MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 3048–3055
0270-7306/90/063048-08$02.00/0

Vol. 10, No. 6

Epidermal Growth Factor Receptor Cytoplasmic Domain Mutations Trigger Ligand-Independent Transformation

SHARON MASSOGLIA,[1] ALANE GRAY,[1] THOMAS J. DULL,[1] SUSAN MUNEMITSU,[1] HSING-JIEN KUNG,[2] JOSEPH SCHLESSINGER,[3] AND AXEL ULLRICH[1]†*

Department of Developmental Biology, Genentech, Inc., South San Francisco, California 94080[1]; Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106[2]; and Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406[3]

Received 27 June 1989/Accepted 8 March 1990

The transforming gene product of avian erythroblastosis virus, v-erbB, is derived from the epidermal growth factor (EGF) receptor but has lost its extracellular ligand-binding domain and was mutated in its cytoplasmic portion, which is thought to be responsible for biological signal generation. We have repaired the deletion of extracellular EGF-binding sequences and investigated the functional consequences of cytoplasmic erbB mutations. Within the resulting EGF receptors, the autophosphorylation activities of the cytoplasmic domains of v-erbB-H and v-erbB-ES4 were fully ligand dependent in intact cells. However, the mitogenic and transforming signaling activities of an EGF receptor carrying v-erbB-ES4 (but not v-erbB-H) cytoplasmic sequences remained ligand independent, whereas those of a receptor with a v-erbB-H cytoplasmic domain were regulated by EGF or transforming growth factor α. Thus, structural alterations in the cytoplasmic domain of growth factor receptor tyrosine kinases may induce constitutive signaling activity without autophosphorylation. These findings provide new insight into the mechanism of receptor-mediated signal transduction and suggest a novel alternative for subversion of cellular control mechanisms and proto-oncogene activation.

The genomes of acutely transforming avian erythroblastosis viruses (AEV) contain derivatives of a normal cellular gene that are directly responsible for the erythroleukemias and fibrosarcomas induced in infected chicks (13, 33). This retroviral transforming gene, termed v-*erbB*, encodes a membrane-associated tyrosine kinase that originated by recombination of chicken epidermal growth factor (EGF) receptor (CER) sequences with the viral genome (11, 22, 30). When compared with EGF receptor, the *erbB* proteins of all currently characterized AEV strains have lost most of their amino-terminal, extracellular ligand-binding domain, which is thought to result in constitutive activation of its cytoplasmic tyrosine kinase activity (6, 9, 25, 34). Furthermore, during or after the recombination event, other structural alterations that include cytoplasmic C-terminal truncations, internal deletions, and point mutations were introduced into the virus-acquired CER sequences which differ among AEV strains (6, 9, 12, 25, 34, 35). These alterations are likely to be responsible for the characteristic properties of distinct virus isolates with respect to pathogenic potential in vivo and transforming activity in cultured cells (3, 12, 25). Therefore, naturally selected v-erbB variants represent an excellent system with which to evaluate the structural basis for altered biochemical properties and biological signaling activities of growth factor receptor derivatives involved in a variety of distinct neoplastic phenotypes.

We have previously shown that erbB ligand responsiveness can be restored by precise reattachment of human EGF receptor (HER) extracellular sequences to repair the amino-terminal deletion. This type of HER-erbB chimeric protein exhibited EGF-responsive functions at the surface of transfected Rat-1 fibroblasts (26). Here, we have extended this approach to compare the biological signaling activities of the cytoplasmic domains of two distinct, well-characterized v-*erbB* oncogene products: v-erbB-H and v-erbB-ES4. Parallel analyses of the signaling characteristics of the chimeric receptors have revealed pronounced differences between erbB-H and -ES4 cytoplasmic domains on the growth of transfected cells and the potential to transform mouse NIH 3T3 fibroblasts. Whereas both erbB-H and erbB-ES4 autophosphorylation activities become ligand dependent after reattachment of HER extracellular sequences, the normal and transforming signals mediated by the HER-erbB-ES4 chimera remain largely EGF independent, in contrast to HER-erbB-H. This demonstrates that structural alterations within the cytoplasmic domain can induce a partially activated EGF receptor in the absence of its ligand.

