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Antiprogestins prevent progesterone receptor binding to hormone responsive elements *in vivo*

(genomic footprinting/steroid hormone receptors/antihormones/mouse mammary tumor virus)

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ABSTRACT **Antiprogestins inhibit progesterone action by competing for binding to the progesterone receptor and are potentially important pharmaceuticals in fertility control and cancer therapy. Why the complex of antiprogestins and progesterone receptor is functionally inactive is unclear. Present models are based on indirect evidence, such as transfection competition assays and *in vitro* DNA binding studies, partly because of difficulties in visualizing the receptor bound to DNA *in vivo*. Here we used genomic footprinting analysis to show ligand-dependent binding of endogenous progesterone receptor to the hormone responsive elements (HREs) of a chromosomally integrated mouse mammary tumor virus long terminal repeat in a human mammary carcinoma cell line. The antiprogestins RU 486 and ZK 98299 do not promote binding of the progesterone receptor to this natural HRE *in vivo*, even at concentrations that completely inhibit the agonistic effects of potent synthetic progestins. Moreover, antiprogestins cause a rapid disappearance of the agonist-induced progesterone receptor footprint. We conclude that antiprogestins interfere with receptor function by preventing its specific DNA binding.**

Steroid hormones modulate gene activity by binding to their intracellular receptors, which are able to either recognize hormone responsive elements (HREs) in the vicinity of target promoters or influence gene expression by interaction with other transcription factors independently of HREs (1, 2). However, the precise role of the ligand in hormonal gene regulation is not well understood (3). Recently, evidence has accumulated that hormone receptors can be activated in the absence of their physiological ligands by second messenger signal transduction pathways, which probably influence receptor phosphorylation (4). Interestingly, both ligand-induced and ligand-independent receptor activation can be inhibited by hormone antagonists (5).

Antiprogestins, like RU 486, have a great therapeutic potential in fertility control and treatment of hormone-dependent tumors. They compete with natural ligands for binding to the progesterone receptor (PR), but why the complex of receptor and antiprogestins is functionally inactive is not clear (6). It is known that antiprogestins influence the conformation of the receptor (7–10) and may impair the dissociation of PR from heat shock proteins (11). Prevalent models assume the existence of two types of antiprogestins (6). Members of one class, represented by ZK 98299, are considered to be pure antagonists and to prevent binding of the receptor to DNA (12). Other antiprogestins, such as RU 486, can be partial agonists and are supposed to enable DNA binding of the PR but to generate receptor complexes unable to activate hormone responsive genes (13, 14). This model is based on indirect evidence, such as transfection competition assays, and on *in vitro* DNA binding studies, but conflicting results have also been reported (15). Moreover, there is no direct evidence for an effect of antiprogestins on *in vivo* PR binding to HREs.

We have used a cell line that contains a single copy integrate of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) coupled to a luciferase reporter gene and have directly assayed the effects of progestins and antiprogestins on protein–DNA interactions by *in vivo* dimethyl sulfate (DMS) footprinting. Our results provide unambiguous evidence that only agonistic progestins but not antiprogestins, such as RU 486 and ZK 98299, are able to induce binding of PR to the MMTV promoter *in vivo*. Furthermore, antiprogestins cause a rapid disappearance of the agonist-induced footprints of PR as well as footprints of other transcription factors recruited by the receptor to the MMTV promoter. Therefore, our view of the mechanism of action of antiprogestins has to be reformulated to include events taking place before the interaction of the occupied receptor with the HREs.

MATERIALS AND METHODS

Cell Culture and Cell Lines. The cell line T47D-MTVL (unpublished data) is derived from the human mammary carcinoma cell line T47D (16) and contains naturally high levels of PR and a single integrated copy of the plasmid pAGE5MMTVLu, which is composed of a complete MMTV LTR linked to a luciferase reporter gene and the scaffold attachment site of the human interferon β gene (17). Cells were grown in RPMI 1640 medium supplemented with insulin and 10% fetal calf serum at 5% CO_2/95% air at 37°C.

