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The developmentally regulated transcription factor AP-2 is involved in c-*erbB-2* overexpression in human mammary carcinoma

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ABSTRACT **Overexpression of the c-*erbB-2*/HER2 protooncogene in breast carcinoma is controlled not only by the degree of amplification of the gene but also at the level of gene transcription. Thus, whether or not the gene is amplified, the activity of the c-*erbB-2* promoter is enhanced in overexpressing cells through the binding of an additional transcription factor, OB2-1, whose activity is increased in these lines. Here we describe further characterization of OB2-1 and show that it is identical to the developmentally regulated transcription factor AP-2. Functional assays confirm that AP-2 is able to regulate c-*erbB-2* expression in mammary-derived cell lines. Furthermore, although AP-2 is barely detectable in cells with the low c-*erbB-2* expression phenotype, protein levels are clearly elevated in a panel of c-*erbB-2*-overexpressing lines. These findings demonstrate an important role for this transcription factor in human cancer.**

The c-*erbB-2*/HER2 protooncogene is related to the epidermal growth factor receptor (EGFR) and encodes a 185-kDa receptor tyrosine kinase which is overexpressed in a number of human tumors, including 25–30% of breast carcinomas, where it is a marker of poor prognosis (1). Several studies *in vitro* and in transgenic mice have shown that overexpression of c-*erbB-2* or its rodent counterpart, c-*neu*, leads to cellular transformation (2–6), thus implicating this gene in the pathogenesis of breast tumors. Protein overexpression is generally associated with amplification of the c-*erbB-2* gene (1); however, it also occurs from a single-copy gene (7–10). Indeed, further studies in mammary-derived cell lines have shown that, whether or not the gene is amplified, there is a 6- to 8-fold increase in accumulation of c-*erbB-2* mRNA per gene in overexpressing cells (11, 12). This overexpression is due to the increased activity of the c-*erbB-2* promoter resulting from an elevation in the activity of the transcription factor OB2-1, present in all overexpressing lines, which binds and activates the c-*erbB-2* promoter (13).

The c-*erbB-3* gene is a third member of the EGFR family and can also be overexpressed both in mammary tumors and derived cell lines (14, 15), often in parallel with c-*erbB-2* overexpression (16). Increased transcription of c-*erbB-3* in mammary cell lines is also mediated by the transcription factor OB2-1 (17). Comparison of the OB2-1 binding site sequences within the two promoters has now revealed a similarity to the binding site for the developmentally regulated transcription factor AP-2. In this paper we describe our further characterization of OB2-1 and show that it is antigenically and functionally indistinguishable from AP-2, thus demonstrating an additional role for this factor in human cancer.

MATERIALS AND METHODS

DNase Footprinting. The c-*erbB-2* promoter probes were labeled at the *Avr*II site at −300; the reactions were as previously described (18). Both the wild-type promoter and an inactive mutant version lacking 4 bp within the OB2-1 binding site have been described (13). AP-2 expressed in bacteria and AP-2 protein from HeLa cells were affinity purified (18).

Electrophoretic Mobility-Shift Assays. These were carried out as detailed (19) with a double-stranded oligonucleotide representing the OB2-1 binding site within the c-*erbB-2* promoter as the probe (13). Proteins and antisera were coincubated for 2 hr at 4°C prior to addition of probe. Crude nuclear extracts from mammary cell lines were prepared according to Dignam *et al.* (20) and *in vitro* translated AP-2 was prepared from the T7βAP-2 clone (21) with the TNT kit (Promega). Generation of the N-terminal AP-2 antibody has been detailed (22); the C-terminal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