MATERIALS AND METHODS

Reagents. Receptor-grade EGF, human transferrin, and human insulin were obtained from Collaborative Research, Inc. Growth factor bovine serum albumin (BSA) diluent was from Schwartz/Mann. ^{125}I-EGF (100 μCi/μg), L-[^{35}S] methionine (1,000 Ci/mmol), [*methyl*-^{3}H]thymidine (84 Ci/mmol), and [γ-^{32}P]ATP (5,000 Ci/mmol) were from Amersham Corp. Protein A-Sepharose was from Pharmacia, Inc. All cell culture reagents including Geneticin (G418 sulfate), were from GIBCO Laboratories. BSA, phenylmethylsulfonyl fluoride, and aprotinin were from Sigma Chemical Co.; En^{3}Hance and Aquasol scintillation cocktail were from Dupont, NEN Research Products. Purified tissue culture agar was from Difco Laboratories.

Receptor expression plasmids. The expression vector CVN and the HERc expression construct have been described previously (16, 26). Chimeric receptor expression constructs with HER extracellular and transmembrane domains and normal or mutated (v-erbB) cytoplasmic sequences were generated by using either of the *Aha*II or *Dra*III restriction sites within the 100% conserved amino acid sequence of the juxtamembrane domain (19, 22, 30, 34). HER-CER (1,190

* Corresponding author.
† Present address: Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 8033 Martinsried, Federal Republic of Germany.

amino acids) expression vector was generated by ligating *Pvu*I-*Nar*I (3,292 base pairs [bp]) and *Sal*I-*Pvu*I (6,155 bp) fragments from the HERc expression construct to an *Aha*II-*Sal*I (1,747 bp) fragment from pc-erbBNX (23). HER-erbB-H (1,160 amino acids) consists of *Pvu*I-*Dra*III (3,381 bp) and *Eco*RV-*Pvu*I (6,164 bp) HERc expression construct fragments and v-erbB-H sequences (*Dra*III–filled-in *Kpn*I; 2,618 bp) from a pUC18 subclone (*Sma*I site) of a 1.7-kilobase-pair filled *Apa*I-*Xmn*I fragment from pAE7.7 (kindly provided by B. Vennström). The entire v-erbB-H fragment used in this construct was sequenced and found to contain only two amino acid sequence differences at positions 699 and 705 of CER (19, 22) in addition to the C-terminal 34-amino-acid deletion. The HER-erbB-ES4 expression construct coding for a 1,096-amino-acid chimeric receptor was derived from ligation of *Pvu*I-*Nar*I (3,292 bp) and *Eco*RV-*Pvu*I (6,164 bp) HERc vector fragments and a 1,378-bp *Aha*II-*Xmn*I v-erbB-ES4 fragment of pAE11 (provided by B. Vennström).

To construct CVN/HER-CERΔCT (1,160 amino acids, including 4 from the AEV *env* gene), a 1,772-bp *Mst*II-*Sst*I fragment from CVN/HER-CER was joined with a 229-bp *Sst*I-*Sst*II fragment from CVN/HER-erbB-H and a 9,078-bp *Sst*II-*Mst*II fragment from CVN/HER-CER. Similarly, the CVN/HER-erbB-H+CT construct was generated by joining a 9,486-bp *Sst*II-*Dra*II fragment from CVN/HERc with a 1,091-bp *Dra*III-*Dra*III fragment from CVN/HER-erbB-H and a 647-bp *Dra*III-*Sst*II fragment from CVN/HER-CER and encodes an 1,190-amino-acid chimeric receptor precursor with two point mutations in the ATP-binding site.

Cell culture. An NIH 3T3 fibroblast subclone (CL7; kindly provided by C. Sherr) was used for all transfection experiments. The cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 1 g of glucose per liter, 10% fetal bovine serum (FCS), 2 mM L-glutamine, and antibiotics. Stable lines were cultured in this medium plus 400 ng of G418 sulfate per ml. Cells were passaged once a week at 1:10 dilution and used for 10 passages.

Generation of transfected cell lines. NIH 3T3 cells (2×10^5 cells per 35-mm dish) were transfected by the calcium phosphate coprecipitation method (14, 31), using 5 μg of high-molecular-weight NIH 3T3 DNA and 100 ng of control (CVN) or receptor expression plasmid. The following day, cells from each dish were split into two 10-cm dishes with DMEM containing 10% FCS and 400 μg of G418 sulfate per ml. Medium was changed every week. Cells were amplified by selection in DMEM containing 10% dialyzed FCS and 250 nM methotrexate. Medium was changed every week. Cells were cloned at this level and screened for expression by [^{35}S]methionine labeling and immunoprecipitation with an HER-specific monoclonal antibody (MAb 108.1).

