BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 252–256 (1997)
ARTICLE NO. RC977363

A Conditional Version of the Ets Transcription Factor Erm by Fusion to the Ligand Binding Domain of the Oestrogen Receptor

Hélène Pelczar,* Olivier Albagli,† Anne Chotteau-Lelievre,*,‡
Isabelle Damour,* and Yvan de Launoit*,[1]

**Mécanismes du Développement et de la Cancérisation, UMR 319 CNRS/Institut Pasteur de Lille - Institut de Biologie de Lille, 1 rue Calmette, BP 447, 59021 Lille Cedex, France; †INSERM U 124, IRCL, Place de Verdun, 59045 Lille Cedex, France; and ‡Centre de Biologie Cellulaire, Unité Dynamique des Cellules Embryonnaires et Cancéreuses, Bâtiment SN3, USTL, Villeneuve d'Ascq, France*

Received August 9, 1997

The fusion of a wide range of proteins to the ligand-binding domain of nuclear receptors has been shown to impart ligand-dependent inducible activity of the resulting chimera. Transcriptional regulators of the ETS family are involved in both normal and oncogenic processes. In order to address the role of Erm, a "*PEA3* subgroup" member of this family, we generated a chimera between Erm and the widely used ligand-binding domain of the oestrogen receptor (ER). The chimera, ErmER, consists of Erm protein fused at its C-terminal end to the ER domain. We show that ErmER displays a ligand-dependent transcriptional activity on *ets* responsive elements. The efficiency of ErmER mediated transactivation is modulated by the hormone concentration while its weak leakiness is reduced by using the steroidal anti-oestrogen EM-139. Our results define ErmER as the first conditional version of an Ets transcription factor, providing a useful tool to decipher Erm biological role and to identify potential Erm target genes.

Key Words: **Erm; Ets; transcription factor; oestrogen receptor; EM-139; Chimera.**

The *ets* family members are genes encoding transcription factors involved in both transcriptional activation and repression. They share the family hallmark domain named the Ets domain, responsible for their specific DNA binding to sequences centred on the GGAA/T core. Based on sequence identity data, several subgroups of highly related *ets* members have been defined (1, 2). The "PEA3 subgroup" is composed of *pea3* (3), *erm* (4) and *er81* (5) classified by virtue of their close similarity to *pea3*. In addition to the Ets domain, two conserved transactivating domains have been defined within Erm and Er81: the N-terminal acidic domain and the region localised in the C-terminal tail of the proteins just downstream of the Ets domain (6, 7). Furthermore, both Erm and Er81 have been shown to be targets for signalling pathways through MAP kinases (7, 8), while Erm is activated by protein kinase A (8).

Although Erm transcriptional properties *in vitro,* as well as *erm* embryonic spatio-temporal expression (9, for a review, see 10) have been well characterised, little is known about the biological processes in which Erm could be involved. It has been proposed that they could contribute to the metastatic processes in particular in the cancers of the mammary gland (11, 12).The availability of a selective and tightly regulated Erm-inducible system would be helpful to further address the question of Erm role. Such a molecular tool is provided by the translational fusion of proteins to the ligand-binding domain of several nuclear receptors (13). As exemplified for the transcription factors Myc (14), Myb (15), Fos (16), Jun (17), v-Rel (18), p53 (19), MyoD (20) or NF-M (21), the chimeric proteins display a ligand-regulated activity, as they are usually inert without hormone while they become active in the presence of the appropriate ligand.

Following this strategy, we created an inducible version of Erm by fusing the ligand binding domain (ER) of the oestrogen receptor at the C-terminal end of the full-length Erm. Here we describe the basic properties of the resulting chimera ErmER. Our results demonstrate that ErmER transcriptional activity on *ets* sites is oestrogen-dependent. Furthermore, by using the pure steroidal anti-oestrogen EM-139 (22) we were able

[1] To whom correspondence should be addressed. E-mail: delaunoi@infobiogen.fr. Fax: (int) 33 320 87 79 08.

0006-291X/97 $25.00

to reduce ErmER basal activity, suggesting that the weak ErmER leakiness is due to a residual oestrogen-like activity from the culture medium. Taken together, our results define ErmER as a useful tool to clarify the biological role of Erm.