Luciferase Assay. Hormone-dependent transcriptional activity of the MMTV LTR was determined by measuring luciferase activity 24 hr after treating the cells with the indicated concentrations of hormones and antihormones according to ref. 17.

DMS Footprinting by Ligation-Mediated (LMPCR). Cell culture medium was supplemented with various concentrations of R 5020 (10^{-12}–10^{-7} M), RU 486 (10^{-6} M), ZK 98299 (10^{-6} M), or ethanol for 30 min. Intact cells were then treated with 0.2% DMS in prewarmed medium (37°C) for 2 min. Reactions were stopped by removing the DMS-containing medium through four subsequent washes with phosphate-buffered saline. DNA controls were generated by treating protein-free DNA with DMS for 20–60 sec. Methylated DNA was cleaved with piperidine and analyzed by LMPCR as described (18) with minor modifications. Stoffel fragment (Perkin–Elmer) was used instead of *Taq* polymerase and samples were phenol extracted and precipitated before primer

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Abbreviations: HRE, hormone responsive element; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; PR, progesterone receptor; DMS, dimethyl sulfate; LMPCR, ligation-mediated PCR.

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extension with radioactive labeled primer. Sequences of the oligonucleotides are as follows: C-C3H, CCC TCT GAA AGG TGA AGG; A25, AGG ATA AGT GAC GAG CGG AGA CGG G; A, GAC GAG CGG AGA CGG GAT GGC GAA CAG.

Quantitation of Footprints. Quantitation of the genomic footprint experiment was performed with a PhosphorImager (Molecular Dynamics) and GENEIMAGE 3.0 software. The band intensity was corrected for differences in loading by normalizing for the total radioactivity per lane. Correction for the intensity of bands that obviously did not change intensity in any of the methylation protection experiments led to identical results.

RESULTS

Correlation Between Agonist Induction and Protection over the MMTV Promoter. The effects of agonists and antagonists on transcriptional activity and binding of the PR to the MMTV promoter *in vivo* were tested. Induction of MMTV transcription was dependent on the concentration of the synthetic progestin agonist R 5020, with half-maximal effect observed in the nanomolar range (Fig. 1*A*). Analysis of the transcription rate in nuclear runon experiments demonstrated that induction was detectable after 15 min and that near-maximal transcription rates were attained 30 min after addition of R 5020 (data not shown). Using DMS genomic footprinting, we found a dose-dependent protection of the guanines in all four TGTTCT motifs of the HREs 30 min after hormone stimulation (Fig. 1*B*, lanes 3–5). As the protection pattern is very similar to that generated by PR binding to the MMTV promoter *in vitro* (19), we assume that the protection is due to agonist-dependent occupancy of the HREs by the PR. Receptor binding is seen as early as 5 min after hormone addition and the sites remain occupied after 75 min (data not shown; see also Fig. 3). Quantitation of the extent of protection shows a good correlation between PR binding and transactivation (Fig. 1*A*), suggesting that bound PR molecules are required for promoter activation.

Simultaneously with the protection of the HREs we detect protection of the two guanine residues within the binding site for the transcription factor NF-I (Figs. 1*B* and 2 *B* and *C*), which is thought to be recruited to the promoter upon hormone stimulation (20, 21). A weak protection is also found over the octamer motifs (Figs. 1*B* and 2 *B* and *C*), which are known to contribute only partially to hormone-dependent transcription of the MMTV promoter in these cell lines (22).

Antiprogestins Do Not Generate Protection over the MMTV Promoter. Neither RU 486 nor ZK 98299 showed any agonistic activity in T47D-MTVL cells, while either antagonist at 1 mM completely inhibited induction by 10 nM R 5020 (Fig. 2*A*). Thus, in this assay both antiprogestins behave as pure antagonists. Furthermore, no PR binding to the HREs could be detected at high concentrations of RU 486 (Fig. 2*B*). Similar results were obtained with ZK 98299 (Fig. 2*C*). Therefore, under conditions where RU 486 and ZK 98299 behave as pure antagonists, they are not able to elicit significant binding of PR to the MMTV promoter *in vivo*.