Immunoprecipitation and gel electrophoresis. Subconfluent stably transfected cell lines were grown in 6-cm culture dishes. The cells were washed twice with phosphate-buffered saline (PBS). A 3-ml sample of methionine-free medium prepared from a minimal essential medium select amine kit (GIBCO) was added, and the incubation continued for 18 h with 300-μCi of [^{35}S]methionine. ^{35}S-labeled cell monolayers were washed twice with PBS and solubilized in 500 μl of 1% Triton X-100 buffer as described by Kris et al. (17). The 500-μl sample was incubated with an excess of HER-specific MAb 108.1 antiserum for 2 h at 4°C. Then 60 μl of protein A-Sepharose slurry (1:1 in distilled H_2O) was added for 1 h at 4°C. Immunoprecipitates were washed five times in 20 mM HEPES (pH 7.5)–150 mM NaCl–10% glycerol–0.1% Triton X-100. The pellet was then suspended in 30 μl of 2× sodium dodecyl sulfate (SDS)-gel sample buffer. Samples were boiled for 5 min, centrifuged, and analyzed on 8% SDS-polyacrylamide gels (18). Gels were fixed for 1 h in 10% acetic acid–10% trichloroacetic acid–30% methanol, incubated for 1 h in En^{3}Hance (Dupont), precipitated for 1 h in H_2O, and dried under vacuum at 60°C.

^{125}I-EGF binding experiments. Subconfluent, stably transfected cells were plated in 2.2-cm, 12-well culture dishes at 2×10^5 cells per well. The following day, the wells were washed twice with PBS–0.2% BSA and incubated with 1 ml of serum-free cell culture medium containing 0.2% BSA and 50 mM HEPES (pH 7.5) for 2 h at 4°C in the presence of ^{125}I-EGF and various concentrations of unlabeled EGF. Nonbound radioactivity was removed by three washes with cold PBS-BSA, and cells were lysed in 1 N NaOH. The radioactivity of the lysates was determined in a gamma counter. The computer program SCATPLOT (R. Vandlen, unpublished data) was used to analyze the data and determine receptor number and K_d values for high- and low-affinity binding sites (not shown).

Receptor autophosphorylation. Confluent cell monolayers in six-well dishes were placed in DMEM with 0.5% FCS 24 h before induction with EGF at 500 ng/ml for 30 min at 37°C. After rinsing with PBS, cells were lysed in 200 μl of SDS sample buffer, sonicated for 10 s, boiled for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis (7%). Proteins were electrophoretically transferred to nitrocellulose and subsequently incubated with an antiphosphotyrosine antibody. ^{125}I-protein A was then used to detect immunocomplexes (4).

Characterization of cell growth. To determine the extent of DNA synthesis stimulation by various concentrations of receptor-grade EGF, confluent cell monolayers in 24-well culture dishes were starved for 24 h in medium containing 0.5% FCS. At 18 h after growth factor addition, cells were labeled with 0.5 μCi of [*methyl*-^{3}H]thymidine. Subsequently cells were washed three times with PBS, followed by precipitation and washing with 5% trichloroacetic acid for 30 min at 4°C. The precipitate was then washed twice with PBS and solubilized in 500 μl of 0.2 N NaOH. After addition of 10 ml of Aquasol (Dupont), the incorporated radioactivity was determined in a scintillation counter.

For growth rate determinations, cells were plated at 2×10^4 in 35-mm culture dishes in DMEM and 10% FCS or in DMEM and 0.5% FCS with or without EGF (10 ng/ml). Cells were counted each day in a Coulter Counter. EGF was added again at day 4.

To examine cell growth under serum-free conditions, 4×10^4 NIH 3T3 cells, mock-transfected control NIH 3T3 cells, and cells expressing each construct were plated in 35-mm gelatin-coated tissue culture dishes in DMEM with 10% FCS. The following day, the cells were washed with 10 ml of PBS, and medium was changed to serum-free DMEM in the presence of either transferrin (10 μg/ml), transferrin and insulin (1 μg/ml), transferrin and EGF (10 ng/ml), or transferrin, insulin, and EGF in a DMEM–0.5% BSA diluent. Cell numbers were determined after 6 days by using a Coulter Counter.