MATERIALS AND METHODS

Plasmids. The Erm derivatives were expressed from expression vectors pTL1 and pCDNA3 (InVitrogen).

erm EcoRI cDNA sequence (4) was cloned into the EcoRI site of the pCDNA3. To construct the chimera ErmER, the stop codon within the EcoRI fragment of *erm* cDNA was replaced by a BamHI site using PCR with oligonucleotide 5′-GGGATCCCAAGCAAAGCC-TTCGGCATAGGGGAG-3′ as antisense primer; the BamHI-SstI fragment derived from the HE14 construction (23) and containing the ER sequence was cloned in-frame with Erm modified sequence using the new BamHI site. The ErmER sequence was inserted into the EcoRI and SstI sites of the pTL1. It was inserted into the EcoRI and XhoI sites of the pCDNA3 plasmid by using an adaptator between the ER 3′end SstI site and the pCDNA3 XhoI site (Detailed information upon request).

The TORU-luc plasmid (8) was used as a reporter plasmid.

Cell culture and transfection. Human cervical carcinoma HeLa cells were grown at 37°C and in water-saturated 5% CO_2 atmosphere, in Dulbecco's modified medium (DMEM) supplemented with 10% foetal calf serum (Gibco BRL). These cells do not express the endogenous oestrogen receptor, as assessed by dose response experiments to an oestrogen treatment on cells transfected by a reporter plasmid containing an oestrogen responsive element (data not shown).

Transfection experiments were carried out in 6 well-plates using either optiMEM (Gibco BRL) or DMEM without red phenol (Gibco BRL) containing 4g/l glucose. Before transfection, cells grown at 50% confluence were washed twice (5 min and 1 h) in the corresponding transfection medium. Cells were then transfected for 5 h using 500 ng expression vector and 150 ng reporter plasmid in 1 μg total DNA and 10 μl lipofectamine (Gibco BRL) in 1ml transfection medium. Finally, transfection medium was replaced by fresh medium containing 17β-oestradiol (Sigma) or EM-139 (22) from 1000-fold concentrated stock solutions in ethanol at the concentration mentioned in the figure legends.

For the immunofluorescence analyses, cells were grown on 14mm coverslips before transfection.

Luciferase assay. Cells were lysed in 500 μl of Reporter Lysis Buffer (Promega) 24h to 48h after transfection. Cell extracts were analysed as described (6). The data presented here are the mean ± sem of at least 3 independent experiments.

Immunoprecipitation. Twenty four h after transfection, cells were starved for 1h in Met+Cys free MEM (ICN) and then labelled with 100μCi/ml (^{35}S)Met+(^{35}S)Cys (Amersham) for 3h in 0.5ml Met+Cys free medium. Cells were lysed and immunoprecipitations were performed as described (12), by using anti-$Erm_{12\text{-}226}$ (8) and anti-$Erm_{355\text{-}510}$ (12) antibodies.

Immunofluorescence analysis. All the following steps were separated by three PBS 1X washes at room temperature. Twenty four h after transfection, cells growing on 14mm coverslips were fixed in 4% paraformaldehyde PBS 1X for 15 min at room temperature, permeabilized with 0.25% Triton PBS 1X for 5 min at room temperature, and neutralised in PBS 1X containing 50 mM NH_4Cl for 5 min at room temperature. Preparations were blocked in 2% foetal calf serum PBS 1X (blocking buffer) for 30 min at 4°C. The rabbit polyclonal anti-$Erm_{12\text{-}226}$ antibody raised against Erm (8) was applied at 1/500 dilution in blocking buffer for 60 min at 4°C. Preparations were washed 3 times in blocking buffer and incubated with 1/200 diluted mouse anti rabbit FITC conjugated antibody (Jackson) in blocking buffer for 45 min at 4°C. Cell nuclei were then stained with Hoechst 33258 (Sigma) at 4 mg/l in PBS 1X for 5 min (data not shown). Cells were mounted in Moviol 4.88 (Sigma) containing 25 mg/ml 1,4-diazabicyclo(2.2.2)octane (Sigma) as an antiquentching agent and examined at x63 magnification with a Zeiss Axiovert microscope equipped with epi-illumination.