OB2-1 Antibodies. Crude nuclear extract (20) from the c-*erbB-2*-overexpressing mammary line ZR75-1 was dialyzed versus buffer D (20 mM Hepes·KOH, pH 8.0/1 mM $MgCl_2$/0.5 mM EDTA/0.03% lauryldimethylamine oxide/0.5 mM phenylmethanesulfonyl fluoride/2 mM dithiothreitol) containing 150 mM KCl. This was applied to a Bio-Rex 70 column (Bio-Rad), washed and step eluted with buffer D containing successively 0.33, 0.68, and 1.0 M KCl. The 0.68 M peak, containing OB2-1 binding activity, was dialyzed versus buffer D/0.15 M KCl, passed over a nonspecific DNA column, and subjected to two rounds of affinity chromatography using the OB2-1 binding site in the c-*erbB-2* promoter immobilized on Sepharose. Peak fractions were identified by electrophoretic mobility-shift assay. OB2-1 antiserum was raised in C57Bl × BALB/c mice using 200 ng of purified OB2-1 binding activity per injection in an alum precipitate (19). The specificity of the antiserum was confirmed by mobility-shift and Western blot assays on crude and purified protein.

Western Blotting. Proteins were separated by SDS/PAGE and transferred to nitrocellulose. Binding of the primary antibodies was visualized with the ECL system (Amersham).

Transfection Assays. Calcium phosphate transfections were carried out as described (13) with the addition of 1–3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) as an internal control. Chloramphenicol acetyltransferase (CAT) activity in each extract was corrected for transfection efficiency by using the β-galactosidase activity from the same extract. Each set of transfections was repeated at least three times, and the mean results are presented together with the standard errors. Whole cell lysates from additional transfections were harvested for Western blot analysis. The p300CAT construct contains c-*erbB-2* promoter sequence between −300 and +40 and shows full promoter activity in overexpressing cells (13). The pSS0.2CAT control plasmid contains the basal promoter for the human glutathione transferase π gene (13, 23). The construction of AP-2 expression plasmids has been detailed (21). MTSV1.7neo is a nontumorigenic, immortalized

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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mammary epithelial cell line (6) and has the low c-*erbB-2* expressor phenotype (13).

RESULTS

The core binding site for OB2-1 within the c-*erbB-3* promoter is GCCTCTGGC (17), which conforms to the consensus binding site, GCCNNNGGC, determined for the transcription factor AP-2 (21). Inspection of the OB2-1 binding site within the c-*erbB-2* promoter, GCTGCAGGC, revealed that it too is closely related to the consensus AP-2 site (13). The functional significance of these sequence similarities was tested by binding studies. DNase footprinting showed that AP-2 protein, whether purified from HeLa cells or synthesized in bacteria, specifically bound to the c-*erbB-2* promoter (Fig. 1, lanes 6–8 and 2–4) but was unable to bind to an inactive, mutant promoter with a 4-bp deletion within the OB2-1 site (lanes 11–13). Thus, the OB2-1 site can function as a specific AP-2 binding site.

To show that OB2-1 and AP-2 are related proteins (rather than distinct species with overlapping DNA-binding specificities), we used a panel of antisera to challenge protein–DNA complexes containing either OB2-1 from the c-*erbB-2*-overexpressing ZR75-1 mammary cell line or AP-2 synthesized *in vitro*. Antisera raised against N- and C-terminal peptides of AP-2 were able to supershift equivalently DNA complexes containing either OB2-1 (Fig. 2, lanes 2 and 3) or AP-2 (lanes 7 and 8). Moreover, antiserum raised in mice against purified OB2-1 (see *Materials and Methods*) prevented DNA binding by both OB2-1 (lane 4) and AP-2 (lane 9). These results indicate that OB2-1 and AP-2 are highly related or identical proteins.

OB2-1 binding activity is confined to mammary tumor lines which overexpress c-*erbB-2* (13). The distribution of AP-2 within mammary-derived cells was therefore determined. A Western blot of nuclear extracts from 10 mammary cell lines was probed successively with the OB2-1 mouse polyclonal antiserum (Fig. 3*A*) and the antiserum against the N-terminal peptide of AP-2 (Fig. 3*B*). An apparently identical 47-kDa protein was readily detected by both antisera in all c-*erbB-2*-overexpressing lines but was not observed in any of the low-expressing cells (Fig. 3, compare lanes 7–11 with lanes 2–6). Bacterially synthesized AP-2 (lanes 1) was used as a control. The OB2-1 serum also recognized

