Overexpression of *ras* genes may cause oncogenic transformation[19,20] and the c-H-*ras* proto-oncogene has been shown to play a prominent role as a metastasis-modulating gene in breast cancer.[21] Additionally, H-*ras* cooperates with glucocorticoid hormones in cell transformation[22] and with estrogens in the development of breast carcinomas in rats.[23]

In a previous study,[24] we showed that the human H-*ras* gene contains, within its first and fourth introns, sequences that specifically bind the human glucocorticoid receptors (GRs) and estrogen receptors (ERs), respectively. In this study, using nuclear extracts from human endometrial and ovarian lesions in gel retardation assays, we examined the levels of GR and ER binding in the respective H-*ras* DNA elements. We found elevated binding of GRs and ERs in H-*ras* in human tumors tested, as compared to the adjacent normal tissue, suggesting that H-*ras* proto-oncogene is directly implicated in human genital cancer through abnormal hormone regulation and that steroid receptor DNA-binding levels may be of prognostic significance in hormonally responsive human cancers.

MATERIALS AND METHODS

Biopsy material Endometrial and ovarian tumor tissues were obtained from Alexandra Hospital, Athens, Greece.

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Breast tumor tissues used as control were obtained from the First Surgical Department of H. Venizelou Hospital, Athens, Greece. There was no statistically significant correlation between tumor cases tested and pathological type, degree of differentiation, stage or age.

Nuclear extract preparation Tumors were cut into small pieces on dry ice and homogenized in hypotonic buffer (25 m*M* Tris HCl, pH 7.5, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.5 m*M* PMSF). The nuclei were pelleted, washed with isotonic buffer (25 m*M* Tris HCl, pH 7.5, 5 m*M* KCl, 0.5 m*M* $MgCl_2$, 0.5 m*M* DTT, 1 m*M* PMSF, 0.2 m*M* sucrose) and lysed with an extraction buffer (25 m*M* Tris HCl, pH 7.5, 1 m*M* EDTA, 0.1% Triton, 0.5 m*M* DTT, 0.5 m*M* 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.[25]

Oligonucleotides and labeling The *ras* GR element (GRE) and *ras* ER element (ERE) probes encompassing the H-*ras* glucocorticoid and estrogen binding elements respectively, have been previously described.[24] The hMTIIA GRE probe, containing the GRE of the human metallothionein IIA gene promoter and the vitellogenin ERE probe, encompassing the ERE of the *Xenopus* vitellogenin A2 gene, have also been described.[24] SP-1 was used as an HRE-unrelated oligonucleotide for competition experiments.[26] Radioactive end labeling was performed using T4 polynucleotide kinase (Boehringer, Mannheim) and [γ-^{32}P]ATP.[27]

Gel retardation assay GR-related DNA-binding reac-

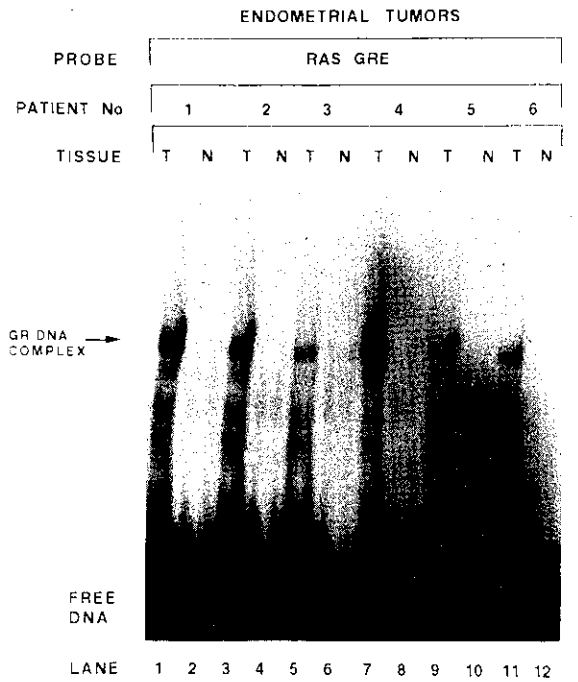

Fig. 2. Elevated binding of glucocorticoid receptor in the H-*ras* element in human endometrial tumors, as compared to adjacent normal tissue. ^{32}P-End-labeled *ras* GRE probe (0.01 pmol) was incubated with 20 μg of nuclear extracts from endometrial tumor and adjacent normal tissue. The reactions were resolved on native polyacrylamide gels and visualized by autoradiography. Protein-DNA complex is indicated by an arrow. T, tumor: N, normal tissue.