Antiprogestins Inhibit Protection over the MMTV Promoter Generated by the Agonist-Receptor Complex. The inability of antiprogestins to promote PR binding to the HREs of the MMTV promoter prompted us to investigate whether they are able to displace prebound agonist-PR complexes from DNA. In cells preincubated with R 5020, addition of RU 486 led to a rapid disappearance of PR footprints on the MMTV promoter, with half-maximal effect seen after 30 min (Fig. 3). We therefore conclude that antiprogestins interfere with steps in the process of PR action that are needed for specific DNA binding and/or maintenance of PR-DNA complexes.

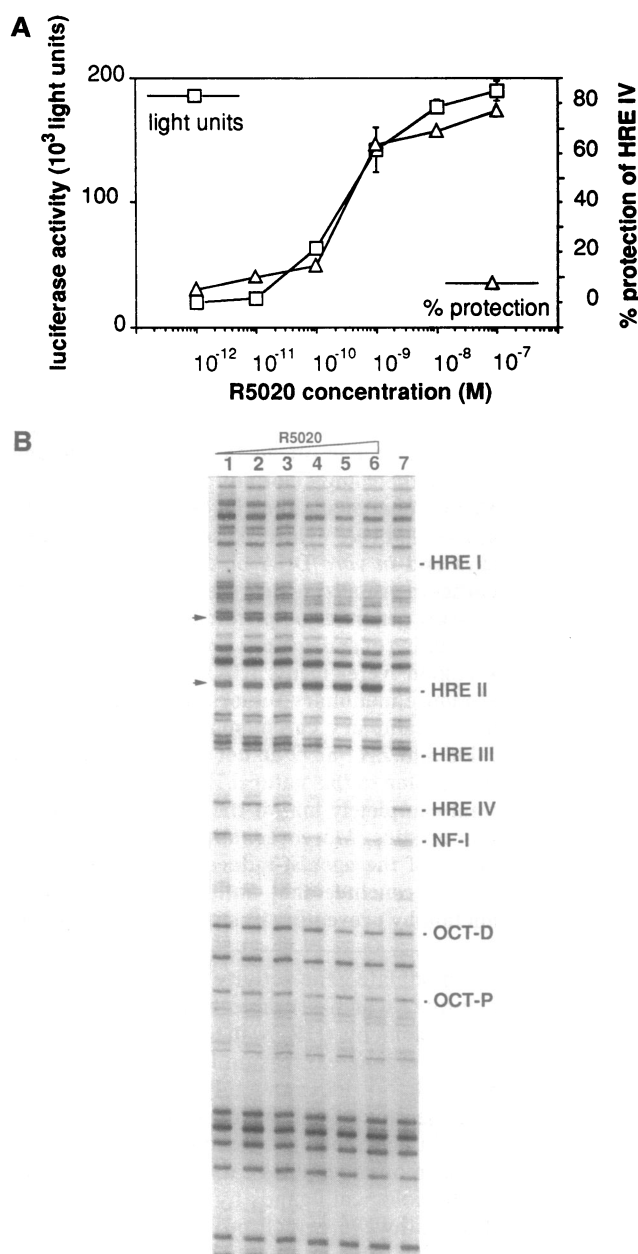
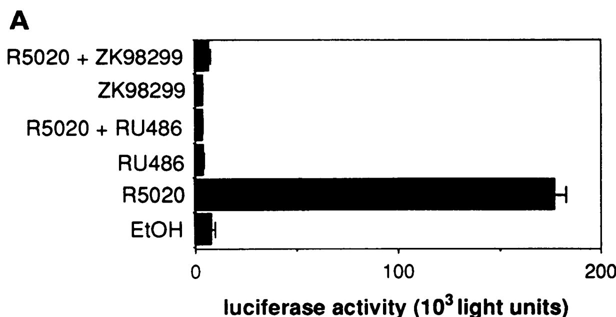