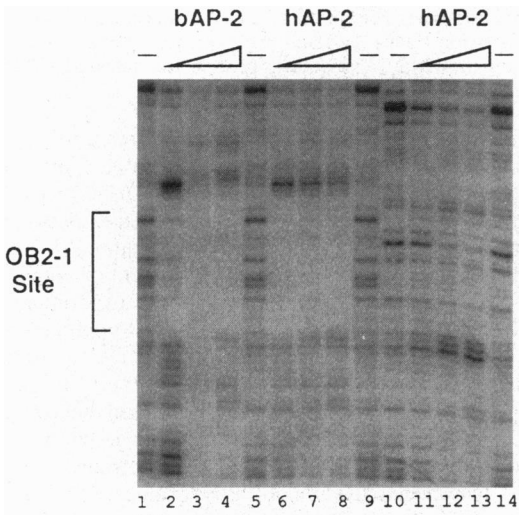

FIG. 1. DNase footprinting of AP-2 on the c-*erbB-2* promoter. Various amounts of bacterially expressed AP-2 (bAP-2; lanes 2–4) or AP-2 purified from HeLa cells (hAP-2; lanes 6–8) were prebound to the wild-type c-*erbB-2* promoter prior to DNase I digestion; control DNase reactions lacked AP-2 (lanes 1, 5, and 9). The position of the known OB2-1 binding site is indicated. As a control, AP-2 was also incubated with a mutant c-*erbB-2* promoter which has a 4-bp deletion within the OB2-1 binding site: incubations with hAP-2 are shown (lanes 11–13) with DNA-only digests (lanes 10 and 14).

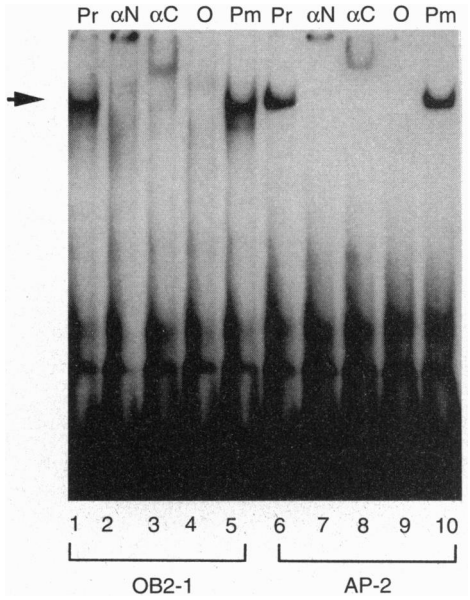

FIG. 2. DNA complexes containing OB2-1 or AP-2 interact identically with a panel of antibodies. The arrow marks specific protein/DNA complexes containing either OB2-1 present in nuclear extract from ZR75-1 cells (lanes 1–5) or *in vitro* synthesized AP-2 (lanes 6–10). The complexes were incubated with various antisera as follows: rabbit preimmune serum, Pr (lanes 1 and 6); AP-2 N-terminal antibody, αN (lanes 2 and 7); AP-2 C-terminal antibody, αC (lanes 3 and 8); anti-OB2-1 mouse antiserum, O (lanes 4 and 9); and mouse pre-immune serum, Pm (lanes 5 and 10).

a contaminating 110-kDa protein present in the purified OB2-1 preparation; this protein was probably a nonspecific DNA-binding protein (data not shown) but served as a useful control of extract integrity, as this species was present in all the cell lines examined. Thus, the abundance of the 47-kDa AP-2 protein reflects the known distribution of OB2-1 binding activity (13) within a range of breast tumor lines.