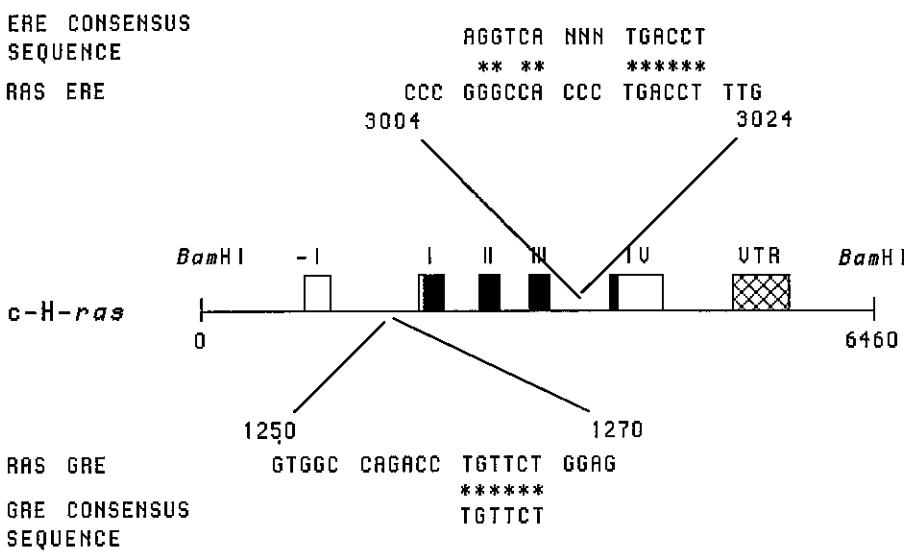

Fig. 1. Organization of the human H-*ras* gene and position of the H-*ras* GRE and ERE. Exons, rectangles; coding sequences, filled rectangles; VTR, crosshatched box. Homology of the H-*ras* HREs to the respective consensus sequences is indicated by the asterisks.

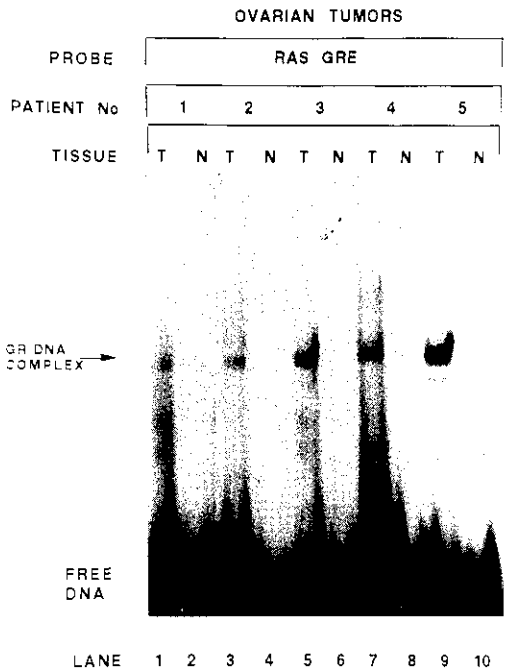

Fig. 3. Elevated binding of glucocorticoid receptor in the H-*ras* element in human ovarian tumors, as compared to adjacent normal tissue. ^{32}P-End-labeled *ras* GRE probe (0.01 pmol) was incubated with 20 μg of nuclear extracts from ovarian tumor and adjacent normal tissue. Protein-DNA complexes were separated as in Fig. 2. T, tumor; N, normal tissue.