FIG. 1. (*A*) Hormone-dependent transcriptional activity of a single integrated copy of the plasmid pAGE5MMTVLu. Graph indicates luciferase activity (light units) and extent of protection over the HREs (% protection) determined by genomic footprinting (see below) as a function of the concentration of R 5020. Luciferase measurements represent mean ± SD of triplicate experiments. The extent of protection was quantitated by using a PhosphorImager (Molecular Dynamics). (*B*) Binding of PR and other transcription factors to the MMTV promoter as determined by genomic footprinting (16) after treating the cells for 30 min with increasing concentrations of R 5020. Lanes: 1, 10^{-12} M; 2, 10^{-11} M; 3, 10^{-10} M; 4, 10^{-9} M; 5, 10^{-8} M; 6, 10^{-7} M R 5020; 7, ethanol control; 8, naked DNA control. Positions of guanine residues within transcription factor binding sites are indicated on the right. HRE I to HRE IV indicate the TGTTCT hexanucleotide motifs. Positions of relevant guanine residues are as follows: HRE I, −174; HRE II, −118; HRE III, −97; HRE IV, −82. NF-I indicates the NF-I binding site with the relevant guanines at −73 and −74. OCT-D indicates the promoter distal octamer motif with the relevant guanine at −62. OCT-P indicates the promoter proximal octamer motif with the relevant guanine at −48. Positions of guanine residues that become hypermethylated upon factor binding are indicated by arrows. Protection of the G within HRE II is masked by the proximity of one of these hypermethylated residues.



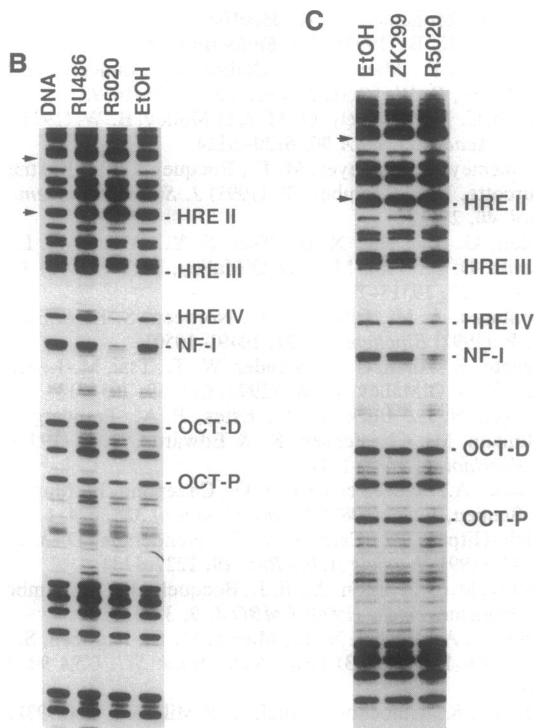


FIG. 2. Effect of progestins and antiprogestins on transcription of the MMTV promoter and DNA binding of PR. (A) Cells were treated with ethanol (control), with the synthetic progestin R 5020 (10^{-8} M), with the antiprogestin RU 486 (10^{-6} M) or ZK 98299 (10^{-6} M), or combinations thereof. Luciferase activity was determined after 24 hr. Results are representative of at least three independent experiments and show means ± SD of three plates processed in parallel. (B) Genomic footprinting experiments with cells exposed to RU 486 (10^{-6} M), R 5020 (10^{-8} M), and ethanol (control). Analysis of these samples and of *in vitro* methylated control DNA was performed by LMPCR (16). (C) Same experiment performed with ZK 98299 (10^{-6} M) instead of RU 486.