RESULTS AND DISCUSSION

ErmER Fusion Protein

The hormone binding domain (ER) of the human oestrogen receptor HE0 (23) was fused to the C-terminus of the human Erm protein as shown in figure 1A. This chimeric fusion was cloned in two expression vectors : the pTL1 and the commercial pCDNA3 vectors. These constructions were efficiently expressed in rabbit reticulocyte lysates (data not shown), as well as in radiolabelled transiently transfected HeLa cells (fig. 1B). Figure 1B shows that HeLa cells appeared to be devoid of any detectable Erm, as no specific common band was revealed after immunoprecipitation using two different anti-Erm polyclonal antibodies, and SDS-PAGE analysis. In this cellular context, the proteins encoded by the constructions displayed the expected migration profile, i.e. 72kDa for Erm and 90kDa for ErmER, approximately. We next performed immunofluorescence analyses to investigate the subcellular location of the chimera upon oestrogen and anti-oestrogen treatments. Figure 1C shows the diffuse nuclear location of the ErmER chimera, as compared to the cells transfected by the empty vector (fig. 1C, control). This location was not affected by the presence of either 10^{-7}M 17β-oestradiol or 10^{-7}M EM-139 (data not shown) and was comparable to the Erm location already described (8).

ErmER Oestrogen-Dependent Transcriptional Activity

The ability of ErmER to transactivate through an *ets* responsive element was tested by cotransfecting ErmER expression plasmids with the TORU-Luc reporter plasmid containing the well characterised *ets* responsive element derived from the polyoma virus enhancer (24).

All the pCDNA3 constructions were transfected in parallel. Figure 2A shows that the ErmER-mediated transactivation activity was hormonally induced in a dose-dependent manner, as compared to the luciferase reporter activity corresponding to cells transfected by the empty plasmid without 17β-oestradiol addition. At high concentration of 17β-oestradiol ErmER was as efficient as wild-type Erm. Similar results were obtained when using a reporter sequence derived from the LTR of the HTLV-1 virus (25) as an *ets* responsive element (data not shown).

We also tested oestrogen free controlled conditions by placing the cells in red phenol/serum free MEM for 20h after transfection. In this context, luciferase reporter activity due to endogenous transcriptional activ-