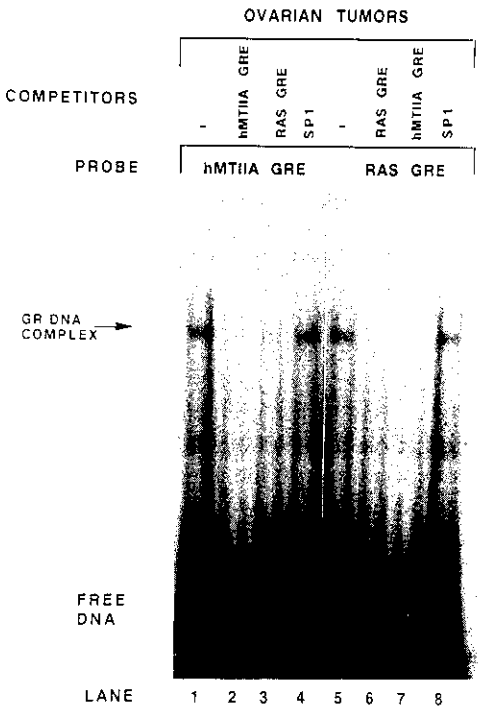

Fig. 4. Human glucocorticoid receptor binds the H-*ras* element specifically. ^{32}P-End-labeled hMTIIA GRE (lanes 1–4) and *ras* GRE (lanes 5–8) probes (0.01 pmol) were incubated with nuclear extracts from ovarian tumor tissue. A 200-fold excess of unlabeled competitor DNA was added (lanes 2–4 and 6–8). Protein-DNA complexes were separated as in Fig. 2.

tions were carried out in binding buffer containing: 50 m*M* Hepes, pH 8.0, 500 m*M* NaCl, 0.5 m*M* PMSF, 0.5 μg/ml BSA, 20% glycerol, 1 m*M* EDTA, 1 m*M* DTT and 150 mg/ml poly (dI-dC).[24] ER-related DNA-binding reactions were carried out in 10 m*M* Tris-HCl pH 7.5, 0.5m*M* EDTA, 80 m*M* KCl, 5% glycerol, 0.5 m*M* DTT and 150 mg/ml poly (dI-dC).[28] Reactions were carried out as described.[24] A 200-fold molar excess of unlabeled oligonucleotides or 0.5 μg of polyclonal anti-human GR antibody (PA1-510, Affinity Bioreagents, NJ), or monoclonal anti-human ER antibody (F3, kindly provided by Dr. L. Tora, IGBMC, Strasbourg, France), was included prior to the addition of the radiolabeled probe in the reaction mixture, where indicated, and incubation was carried out at 0°C for 15 min.

McGuire test Control breast tumors were determined as ER-negative by use of the McGuire test.[29]

RESULTS

The positions of the H-*ras* GR and ER binding motifs[24] are shown in Fig. 1.

GR activity in endometrial and ovarian lesions The level of GR binding in the H-*ras* binding site in human endometrial and ovarian tumors, as compared to the adjacent normal tissues, was examined by means of gel retardation assays. The radiolabelled *ras* GRE oligonucleotide was incubated with nuclear extracts from endometrial tumor (T) and from adjacent normal (N) tissues (Fig. 2). By "normal tissue" we mean macroscopically normal endometrium and ovary, which was microscopically composed of purely benign tissue. In 10/11 pairs tested, an elevated binding of GR on H-*ras* GRE in tumor compared to the normal tissue was observed.

Additionally, in 10/10 pairs of ovarian tumor (T) and adjacent normal (N) tissues tested, elevated binding of glucocorticoid receptor on *ras* GRE in tumor versus normal tissue was observed (Fig. 3).

To determine if recognition of the ^{32}P-labeled H-*ras* DNA by the human GR was sequence-specific, a 200-fold excess of unlabeled oligonucleotide competitors and 0.5 μg of anti-GR antibody were included in the binding reaction. The control hMTIIA GRE probe and *ras* GRE