Transformation assays. To examine the ability of transfected cells to form colonies in soft agar, 10^5 cells were plated in a 6-cm dish in the presence or absence of 50 ng of EGF per ml in a top layer of 3 ml of DMEM containing 10% FCS and 0.2% agar. The bottom layer (4 ml) contained DMEM, 10% FCS, and 0.4% agar with or without EGF. Visible colonies were scored after 21 days.

To measure the potential of various receptor expression plasmids to form foci in NIH 3T3 monolayers, 2×10^5 cells

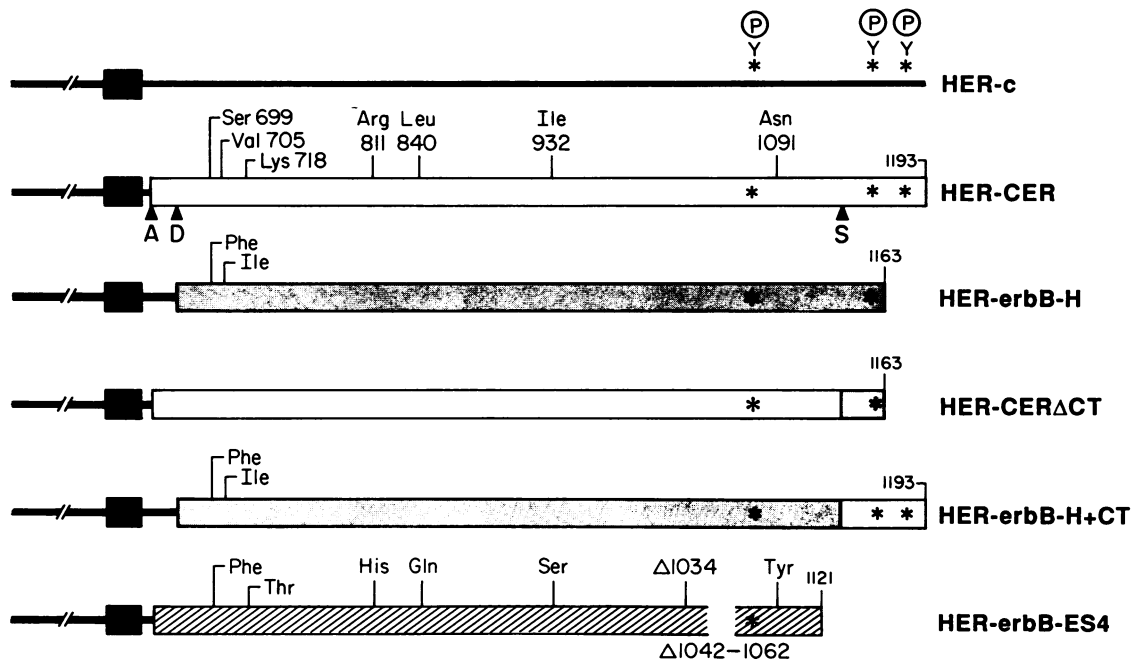

FIG. 1. Structures of EGF receptor chimeras. Plasmids were constructed as described in the text. The transmembrane domains and specific point mutations, as well as the chimeric makeup of each construct, are indicated. Δ indicates internal deletions of one (Δ1034) and 21 (Δ1042-1062) amino acids in the HER-erbB-ES4 sequence. Positions of HERc tyrosine phosphorylation sites at positions 1068, 1148, and 1173 are indicated by asterisks, and corresponding tyrosine residues in CER are marked. HER-erbB-H and HER-CERΔCT constructs encode four retroviral *env* protein amino acids at their C termini in addition to the 1,155 HER-CER amino acids. Amino acid numbering is based on CER sequence (19). The actual coding sequences of chimeric constructs are three amino acids shorter because of differences in HER versus CER extracellular sequences and 22 residues shorter in HER-erbB-ES4 as a result of internal deletions. Sequence analysis of clones used in our experiments did not confirm the differences reported for v-erbB-H (34) and v-erbB-ES4 (6) at positions 1091 and 716, respectively, of CER; the v-erbB-H DNA used in these experiments contained a mutation at position 699 (Phe for Ser), in contrast to the sequence reported by Yamamoto et al. (34).