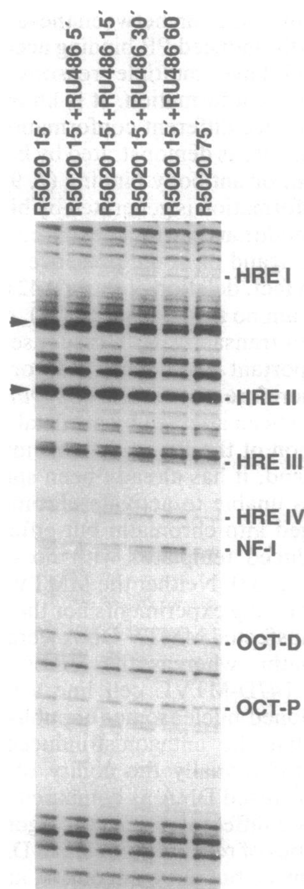


FIG. 3. RU 486 treatment leads to a rapid disappearance of all agonist-induced protein–DNA interactions on the MMTV promoter. Cells were treated with 10^{-7} M R 5020 for 15 min (R 5020 15′) to induce DNA binding of PR, NF-I, and OTF-1, followed by a 10-fold excess of the antihormone RU 486. After 5, 15, 30, and 60 min, binding of PR and the other transcription factors was analyzed by genomic footprinting (16). As a control, ethanol was added to cells treated for 15 min with R 5020, and incubation was continued for an additional 60 min (R 5020 75′).

Simultaneously with the disappearance of the PR footprint, antiprogestins also eliminate the footprint over the NF-I binding site and the weak protection over the octamer motifs (Fig. 3). It could be argued that antiprogestins do not induce a PR footprint because, contrary to agonists, they generate a conformation of the PR unable to cooperate with NF-I for DNA binding. However, in similar experiments with stably transfected cells, mutation of the NF-I binding site does not eliminate the *in vivo* footprint over the HREs (data not shown), suggesting that interaction with NF-I is not responsible for the agonist-induced receptor footprint.

DISCUSSION

T47D cells have been widely used for the study of PR action on the MMTV promoter in response to progestins and as a source of extracts for cell-free transcription and DNA binding studies, because they contain high levels of PR (12, 23–26). None of these experiments has provided evidence for proteins other than the receptor that might have the potential to bind to the four HREs of the MMTV promoter. This is important because of previous controversies concerning the nature of the protein protecting one of the HREs of the rat tyrosine aminotransferase gene in hepatocytes (27). We therefore think that our T47D-MTVL cell line is particularly suitable to study hormone or antihormone effects on PR binding to the MMTV promoter *in vivo*. The good correlation between induction by the synthetic progestins R 5020 and protection against DMS methylation over the corresponding guanine residues of the HREs and the similarity to the methylation protection pattern observed with purified PR (19) strongly suggest that the factor responsible for this protection *in vivo* is the PR.

Our results that RU 486 and ZK 98299 are unable to induce DNA binding of PR represent direct evidence on the effects of antiprogestins on the ability of endogenous PR to bind to a HRE *in vivo*. On the other hand, it is known that the partially purified PR can bind to HREs in the presence of antiprogestins *in vitro* (23, 28, 29) and, after transient cotransfection of a wild-type PR, RU 486 treatment prevented transcriptional activation by a constitutive PR lacking the steroid binding domains. This was interpreted as a competition for DNA binding (30).

The apparent contradiction between these findings and the lack of antiprogestin-induced PR binding according to *in vivo* footprinting could have multiple reasons related to the changes in receptor conformation. It is known that agonists and antagonists induce different conformations of the C-terminal region of the PR, as demonstrated by band shift assays, protease digestion, or antibody binding (7, 9). While *in vivo* the active PR conformation is generated by binding of agonist ligands, *in vitro* conditions can induce this conformation even in the absence of ligand or in the presence of antihormones (23, 28, 29, 31). In fact, deletion of the last 42 amino acids (9), and even a single amino acid exchange (32), converts the PR into a form able to transactivate in response to antagonists, suggesting an important role of the receptor C terminus for DNA binding. Therefore, the results of *in vitro* DNA binding studies could have been affected by artificial induction of the active conformation of the PR by *in vitro* manipulations.

On the other hand, it has already been shown that newly synthesized PR is unable to activate chromosomal MMTV templates packaged into chromatin but efficiently activates transiently introduced templates with no or a disordered chromatin structure (33). Neither the MMTV fragments used for *in vitro* DNA binding experiments nor the binding sites on the transiently transfected MMTV DNA were properly packaged into chromatin, whereas the HREs of the MMTV promoter in the T47D-MTVL cell line are covered by a specifically positioned nucleosome (unpublished data). One could envision that the antagonist-induced conformation could influence differentially the ability of PR to bind to nucleosomally organized DNA as compared to naked DNA.