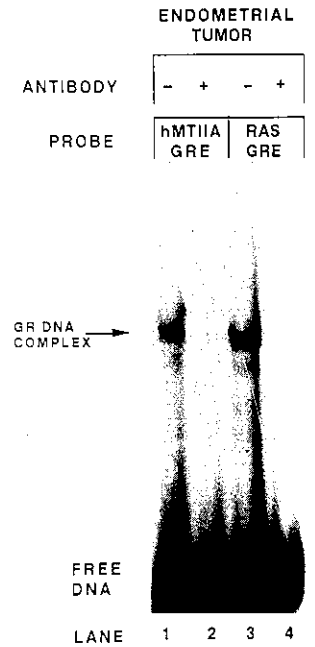

Fig. 5. Human glucocorticoid receptor binds the H-*ras* element specifically. ^{32}P-End-labeled *ras* GRE (lanes 3, 4) and hMTIIA GRE (lanes 1, 2) probes (0.01 pmol) were incubated with 20 μg of nuclear extracts from endometrial tumor tissue. Anti-GR antibody (0.5 μg) was added (lanes 2, 4). Protein-DNA complexes were separated as in Fig. 2.

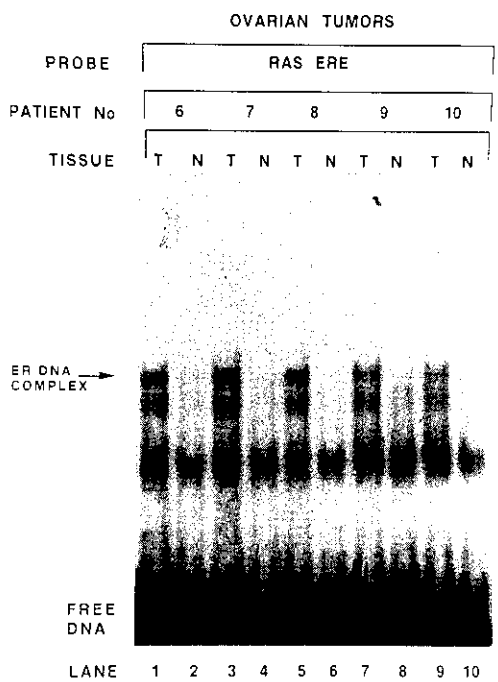

Fig. 6. Elevated binding of estrogen receptor in the H-*ras* element in human ovarian tumors, as compared to adjacent normal tissue. ^{32}P-End-labeled *ras* ERE probe (0.01 pmol) was incubated with 20 μg of nuclear extracts from ovarian tumor and adjacent normal tissue. Protein-DNA complexes were separated as in Fig. 2. T, tumor; N, normal tissue.

probe were incubated with ovarian tumor nuclear extract (Fig. 4), GR/DNA binding (lanes 1 and 5) was competed out by oligonucleotides containing a GR-binding site (lanes 2, 3 and 6, 7, respectively), but not by oligonucleotide SP-1, which does not contain a GR-binding site (lanes 4 and 8). The control hMTIIA GRE probe and *ras* GRE probe were incubated with nuclear extracts derived from endometrial tumor tissue (Fig. 5). A common retarded band was observed (lanes 1 and 3), which was abolished when anti-human GR antibody, which interferes with the DNA-binding domain of the hGR, was included in the reaction mixture, thus indicating the sequence specificity of the GR/DNA interaction. Competition and antibody experiments were also performed for the extracts of endometrial and ovarian cancer, with corresponding results (data not shown).

ER activity in ovarian lesions The level of ER binding in the H-*ras* element in human ovarian tumor as compared to the adjacent normal tissue, was also examined. The radiolabeled *ras* ERE oligonucleotide was incubated with nuclear extracts from ovarian tumor (T) and from adjacent normal (N) tissues (Fig. 6). In 5/5 pairs tested, an elevated binding of ER on *ras* ERE in tumor compared to normal tissue was observed.

To determine if recognition of the radiolabeled H-*ras* DNA by the human ER was sequence-specific, vitellogenin ERE and *ras* ERE probes were incubated with ovarian tumor nuclear extract (Fig. 7) in the absence (lanes 1 and 5) or presence of a 200-fold excess of unlabeled oligonucleotide competitors. Binding was competed out by oligonucleotides encompassing an ER-binding motif (lanes 2, 3 and 6, 7) but not by unrelated competitors (lanes 4 and 8). The ER/DNA complex was supershifted when 0.5 μg of anti-human ER antibody, which interferes with the hER in a domain distinct from the DNA-binding region,[28] was included in the reaction mixture, thus indicating the sequence specificity of the ER/DNA interaction (Fig. 8).