(NIH 3T3 CL7) were plated in 35-mm dishes. The medium was changed the following day, and 4 h later the cells were transfected by calcium phosphate coprecipitation (14) with 5 μg of NIH 3T3 high-molecular-weight carrier DNA and 100 ng of a control (pCVN) expression construct, a v-*fms* expression construct, or the human EGF chimeric receptor expression plasmids. In parallel, cells were cotransfected with 100 ng of a tumor growth factor α (TGF-α) expression plasmid (27). The following day, cells from each dish were split into three 6-cm dishes with DMEM containing 5% FCS. Medium was changed every 3 to 4 days, and foci were scored on day 21. Visible foci were isolated on day 14 and grown in DMEM–10% FCS plus 400 μg of G418 sulfate per ml. Neomycin-selected stable foci lines were subsequently screened for expression of their EGF chimeric receptor plasmid as well as for TGF-α production.

RESULTS

Construction of chimeric expression vectors. Ligand binding to the ectodomain of receptor tyrosine kinases leads to activation of an intracellular tyrosine kinase activity and generation of a signal that is thought to be specified by the cytoplasmic domain (35). Structural alterations in this domain would be expected to alter or abolish this signal or, alternatively, to subvert intrinsic molecular control mechanisms. While v-*erbB* oncogene products share the same 554-amino-acid amino-terminal deletion, they differ in their cytoplasmic sequence alterations (Fig. 1; 6, 12, 25, 34). In addition to C-terminal deletions of 34 and 72 amino acids in v-erbB-H and v-erbB-ES4, respectively, these oncogene products exhibit characteristic point mutations and internal sequence deletions. The only cytoplasmic sequence alteration shared between v-erbB-H and v-erbB-ES4, Phe-669, is located within the ATP-binding site consensus sequence (GXGXXG) between the first two glycine residues. Further mutations are found in both oncogene products near this location (Ile-705 in erbB-H; Thr-718 in erbB-ES4) and downstream in the erbB-ES4 sequence (Fig. 1).

To focus on the various mutations in erbB and their effects on biochemical properties and biological activities, we eliminated the influence of the amino-terminal deletion by repairing it with intact HER ligand-binding sequences. Figure 1 shows schematically the structural differences between wild-type CER cytoplasmic sequences (19, 22) and those of v-erbB-H and v-erbB-ES4 and the design of the EGF receptor constructs used. CER and v-erbB cytoplasmic sequences were fused to HER sequences by using either *Dra*III or *Apa*I restriction endonuclease cleavage sites that are conserved between the two receptor genes. These sites are located in a region that encodes juxtamembrane domain sequences and is identical in HER and CER. v-erbB-H, v-erbB-ES4, and CER sequences were fused with HER sequences to yield the chimeric receptors, HER-erbB-H, HER-erbB-ES4, and HER-CER. Two additional expression vectors were constructed in which the C termini of v-erbB-H and CER were exchanged. The portions exchanged include the CER tyrosine residue (Tyr-1179) that corresponds to Tyr-1173, the major HER autophosphorylation site (10)

TABLE 1. Receptor number per cell in transfected NIH 3T3 cell lines[a]

Cell line	Receptors/cell
CVN-transfected 3T3	2.0×10^4
HER-c CL1	4.3×10^5
HER-c CL2	1.2×10^6
HER-CER CL1	2.8×10^5
HER-CER CL2	1.2×10^5
HER-erbB-H CL2	4.7×10^5
HER-erbB-H CL3	8.0×10^5
HER-CERΔCT CL4	5.4×10^5
HER-CER CL5	4.7×10^5
HER-erbB-H+CT CL1	8.5×10^5
HER-erbB-H+CT CL3	8.7×10^5
HER-erbB-ES4 CL1	1.2×10^5
HER-erbB-ES4 CL5	1.6×10^5

[a] Receptor numbers were determined as described in Materials and Methods by using ^{125}I-EGF and correlated well with relative quantities estimated by metabolic labeling of cells with [^{35}S]methionine and subsequent immunoprecipitation with an HER-specific antibody. In contrast to the cell lines transfected with HER constructs, the CVN-transfected 3T3 lines expressed only mouse EGF receptor.