To establish whether AP-2 is functionally equivalent to OB2-1 at the transcriptional level, we performed a series of transfection experiments using a c-*erbB-2* promoter–CAT gene reporter construct, p300CAT (see *Materials and Methods*). If the activity of the c-*erbB-2* promoter is dependent on the relative abundance of AP-2 in mammary cells, then specific interference with AP-2 activity in cells overexpressing c-*erbB-2* should reduce c-*erbB-2* promoter activity. Conversely, expression of exogenous AP-2 in cells with low expression of c-*erbB-2* should increase promoter activity in these cells. AP-2 has been shown to bind to DNA as a dimer with the binding and dimerization domains located within the C-terminal half of the protein (21). However, an N-terminally truncated form, ΔN278, which is itself unable to bind DNA, still retains the ability to dimerize with the wild-type protein, thereby preventing its binding to DNA (21). Consequently, exogenous expression of ΔN278 in overexpressing cells specifically interferes with endogenous AP-2 in a dominant negative manner. Fig. 4*A* shows the results obtained when various amounts of a ΔN278 expression plasmid were cotransfected with p300CAT into the c-*erbB-2*-overexpressing line ZR75-1. Expression of ΔN278 (as confirmed by Western blot analysis, Fig. 4*C*, lanes 1–3) specifically suppressed the activity of p300CAT, while the activity of an unrelated, control promoter in parallel transfections was unaffected by coexpression of ΔN278 (Fig. 4*A*). In the converse experiment (Figs. 4 *B* and *C*), various amounts of a wild-type AP-2 expression plasmid were cotransfected with p300CAT into a mammary cell line with the low c-*erbB-2* expression phenotype. A specific 5-fold stimulation of c-*erbB-2*