There was also evidence for elevated ER activities in three pairs of endometrial tumor/adjacent normal tissue tested (our unpublished results).

Control DNA-binding assays Four ER-negative breast tumor-adjacent normal tissue pairs were incubated with *ras* ERE probe in a control DNA-binding reaction. A weak DNA-binding signal was observed in two cases, after overexposure, in both tumor and normal tissue, with no differences in DNA-binding levels in tumor

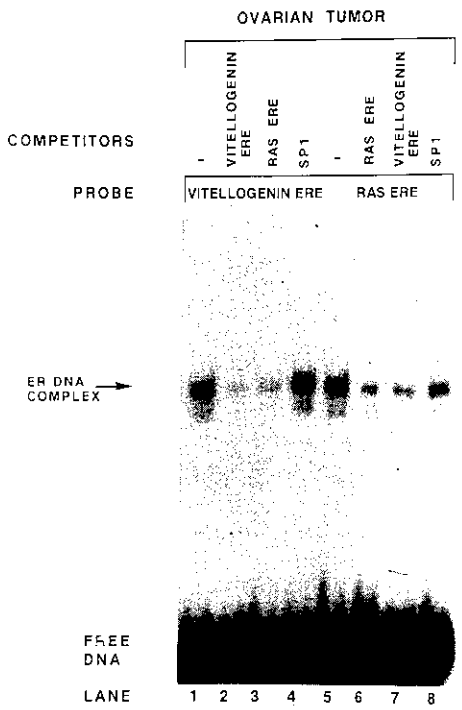

Fig. 7. Human estrogen receptor binds the H-*ras* element specifically. ^{32}P-End-labeled *ras* ERE (lanes 1–4) and vitellogenin ERE (lanes 5–8) probes (0.01 pmol) were incubated with 20 μg of nuclear extracts from ovarian tumor tissue. A 200-fold excess of unlabeled competitor DNA was added (lanes 2–4 and 6–8). Protein-DNA complexes were separated as in Fig. 2.

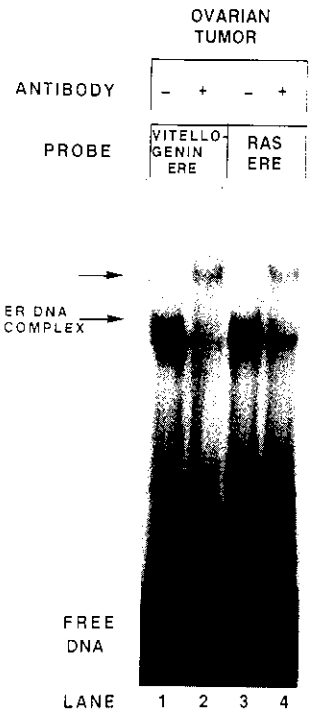

Fig. 8. Human estrogen receptor binds the H-*ras* element specifically. ^{32}P-End-labeled *ras* ERE (lanes 3, 4) and vitellogenin ERE (lanes 1, 2) probes (0.01 pmol) were incubated with 20 μg of nuclear extracts from ovarian tumor tissue. F3 anti-ER antibody (0.5 μg) was added (lanes 2, 4). Protein-DNA complexes were separated as in Fig. 2.

versus normal tissue. In the remaining pairs, there was no DNA-binding activity (data not shown).

DISCUSSION

Hormone regulation of cellular genes is of great importance in cell differentiation and growth.[1,2] Steroid hormones act as tumor promoters[5] and their levels influence the cure rates and the survival of patients with genital tumors,[7–9] as does hormone-replacement therapy of postmenopausal women.[10–12] In the present study, using a sensitive gel retardation assay, we found that GR and ER activity is elevated in human endometrial and ovarian lesions, as compared to the adjacent normal tissue.

The molecular mechanisms of tumor promotion by steroids involve regulation of cellular oncogenes such as c-*fos*, c-*jun*, c-*myc* and cyclin D1 gene.[13–15] Regulation of the murine H-*ras* by glucocorticoids has also been reported.[30] In addition, overexpression of the *ras*-encoded p21 protein was found in ovarian tumors, as compared to normal or benign tumor tissue.[31,32]

Our results suggest that steroid hormones increase the oncogenic potential of the human H-*ras* gene through specific receptor binding in human genital lesions. The *ras* gene family is one of the most frequently detected families of transformation-inducing genes in human tumors.[16,17] Therefore, it would be of great interest to examine *ras* expression levels in these tumors in order to look for a correlation between elevated binding of steroid receptors in H-*ras* DNA elements and H-*ras* overexpression.