(HER-erbB-H+CT), and allowed us to examine the significance of the two erbB-H point mutations within the ATP-binding domain (at positions 699 [Phe versus Ser] and 705 [Ile versus Val]) and the C-terminal deletion itself (HER-CERΔCT). All chimeric receptor constructs were inserted into a simian virus 40-based expression vector that included simian virus 40 early promoter-controlled genes for neomycin (G418) resistance and dihydrofolate reductase, which allowed amplification of plasmid sequences in transfected cells under methotrexate selection (8, 16, 29). Mouse NIH 3T3 fibroblasts containing about 2×10^4 endogenous EGF receptors were transfected and amplified by standard procedures. At least two independently established, stable cell lines were used for each assay and yielded analogous results.

Biosynthesis of ligand binding-competent chimeric receptors. We first examined whether transfected NIH 3T3 cells correctly synthesized and processed the various chimeric receptors and transported the properly folded molecules to the cell surface. All transfected cell lines produced a protein of about 170 kilodaltons that was immunoprecipitated by MAb 108 (not shown), a HER ectodomain-specific antibody (15); the CVN control that expresses only mouse EGF receptors showed no protein of this mobility. Expected minor size differences between HER-CER and its truncated derivatives, HER-CERΔCT, HER-erbB-H, and HER-erbB-ES4 were detected but were not very pronounced on the 8% acrylamide SDS gels used for this analysis (not shown).

Ligand binding analyses using ^{125}I-EGF showed that all transfected cell lines exhibited substantially increased EGF surface binding. Scatchard analysis of the binding data revealed that all receptor chimeras formed high- and low-affinity binding sites, with distribution and K_d values similar to those of the HERc control (not shown; 24). Expression levels of surface-localized, ligand binding-competent receptors ranged between 1.2×10^5 and 1.2×10^6 per cell (Table 1).

EGF-stimulated autophosphorylation of chimeric receptors. Ligand binding to the extracellular domain of the EGF receptor triggers rapid phosphorylation of several tyrosine residues, three of which are located within the EGF receptor C-terminal tail region (10). Kinase activity is essential for most receptor functions (5, 15, 20) and can be detected in detergent lysates as well as in intact cells. We tested whether HER-erbB chimeras demonstrated EGF-dependent autophosphorylation to determine whether fusion of the HER ectodomain to the cytoplasmic domain of *erbB* oncogene products was sufficient to reinstate this important signaling control. Furthermore, it was possible that the response of distinct chimeras to ligand stimulation would provide clues to their distinct biological signaling properties.

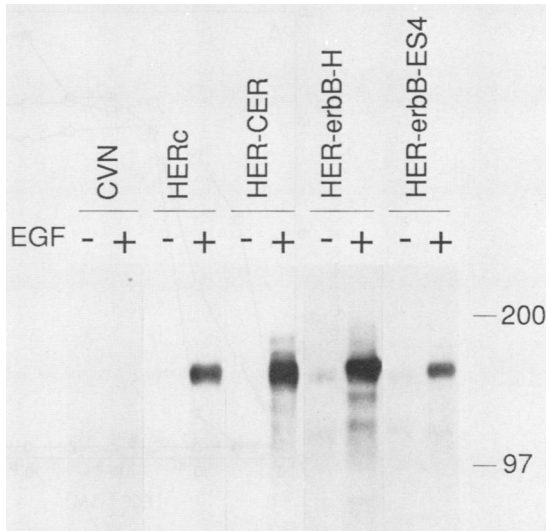

FIG. 2. Receptor autophosphorylation in intact cells. Serum-starved confluent monolayers were incubated for 30 min with (+) or without (−) EGF. After electrophoresis on 8% SDS polyacrylamide gels and transfer to nitrocellulose, bands were detected with anti-phosphotyrosine antibody 5E2 and ^{125}I-protein A. In vivo autophosphorylation of HER-CERΔCT and HER-erbB-H+CT was fully ligand dependent (not shown). CVN is a vector-transfected control cell line. Sizes of molecular weight markers are indicated in kilodaltons on the right.

EGF strongly stimulated the phosphorylation of all chimeric proteins both in vitro and in vivo. Under in vitro conditions, all chimeric receptors and the HERc control exhibited an EGF-inducible autophosphorylation activity (not shown). The basal activity detected in vitro for all receptor constructs was virtually undetectable in intact cells (see below) and was likely a consequence of the experimental protocol used. To examine directly the tyrosine phosphorylation state of the receptors in intact cells, we carried out immunoblot analyses of cell lysates both before and after ligand exposure, using an antiphosphotyrosine monoclonal antibody. Both HER-erbB chimera and control receptors displayed a very strong induction of autophosphorylation upon EGF addition (Fig. 2). Similar results were obtained for the HER-erbB-H+CT and HER-CERΔCT constructs (data not shown). Together, these results demonstrate that the human EGF-binding domain controls the autophosphorylation activity of normal and mutated CER cytoplasmic domains, including the erbB-H and erbB-ES4 oncogene kinases, in the chimeric receptor constructs.