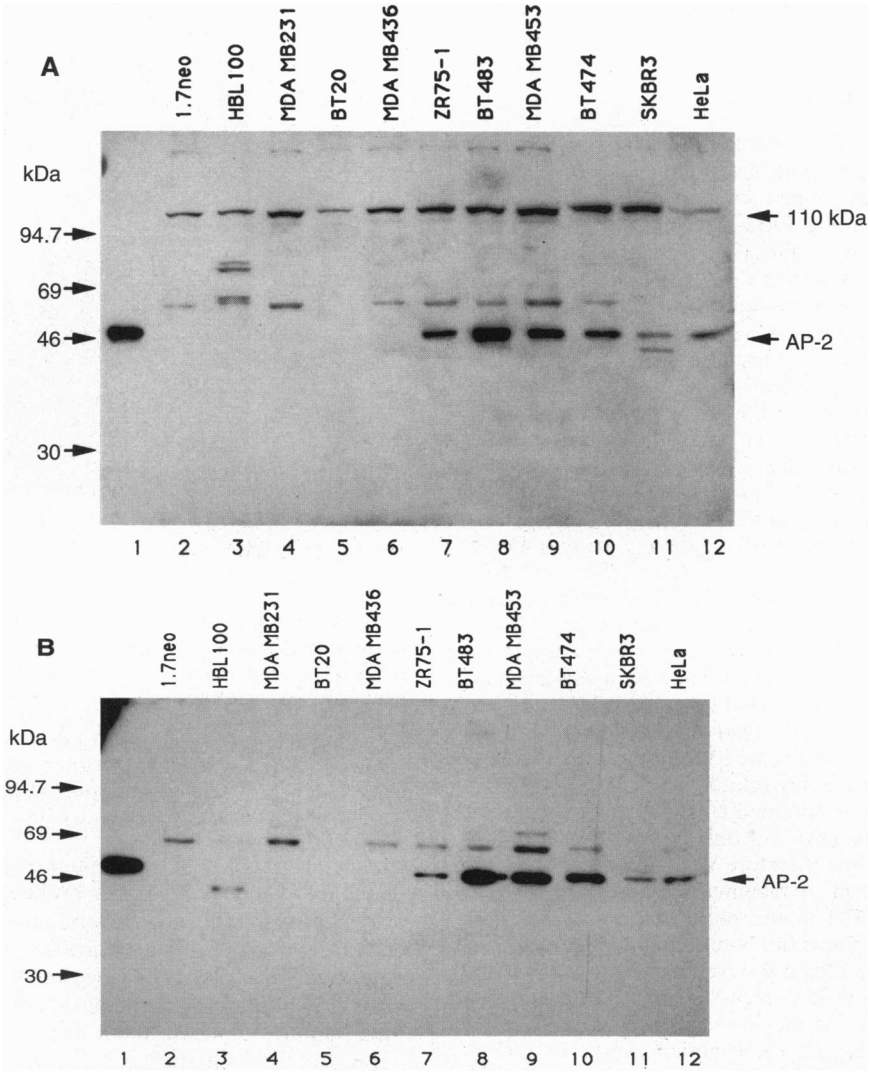

FIG. 3. AP-2 protein is expressed at high levels in mammary lines which overexpress c-*erbB-2*. (*A*) Western blot probed with the mouse OB2-1 antiserum. Bacterially expressed AP-2 was run as a marker (lane 1), and the remaining lanes contained 25 μg of crude nuclear extract from mammary-derived (lanes 2–11) or HeLa (lane 12) cells. Lines with low c-*erbB-2* expression (lanes 2–6) and overexpressers (lanes 7–11) were as indicated. Positions of molecular size markers are shown, and the 47-kDa AP-2 band and the 110-kDa ubiquitous protein are indicated. (*B*) Western blot from *A* was stripped and reprobed with the rabbit antiserum raised against the AP-2 N-terminal peptide.

promoter activity was observed in these cells, confirming that AP-2 can activate this promoter.

DISCUSSION

Overexpression of the c-*erbB-2* protooncogene is an important phenomenon in breast cancer, as it is widely thought to contribute significantly to the poor clinical outcome experienced by the 20–30% of patients that exhibit this phenotype (24). Consequently, an understanding of the molecular mechanisms leading to c-*erbB-2* overexpression is likely to provide insights for development of specific therapies to offer this group of patients. The data reported here show that the transcription factor OB2-1, which is required for increased activity of the c-*erbB-2* promoter in c-*erbB-2*-overexpressing cells (13), is antigenically and functionally identical to the developmentally regulated factor AP-2. This factor has been implicated in the control of gene expression within neural crest and epidermal cell lineages (25, 32) and its expression levels have been shown to be sensitive to retinoic acid (22, 26). Moreover, unlike many transcription factors, AP-2 does not appear to be a member of a large gene family (18, 27, 28), though a non-DNA-binding splice variant has been described (26).

AP-2 has not previously been reported in breast epithelial cells and, as it is not present in lines which represent immortalized "normal" epithelium (see Fig. 3, lanes 2 and 3), it is probable that AP-2 is not normally expressed in adult breast. However, it is unclear how c-*erbB-2*-overexpressing cells acquire AP-2 activity. We have not detected any gross rearrangement or amplification of the AP-2 gene, and Northern blotting experiments have indicated that, although some transcriptional upregulation may occur, posttranscriptional mechanisms must also contribute to the high levels of AP-2 protein observed in c-*erbB-2*-overexpressing cells. Recent data have suggested that overexpression of AP-2 in human teratocarcinoma cells in culture may be induced through activation of the Ras signal transduction pathway (29). However, activation of Ras is considered to occur rarely in breast carcinoma (30). In addition, expression of activated Ha-*ras* in the immortalized MTSV1.7 line leads to full transformation of the cells (31), but with no alteration in their levels of AP-2 protein (unpublished results). Further analysis will therefore be required to elucidate the precise mechanism of AP-2 upregulation in c-*erbB-2*-overexpressing mammary cells.

Nevertheless, the identification of OB2-1 as AP-2 implies a potential role for this factor in human tumors. If examination of tumor biopsy material reveals a close relationship between over-