Moreover, there is evidence for correlation of steroid hormone receptor status in human endometrial and ovarian tumors with prognosis of the disease,[8,9] although this is not as well established as for breast cancer.[33,34] Our results are in agreement with previous data[8] where 89% of endometrial tumors, measured by ligand-binding assay (LBA), or 77% of endometrial tumors, measured by enzyme immunoassay (EIA), were ER-positive. In addition, we suggest that the gel retardation assay we used in our study may be of general application for the determination of hormone receptor levels in human tumors,

since it can evaluate the fraction of receptor molecules that is both hormone-activated and functionally capable of DNA binding. LBA and EIA do not provide information on whether the measured receptor molecules are capable of activating gene expression by specific DNA binding, or are inactivated by mutations, by specific cellular factors, or by exclusion from the nucleus, so gel retardation assays give more accurate information on the oncogenic potential of the measured steroid receptor levels. Correlation of clinical and molecular data will be helpful in understanding the causes of the disease, providing useful prognostic, diagnostic and therapeutic information.

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REFERENCES

1) Beato, M. Gene regulation by steroid hormones. *Cell,* **56,** 335–344 (1989).
2) Beato M., Herrlich, P. and Schutz, G. Steroid hormone receptors: many actors in search of a plot. *Cell,* **83,** 851–857 (1995).
3) Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P. and Cato, A. C. B. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J.,* **13,** 4087–4095 (1994).
4) Thummel, C. S. From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear factor superfamily members. *Cell,* **83,** 871–877 (1995).
5) Sekeris, C. E. Hormonal steroids act as tumor promoters by modulating oncogene suppression. *J. Cancer Res. Clin. Oncol.,* **117,** 96–101 (1991).
6) Lauritzen, C. Cancer risk under hormone therapy. *Ther. Umsch.,* **51,** 755–766 (1994).
7) Geisler, J. P., Wiemann, M. C., Miller, G. A., Zhou, Z. and Geisler, H. E. Estrogen and progesterone receptors in malignant mixed mesodermal tumors of the ovary. *J. Surg. Oncol.,* **59,** 45–47 (1995).
8) Nyholm, H. C., Nielsen, A. L., Lyndrup, J., Greene, G. L. and Thorpe, S. M. Estrogen receptor determination in endometrial carcinoma: ligand binding assay versus enzyme immunoassay. *Anticancer Res.,* **15,** 649–654 (1995).
9) Morris, P. C., Anderson, J. R., Anderson, B. and Buller, R. E. Steroid hormone receptor content and lymph node status in endometrial cancer. *Gynecol. Oncol.,* **56,** 406–411 (1995).
10) Palacios, S. Cancer surveillance during HRT. *Int. J. Fertil. Menopausal. Stud.,* **39,** 93–98 (1994).
11) Grady, D., Gebredsadik, T., Kerlikowske, K., Ernster, V. and Pettiti, D. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. *Obstet. Gynecol.,* **85,** 304–313 (1995).
12) Breckwoldt, M., Keck, C. and Karck, U. Benefits and risks of hormone replacement therapy (HRT). *J. Steroid Biochem. Mol. Biol.,* **53,** 205–208 (1995).
13) Musgrove, E. A. and Sutherland, R. L. Cell cycle control by steroid hormones. *Semin. Cancer Biol.,* **5,** 381–389 (1994).
14) Gordeladzer, J. O., Johansen, P. W., Paulssen, R. H., Paulssen, E. J. and Gautvik, K. M. G-proteins: implications for pathophysiology and disease. *Eur. J. Endocrinol.,* **131,** 557–574 (1994).
15) Hyder, S. M., Stancel, G. M. and Loose-Mitchell, D. S. Steroid hormone-induced expression of oncogene encoded nuclear proteins. *Crit. Rev. Eucaryotic Gene Exp.,* **4,** 55–116 (1994).
16) Shekhar, P. V. and Miller, F. R. Correlation of differences in modulation of *ras* expression with metastatic competence of mouse mammary tumor subpopulations. *Invasion Metastasis,* **14,** 27–37 (1994).
17) Spandidos, D. A. "The Superfamily of *ras*-Related Genes," pp. 1–338 (1991). Plenum Press, New York.
18) Abdellatif, M., MacLellan, W. R. and Schneider, M. D. p21 Ras as a governor of global gene expression. *J. Biol. Chem.,* **269,** 15423–15426 (1994).
19) Spandidos, D. A. and Wilkie, N. M. Malignant transformation of early passage rodent cells by a single mutated human oncogene. *Nature,* **310,** 469–475 (1984).
20) Spandidos, D. A. The human T24 Ha-*ras*1 oncogene: a study of the effects of overexpression of the mutated *ras* gene product in rodent cells. *Anticancer Res.,* **6,** 259–262 (1986).
21) Spandidos, D. A. and Agnantis, N. J. Human malignant tumors of the breast, as compared to their respective normal tissue, have elevated expression of the Harvey *ras* oncogene. *Anticancer Res.,* **4,** 269–272 (1984).
22) Marshall, H., Martens, I., Svensson, C., Akusjärvi, G. and Linder, S. Glucocorticoid hormones may partially substitute for adenovirus E1A in cooperation with *ras*. *Exp. Cell Res.,* **194,** 35–41 (1991).
23) Kumar, R., Sukumar, S. and Barbacid, M. Activation of *ras* oncogenes preceding the onset of neoplasia. *Science,* **248,** 1101–1104 (1990).
24) Zachos, G., Zoumpourlis, V., Sekeris, C. E. and Spandidos, D. A. Binding of the glucocorticoid and estrogen receptors to the human H-*ras* oncogene sequences. *Int. J. Oncol.,* **6,** 595–600 (1995).
25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–