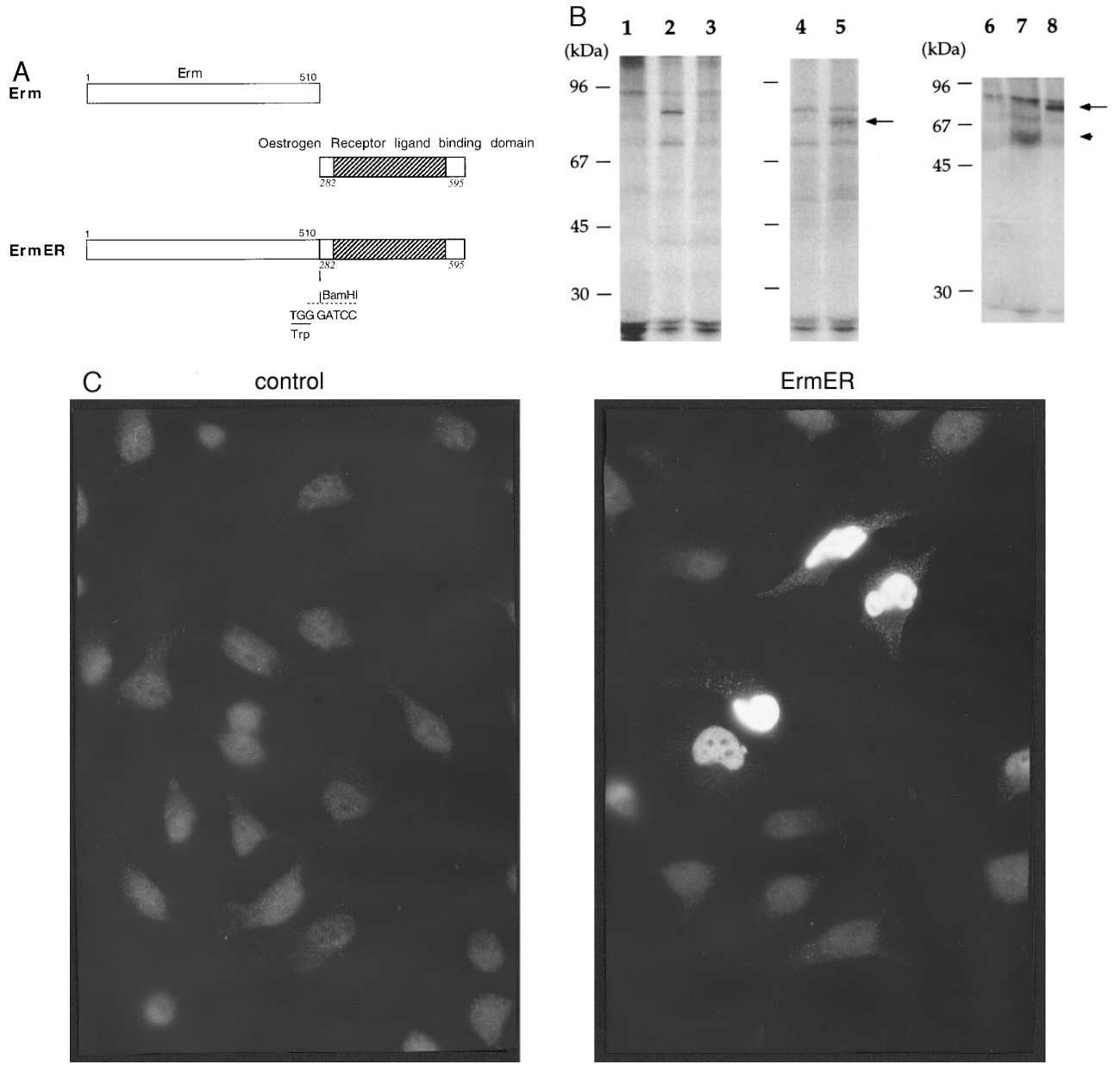

FIG. 1. ErmER fusion protein. (**A**) Schematic representation of ErmER fusion. The ligand-binding domain of the oestrogen receptor (hatched box) was fused to the full length Erm protein (empty box). The numbers indicate amino acid positions of the protein moieties. The translational fusion was made by using a BamHI site (see Materials and Methods section). The tryptophan mentioned (Trp) is at the Erm C-terminal amino acid position in place of the wild-type tyrosine. (**B**) Erm and ErmER expression in transiently transfected HeLa cells. Lanes 1-3, untransfected control HeLa cell extracts devoid of any detectable Erm. Lanes 4-8, HeLa cell extracts after transfection by: pTL1 (lane 4), pTL1-ErmER (lane 5), pCDNA3 (lane 6), pCDNA3-Erm (lane 7), pCDNA3-ErmER (lane 8); migration positions of the encoded proteins are indicated either by an arrow head for Erm or by an arrow for ErmER. Radiolabelled cell lysates were twice immunoprecipitated using two polyclonal antibodies raised against the N-terminal part, anti-$Erm_{12\text{-}226}$, (lane 1) or the C-terminal part, anti-$Erm_{355\text{-}510}$, (lanes 2-8) of Erm. In lane 3, immunoprecipitation was performed in the presence of 10 μg of the corresponding antigen as described previously (12). The positions and sizes of the molecular weight standards are indicated at the left of each gel exposure. (**C**) Diffuse nuclear location of ErmER. HeLa cells transiently expressing ErmER were compared to HeLa cells transfected by the corresponding empty expression plasmid (control). Immunofluorescence analyses using anti-$Erm_{12\text{-}226}$ antibody were carried out 24h after transfection. The same location of ErmER chimera was observed after 10^{-7}M 17β-oestradiol, 10^{-7}M EM-139 or no hormonal treatment.

ity was 20-fold lower than in optiMEM conditions (data not shown) and ErmER transcriptional activity displayed a 14-fold induction upon 10^{-7}M 17β-oestradiol addition (fig. 2B).

Thus, as for other proteins fused to the ligand-binding domain of a nuclear receptor (13), the function of Erm displayed an oestrogen dose-dependent inducible activity, upon its fusion to the ER domain.

Anti-Oestrogen EM-139 Blocked ErmER Activity

When using the pTL1-ErmER construction, we observed a significant basal (without 17β-oestradiol addition) luciferase activity (fig. 3).

Such a leaky behaviour has been already described for other chimeric proteins using ER, as exemplified by TLS-CHOP-ER (26) or p53-ER (19). This basal activity can reflect a residual oestrogen-like activity from the culture medium and/or an intrinsic leakiness of the chimera.