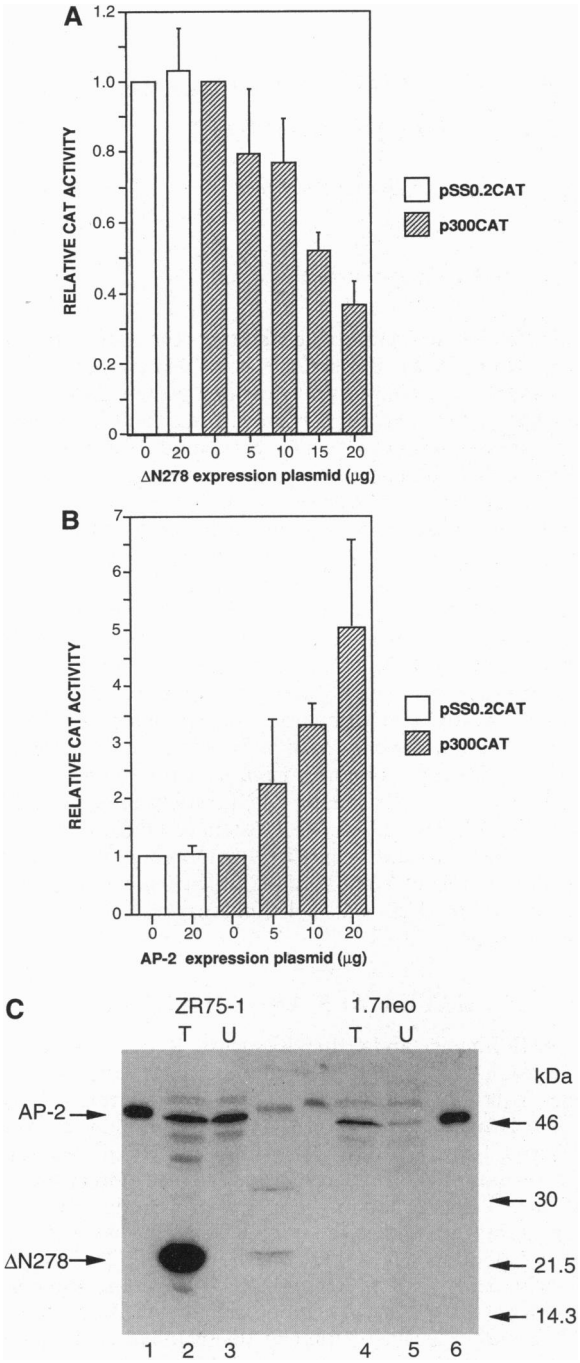

FIG. 4. AP-2 is required for efficient transcription from the c-*erbB-2* promoter. (*A*) Transfection of the ΔN278 expression vector into ZR75-1 cells. Parallel transfection was done with 5 μg of either the c-*erbB-2* reporter plasmid p300CAT or a control promoter, pSS0.2CAT, together with various amounts of the pSPRSVΔN278 expression plasmid as shown. The CAT activity obtained in each transfection set was normalized to the activity obtained from transfection of either reporter alone. (*B*) Transfection of the AP-2 expression vector into MTSV1.7neo cells. Parallel assays were done with 5 μg of either p300CAT or pSS0.2CAT as above, together with various amounts of an expression plasmid for full length AP-2, pSPRSVAP-2. Again, CAT activity was normalized to the values obtained with either reporter alone. (*C*) Western blots to demonstrate expression of either AP-2 or ΔN278 in the appropriate transfected cell lines. Bacterially expressed AP-2 was run as a marker (lanes 1 and 6) with pSPRSVΔN278-transfected (T) (lane 2) and untransfected (U) (lane 3) ZR75-1 cells or pSPRSVAP-2-transfected (lane 4) and untransfected (lane 5) MTSV1.7neo cells. The primary antibody was against the C-terminal peptide of AP-2.

expression of AP-2 and c-*erbB-2* proteins in patient samples, it should be possible to target specifically the activity of AP-2 in a manner which may be developed for therapeutic downregulation of c-*erbB-2* expression. This could provide an anti-c-*erbB-2* strategy to complement other, mainly antiprotein, approaches currently in development in other laboratories (reviewed in ref. 24).