Growth characteristics of transfected NIH 3T3 cells. The mitogenic response of mammalian cells is thought to be strictly controlled by signals that are generated upon interaction of growth factors with specific cell surface receptors (35). Subversion of this control at any stage of signal transmission may lead to aberrant behavior of cells in culture and possibly tumorigenesis in vivo. To further ascertain the signaling differences between various v-erbB isolates, we investigated the growth requirements and transformed state of the chimera-transfected cell lines.

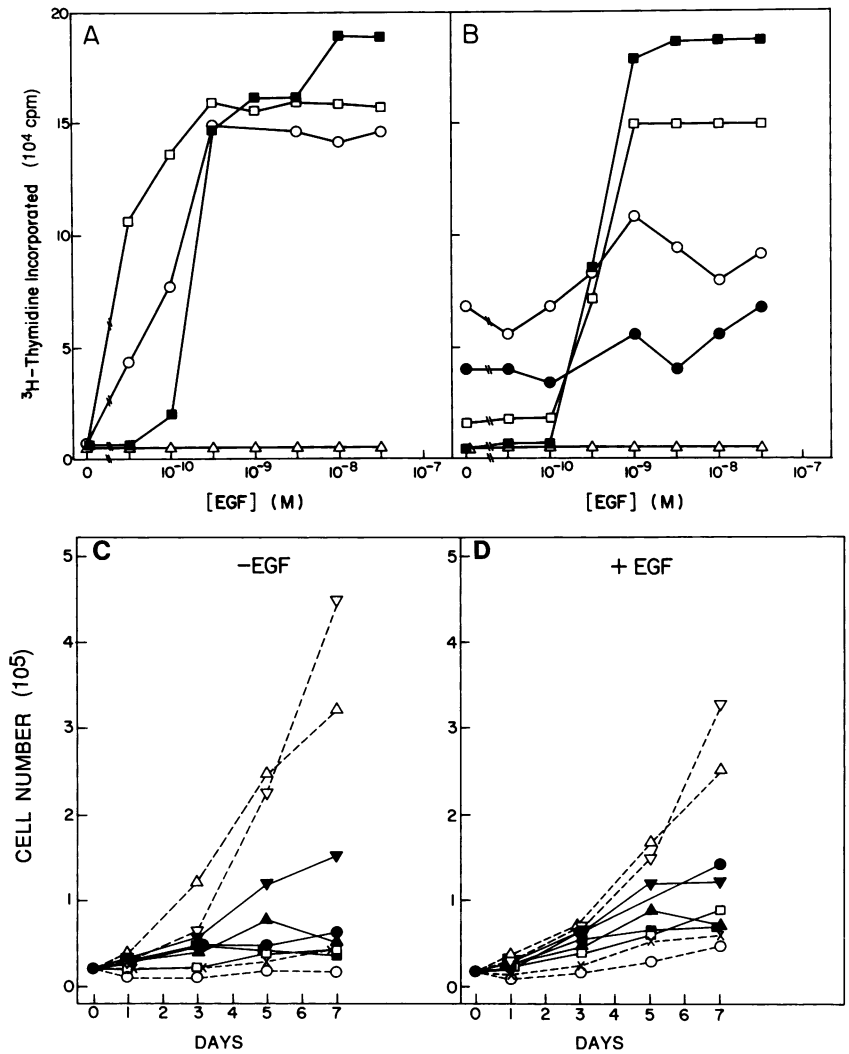

FIG. 3. (A and B) Mitogenic response of transfected NIH 3T3 fibroblasts. (A) HERc CL2 (○), HER-CER CL1 (□), HER-CERΔCT CL5 (■), and CVN control (△). (B) HER-erbB-H CL3 (□), HER-erbB-H+CT CL1 (■), HER-erbB-ES4 CL1 (●), HER-erbB-ES4 CL5 (○), and CVN control (△). Confluent cell monolayers in 24-well dishes were brought to quiescence for 24 h in medium containing 0.5% FCS. Stimulation of DNA synthesis by various concentrations of receptor-grade EGF was monitored 18 h after growth factor addition by 4-h pulse-labeling with 0.5 μCi of (*methyl*-[^{3}H])thymidine. Cells were then washed three times with PBS, and soluble radioactivity was extracted with 1 ml of 5% trichloroacetic acid for 30 min at 4°C. Cells were washed twice with PBS and solubilized in 500 μl of 0.2 N NaOH, and incorporated radioactivity was determined. The absence of an EGF response for CVN-transfected NIH 3T3 cells (△) despite the presence of EGF receptors has been observed in several independently isolated cell lines and cannot be explained at this point. (C and D) Growth of the following EGF receptor wild-type and mutant lines in low serum: HERc CL1 (■), HER-CER CL1 (□), HER-CERΔCT CL4 (▲), HER-erbB-H CL2 (●), HER-erbB-H+CT CL1 (▼), HER-erbB-ES4 CL1 (▽), HER-erbB-ES4 CL5 (△), CVN control (○), and NIH 3T3 CL7 (×). Cells were plated at 2×10^4 in 35-mm dishes in DMEM and 0.5% FCS with or without 10 ng of EGF per ml. Cells were counted each day in a Coulter Counter. EGF was added again at day 4.