Also possible is a differential effect of agonists and antagonists on the kinetics of receptor binding to DNA. It has been reported that agonists, but not antagonists, accelerate the on and off rates of PR binding to unspecific DNA (28). In *in vitro* binding experiments or in transient transfections, the concentrations of receptors and HREs are high and may compensate for slow binding kinetics. However, under physiological conditions, with single HREs embedded in a large excess of random DNA, the critical parameter determining HRE occupancy by the PR may be the rate at which the receptor searches the genomic DNA. The efficiency of this process, which involves transfer between DNA sites requiring repeated rounds of association and dissociation, depends on the on and off rates of receptor binding to DNA. In such a scenario, only a PR in its active conformation, as induced by an agonist, would be capable of efficiently searching chromosomal DNA and bind to HREs.

RU 486 is not only an antiprogestin, it also displays antiglucocorticoid activity. In agreement with our findings, RU 486 does not induce binding of glucocorticoid receptor to the HRE of the tyrosine aminotransferase gene *in vivo* (34). This suggests that the antiglucocorticoid and antiprogestin action of RU 486 involves prevention of specific DNA binding, probably by the same mechanism. A partial agonistic effect of RU 486, on both glucocorticoid and progesterone receptors, is observed under conditions when protein kinase A is activated (35–37), suggesting that the DNA binding ability of RU 486-loaded receptors is affected by cAMP treatment. Whether this involves a direct phosphorylation of the receptor molecule or some indirect mechanism is not yet clear.

It is also possible that the two forms of the PR, A and B, respond differently to the added ligand. There are reports suggesting that the A form of PR can be a dominant repressor of MMTV transactivation by the B form in certain cells (38). However, this effect does not seem to require DNA binding and is therefore not incompatible with our conclusions.

While the mechanistic arguments remain hypothetical, our experimental results demonstrate that, under physiological conditions, two widely used antiprogestins are not able to elicit efficient binding of PR to the HRE in the natural context of the MMTV promoter *in vivo*. Therefore, the mechanism of action of antiprogestins, which is of considerable relevance for designing pharmaceutical strategies for hormone therapy, has to be reconsidered.