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1. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & Mcguire, W. L. (1987) *Science* **235,** 177–182.
2. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R. & Aaronson, S. A. (1987) *Science* **237,** 178–182.
3. Bouchard, L., Lamarre, L., Tremblay, P. J. & Jolicoeur, P. (1989) *Cell* **57,** 931–936.
4. Di Marco, E., Pierce, J. H., Knicley, C. L. & Di Fiore, P. P. (1990) *Mol. Cell. Biol.* **10,** 3247–3252.
5. Pierce, J. H., Arnstein, P., Dimarco, E., Artrip, J., Kraus, M. H., Lonardo, F., Difiore, P. P. & Aaronson, S. A. (1991) *Oncogene* **6,** 1189–1194.
6. D'Souza, B., Berdichevsky, F., Kyprianou, N. & Taylor-Papadimitriou, J. (1993) *Oncogene* **8,** 1797–1806.
7. King, C. R., Swain, S. M., Porter, L., Steinberg, S. M., Lippman, M. E. & Gelmann, E. P. (1989) *Cancer Res.* **49,** 4185–4191.
8. Hynes, N. E., Gerber, H. A., Saurer, S. & Groner, B. (1989) *J. Cell. Biochem.* **39,** 167–173.
9. Iglehart, J. D., Kraus, M. H., Langton, B. C., Huper, G., Kerns, B. J. & Marks, J. R. (1990) *Cancer Res.* **50,** 6701–6707.
10. Parkes, H. C., Lillicrop, K., Howell, A. & Craig, R. K. (1990) *Br. J. Cancer* **61,** 39–45
11. Kraus, M. H., Popescu, N. C., Amsbaugh, C. & King, C. R. (1987) *EMBO J.* **6,** 605–610.
12. Pasleau, F., Grooteclaes, M. & Golwinkler, R. (1993) *Oncogene* **8,** 849–854.
13. Hollywood, D. P. & Hurst, H. C. (1993) *EMBO J.* **12,** 2369–2375.
14. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9193–9197.
15. Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J. & Hurst, H. C. (1992) *Br. J. Cancer* **66,** 1116–1121.
16. Gasparini, G., Gullick, W. J., Maluta, S., Dallapalma, P., Caffo, O., Leonardi, E., Boracchi, P., Pozza, F., Lemoine, N. R. & Bevilacqua, P. (1994) *Eur. J. Cancer* **30A,** 16–22.
17. Skinner, A. & Hurst, H. C. (1993) *Oncogene* **8,** 3393–3401.
18. Williams, T., Admon, A., Luscher, B. & Tjian, R. (1988) *Genes Dev.* **2,** 1557–1569.
19. Hurst, H. C., Totty, N. F. & Jones, N. C. (1991) *Nucleic Acids Res.* **19,** 4601–4609.
20. Dignam, J. D., Lebowitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
21. Williams, T. & Tjian, R. (1991) *Genes Dev.* **5,** 670–682.
22. Luscher, B., Mitchell, P. J., Williams, T. & Tjian, R. (1989) *Genes Dev.* **3,** 1507–1517.
23. Cowell, I. G., Skinner, A. & Hurst, H. C. (1992) *Mol. Cell. Biol.* **12,** 3070–3077.
24. Hynes, N. E. (1993) *Semin. Cancer Biol.* **4,** 19–26.
25. Mitchell, P. J., Timmons, P. M., Hebert, J. M., Rigby, P. W. J. & Tjian, R. (1991) *Genes Dev.* **5,** 105–119.
26. Buettner, R., Kannan, P., Imhof, A., Bauer, R., Yim, S. O., Glockshuber, R., Vandyke, M. W. & Tainsky, M. A. (1993) *Mol. Cell. Biol.* **13,** 4174–4185.
27. Gaynor, R. B., Muchardt, C., Xia, Y. R., Kliask, I., Mohandas, T., Sparkes, R. S. & Lusis, A. J. (1991) *Genomics* **10,** 1100–1102.
28. Bauer, R., Imhof, A., Pscherer, A., Kopp, H., Moser, M., Seegers, S., Kerscher, M., Tainsky, M. A., Hofstaedter, F. & Buettner, R. (1994) *Nucleic Acids Res.* **22,** 1413–1420.
29. Kannan, F., Buettner, R., Chiao, P. J., Yim, S. O., Sarkiss, M. & Tainsky, M. A. (1994) *Genes Dev.* **8,** 1258–1269.
30. Bos, J. L. (1989) *Cancer Res.* **49,** 4682–4689.
31. Kyprianou, N. & Taylor-Papadimitriou, J. (1992) *Oncogene* **7,** 57–63.
32. Leask, A., Byrne, C. & Fuchs, E. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7948–7952.