254 (1976).
26) Pintzas, A. and Spandidos, D. A. Sp1 specific binding sites within the human H-*ras* promoter: potential role of the 6 bp deletion sequence in the T24 H-*ras* 1 gene. *Anticancer Res.*, **11**, 2067-2070 (1991).
27) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning: A Laboratory Manual" (1989). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
28) Ali, S., Lutz, Y., Bellocq, J. P., Chenard-Neu, M. P., Rouyter, N. and Mertzger, D. Production and characterization of monoclonal antibodies recognizing defined regions of the human oestrogen receptor. *Hybridoma*, **12**, 391–405 (1993).
29) McGuire, W. L. Quantitation of estrogen receptor in mammary carcinoma. *In* "Methods in Enzymology," vol. 36, pp. 248–254 (1975). Academic Press, New York.
30) Strawhecker, J. M., Bentz, N. A., Neades, R. Y., Houser, W. and Pelling, J. C. Binding of the 97 kDa glucocorticoid receptor to the 5′ upstream flanking region of the mouse c-Ha-*ras* oncogene. *Oncogene*, **4**, 1317–1322 (1989).
31) Katsaros, D., Theillet, C., Zola, P., Louason, G., Sanfilippo, B., Isaia, E., Arisio, R., Giardina, G. and Sismondi, P. Concurrent abnormal expression of *erb*B-2, *myc*, *ras* genes is associated with poor outcome of ovarian cancer patients. *Anticancer Res.*, **15**, 1501–1510 (1995).
32) Van Dam, P. A., Vergote, I. B., Lowe, D. G., Watson, J. V., van Damme, P., van der Auwera, J. C. and Shepherd, J. H. Expression of c-*erb*B-2, c-*myc* and c-*ras* oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma. *J. Clin. Pathol.*, **47**, 914–919 (1994).
33) Schwartz, M. R. Carcinoembryonic antigen and estrogen receptor: a prognostic duo in breast cancer? *Cancer*, **74**, 1519–1522 (1994).
34) Esteban, J. M., Felder, B., Ahn, C., Simpson, J. F., Battifora, H. and Shively, J. E. Prognostic relevance of carcinoembryonic antigen and estrogen receptor status in breast cancer patients. *Cancer*, **74**, 1575–1583 (1994).