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1. Beato, M. (1989) *Cell* **56**, 335–344.
2. Truss, M. & Beato, M. (1993) *Endocr. Rev.* **14**, 459–479.
3. Tung, L., Mohamed, M. K., Hoeffler, J. P., Takimoto, G. S. & Horwitz, K. B. (1993) *Mol. Endocrinol.* **7**, 1256–1265.
4. Power, R. F., Mani, S. K., Codina, J., Conneely, O. M. & O'Malley, B. W. (1991) *Science* **254**, 1636–1639.
5. Smith, C. L., Conneely, O. M. & O'Malley, B. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6120–6124.
6. Gronemeyer, H., Meyer, M. E., Bocquel, M. T., Kastner, P., Turcotte, B. & Chambon, P. (1991) *J. Steroid Biochem. Mol. Biol.* **40**, 271–278.
7. Allan, G. F., Leng, X. H., Tsai, S. Y., Weigel, N. L., Edwards, D. P., Tsai, M. J. & O'Malley, B. W. (1992) *J. Biol. Chem.* **267**, 19513–19520.
8. DeMarzo, A. M., Oñate, S. A., Nordeen, S. K. & Edwards, D. P. (1992) *Biochemistry* **31**, 10491–10501.
9. Vegeto, E., Allan, G. F., Scrader, W. T., Tsai, M.-J., McDonnell, D. & O'Malley, B. W. (1992) *Cell* **69**, 703–713.
10. Weigel, N. L., Beck, C. A., Estes, P. A., Prendergast, P., Altmann, M., Christensen, K. & Edwards, D. P. (1992) *Mol. Endocrinol.* **6**, 1585–1597.
11. Groyer, A., Schweizer-Groyer, G., Cadepond, F., Mariller, M. & Baulieu, E. E. (1987) *Nature (London)* **328**, 624–626.
12. Klein-Hitpass, L., Cato, A. C. B., Henderson, D. & Ryffel, G. U. (1991) *Nucleic Acids Res.* **19**, 1227–1234.
13. Meyer, M. E., Pornon, A., Ji, J., Bocquel, M. T., Chambon, P. & Gronemeyer, H. (1990) *EMBO J.* **9**, 3923–3932.
14. Beck, C. A., Weigel, N. L., Moyer, M. L., Nordeen, S. K. & Edwards, D. P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4441–4445.
15. Delabre, K., Guiochon-Mantel, A. & Milgrom, E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4421–4425.
16. Horwitz, K. B., Zava, D. T., Thilagia, A. K., Jensen, E. M. & McGuire, W. L. (1978) *Cancer Res.* **38**, 2434–2437.
17. Klehr, D., Maass, K. & Bode, J. (1991) *Biochemistry* **30**, 1264–1270.
18. Pfeifer, G. P., Steigerwald, S., Mueller, P. R. & Riggs, A. D. (1989) *Science* **246**, 810–813.
19. Chalepakis, G., Arnemann, J., Slater, E. P., Brüller, H., Gross, B. & Beato, M. (1988) *Cell* **53**, 371–382.
20. Cordingley, M. G., Riegel, A. T. & Hager, G. L. (1987) *Cell* **48**, 261–270.
21. Brüggemeier, U., Rogge, L., Winnacker, E. L. & Beato, M. (1990) *EMBO J.* **9**, 2233–2239.
22. Brüggemeier, U., Kalff, M., Franke, S., Scheidereit, C. & Beato, M. (1991) *Cell* **64**, 565–572.
23. Bagchi, M. K., Elliston, J. F., Tsai, S. Y., Edwards, D. P., Tsai, M. J. & O'Malley, B. W. (1988) *Mol. Endocrinol.* **2**, 1221–1229.
24. Beck, C. A., Weigel, N. L. & Edwards, D. P. (1992) *Mol. Endocrinol.* **6**, 607–620.
25. Berkenstam, A., Glaumann, H. & Gustafsson, J. A. (1988) *Mol. Endocrinol.* **2**, 571–578.
26. Edwards, D. P., Kühnel, B., Estes, P. A. & Nordeen, S. K. (1989) *Mol. Endocrinol.* **3**, 381–391.
27. Rigaud, G., Roux, J., Pictet, R. & Grange, T. (1991) *Cell* **67**, 977–986.
28. Schauer, M., Chalepakis, G., Willmann, T. & Beato, M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1123–1127.
29. El-Ashry, D., Oñate, S. A., Nordeen, S. K. & Edwards, D. P. (1989) *Mol. Endocrinol.* **3**, 1545–1558.
30. Guiochon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M. & Milgrom, E. (1988) *Nature (London)* **336**, 695–698.

31. Willmann, T. & Beato, M. (1986) *Nature (London)* **324**, 688–691.
32. Shemshedini, L., Ji, J. W., Brou, C., Chambon, P. & Gronemeyer, H. (1992) *J. Biol. Chem.* **267**, 1834–1839.
33. Smith, C. L., Archer, T. K., Hamlin-Green, G. & Hager, G. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11202–11206.
34. Becker, P. B., Gloss, B., Schmid, W., Strähle, U. & Schütz, G. (1986) *Nature (London)* **324**, 686–688.
35. Beck, C. A., Weigel, N. L., Moyer, M. L., Nordeen, S. K. & Edwards, D. P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4441–4445.
36. Sartorius, C. A., Tung, L., Takimoto, G. S. & Horwitz, K. B. (1993) *J. Biol. Chem.* **268**, 9262–9266.
37. Nordeen, S. K., Bona, B. J. & Moyer, M. L. (1993) *Mol. Endocrinol.* **7**, 731–742.
38. Vegeto, E., Shahbaz, M. M., Wen, D. X., Goldman, M. E., O'Malley, B. W. & McDonnell, D. P. (1993) *Mol. Endocrinol.* **7**, 1244–1255.