In the case of ErmER, we wish to know whether its basal activity was under oestrogen control or not. To this end, we tested the effect of the anti-oestrogen EM-139 (22). Figure 3 shows that ErmER basal activity was reduced by the addition of 10^{-7}M EM-139 while ErmER induction at 10^{-7}M 17β-oestradiol was not affected. Of note, 10^{-6}M EM-139 fully antagonised ErmER inducible activity. Thus, the antagonistic effect of EM-139 on ErmER transcriptional activity indicates that ErmER basal activity is, at least in part, due to a residual oestrogen-like activity from the culture medium. This conclusion was further strengthened by the fact that when using pCDNA3 constructions, the weak leakiness of ErmER was totally abolished in the presence of 10^{-6}M EM-139 (data not shown).

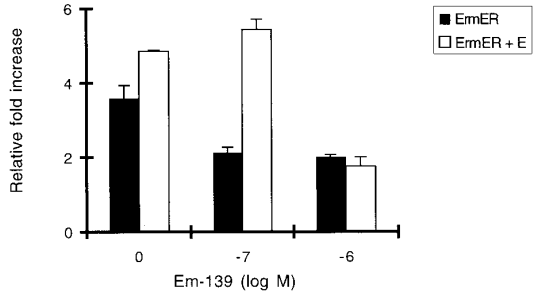

FIG. 3. Antagonistic action of EM-139 on ErmER transcriptional activity. Transfections were performed in optiMEM using pTL1 and pTL1-ErmER (ErmER) constructions. 10^{-7}M 17β-oestradiol (+E) and EM-139 anti-oestrogen at the mentioned concentrations were added 16h after transfection. Twenty-four hours later, the cells were harvested for luciferase assays. The fold-inductions are relative to the empty pTL1 values that did not show any significant differences upon hormonal treatment.

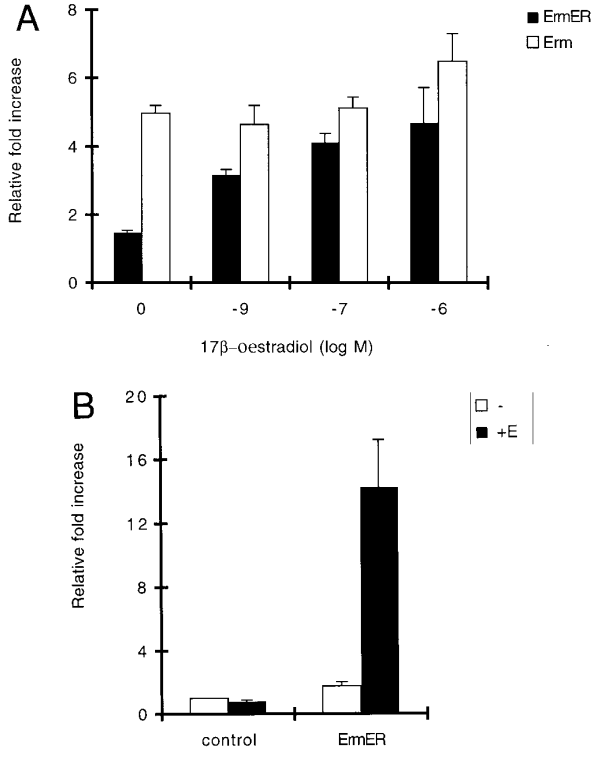

FIG. 2. Oestrogen dependent transcriptional activity of ErmER. (**A**) Dose response of 17β-oestradiol. pCDNA3-ErmER (ErmER) and pCDNA3-Erm (Erm) expression vectors were cotransfected with TORU-Luc reporter plasmid into HeLa cells using optiMEM medium. 17β-oestradiol at the mentioned concentrations was added 16h after transfection. Twenty-four hours later, the cells were harvested for luciferase assays. The fold-inductions are relative to the empty pCDNA3 values that did not show any significant differences upon 17β-oestradiol treatment. (**B**) ErmER transcriptional induction in oestrogen-free conditions. Transfection experiments were performed using pTL1 (control) and pTL1-ErmER (ErmER) plasmids in red phenol and serum free MEM. Twenty-four hours after transfection, 10^{-7}M 17β-oestradiol (+E) was added to the medium, and 20h later the cells were harvested for luciferase assays. The fold-inductions are relative to the control vector without hormone addition.

In conclusion, while many putative target genes have been proposed for the Ets factors, none of them were conclusively shown to be directly regulated as endogenous "resident" genes. Our results demonstrate that the fusion of Erm to the ligand-binding domain of the oestrogen receptor imparts oestrogen dependence to its activity. This defines ErmER as a molecular tool to address the biological functions of Erm. In particular, by combining the oestrogen induction of the chimera and the inhibition of the protein-neosynthesis, ErmER should allow the identification of Erm direct target within chromatin-embedded genes.

ACKNOWLEDGMENTS

The authors thank P. Dhordain, C. Dozier, and J. Coll for their critical reading of this manuscript, T. Lufkind, M. Eilers, and F. Labrie for the gift of reagents. This work was supported by grants from the "Association pour la Recherche contre le Cancer" (France), the "Ligue contre le Cancer" (France), and the "Centre National de la Recherche Scientifique" (France).

REFERENCES

1. Lautenberger, J. A., Burdett, L. A., Gunnell, M. A., Qi, S., Watson, D. K., O'Brien, S. J., and Papas, T. S. (1992) *Oncogene* **7,** 1713–1719.

2. Laudet, V., Niel, C., Duterque-Coquillaud, M., Leprince, D., and Stéhelin, D. (1993) *Biochem. Biophys. Res. Commun.* **190,** 8–14.
3. Xin, J. H., Cowie, A., Lachance, P., and Hassell, J. A. (1992) *Genes Dev.* **6,** 481–496.
4. Monté, D., Baert, J. L., Defossez, P. A., de Launoit, Y., and Stéhelin, D. (1994) *Oncogene* **9,** 1397–1406.
5. Brown, T. A., and McKnight, S. L. (1992) *Genes Dev.* **6,** 2502–2512.
6. Laget, M. P., Defossez, P. A., Albagli, O., Baert, J. L., Dewitte, F., Stéhelin, D., and de Launoit, Y. (1996) *Oncogene* **12,** 1325–1336.
7. Janknecht, R. (1996) *Mol. Cell. Biol.* **16,** 1550–1556.
8. Janknecht, R., Monte, D., Baert, J. L., and de Launoit, Y. (1996) *Oncogene* **13,** 1745–1754.
9. Chotteau, A., Desbiens, X., Pelczar, H., Defossez, P. A., and de Launoit, Y. (1997) *Oncogene* **15,** 937–952.
10. de Launoit, Y., Baert, J. L., Chotteau, A., Monte, D., Defossez, P. A., Coutte, L., Pelczar, H., and Leenders, F. (1997) *Biochem. Mol. Med.*, in press.
11. Kaya, M., Yoshida, K., Higashino, F., Mitaka, T., Ishii, S., and Fujinaga, K. (1996) *Oncogene* **12,** 221–227.
12. Baert, J. L., Monté, D., Musgrove, E. A., Albagli, O., Sutherland, R. L., and de Launoit, Y. (1997) *Int. J. Cancer* **70,** 590–597.
13. Mattioni, T., Louvion, J. F., and Picard, D. (1994) *Methods Cell Biol.* 335–352.
14. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) *Nature* **340,** 66–68.
15. Burk, O., and Klempnauer, K. H. (1991) *EMBO J.* **10,** 3713–3719.
16. Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5114–5118.
17. Fialka, I., Schwarz, H., Reichmann, E., Oft, M., Busslinger, M., and Beug, H. (1996) *J. Cell Biol.* **132,** 1115–1132.
18. Boehmelt, G., Walker, A., Kabrun, N., Mellitzer, G., Beug, H., Zenke, M., and Enrietto, P. J. (1992) *EMBO J.* **11,** 4641–4652.
19. Roemer, K., and Friedman, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9252–9256.
20. Hollenberger, S. M., Cheng, P. F., and Weintraub, H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8028–8032.
21. Muller, C., Kowenz-Leutz, E., Grieser-Ade, S., Graf, T., and Leutz, A. (1995) *EMBO J.* **14,** 6127–6135.
22. de Launoit, Y., Dauvois, S., Dufour, M., Simard, J., and Labrie, F. (1991) *Cancer Res.* **51,** 2797–2802.
23. Kumar, V., Green, S., Staub, A., and Chambon, P. (1986) *EMBO J.* **5,** 2231–2236.
24. Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D., and Stehelin, D. (1990) *Nature* **346,** 191–193.
25. Gegonne, A., Punyammalee, B., Rabault, B., Bosselut, R., Seneca, S., Crabeel, M., and Ghysdael, J. (1992) *New Biol.* **4,** 512–519.
26. Zinszner, H., Albalat, R., and Ron, D. (1994) *Genes Dev.* **8,** 2513–2526.