Figures 3A and B show the effect of EGF on [^{3}H] thymidine incorporation into DNA of serum-starved, confluent cell monolayers. Control plasmid (CVN)-transfected NIH 3T3 cells showed no mitogenic response to EGF, yet this growth factor stimulated [^{3}H]thymidine incorporation into all of the other transfected cell lines. However, whereas HERc, HER-CER, HER-CERΔCT, HER-erbB-H, and HER-erbB-H+CT lines were each dependent on EGF stimulation and displayed similar dose-response profiles (Fig. 3A and B), HER-erbB-ES4-expressing lines appeared to be EGF independent. These cells could not be grown to quiescence and responded only marginally to increasing EGF concentrations (Fig. 3B).

A similar result was obtained when cells were grown either in medium containing only low (0.5%) FCS concentrations or in the presence of 2 nM EGF (Fig. 3C and D). A total of 2×10^4 cells were seeded per 6-cm dish, and the growth rates of the various cell lines were determined by cell counting. As would be expected for normal cells, HERc-, HER-CER-, and HER-erbB-H+CT-expressing cells, as well as nontransfected and CVN-transfected NIH 3T3 controls, did not grow significantly with only 0.5% FCS present (Fig. 3C). Addition of EGF induced two to three rounds of mitosis in HERc-, HER-CER-, and HER-erbB-H+CT-expressing cells over a period of 7 days (Fig. 3D). HER-erbB-H and HER-CERΔCT chimera-expressing lines went through about one round of cell division without EGF and were significantly stimulated by EGF. However, a completely

TABLE 2. Colony formation in soft agar

Cell line	No. of colonies[a]	
	−EGF	+EGF
NIH 3T3 CL7	<10	17
CVN	0	2
HERc CL1	0	496
HER-CER CL1	0	800
HER-CERΔCT CL5	0	904
HER-erbB-H CL2	0	316
HER-erbB-H CL3	<10	332
HER-erbB-H+CT CL1	0	286
HER-erbB-H+CT CL3	0	440
HER-erbB-ES4 CL1	20	246
HER-erbB-ES4 CL5	220	432
HER-erbB-ES4 117	336	480

[a] Colonies were counted after 21 days. Numbers represent averages of two experiments.

TABLE 3. NIH 3T3 focus formation[a]

Construct	No. of foci/μg of DNA	
	−TGF-α	+TGF-α
v-*fms*	2,430	2,160
pCVN	0	0
HERc	0	720
HER-CER	0	1,170
HER-CERΔCT	0	1,260
HER-erbB-H	0	1,680
HER-erbB-H+CT	0	1,740
HER-erbB-ES4	900	930

[a] Subconfluent NIH 3T3 monolayers were transfected with expression plasmids for v-*fms*, control (HERc), and chimeric receptors or expression vector alone (CVN). In parallel, cells were cotransfected with a TGF-α expression construct (+TGF-α). Foci were scored after 21 days in culture.

different behavior was observed for HER-erbB-ES4 cells. In the absence of EGF, these cells displayed a dramatic growth rate (Fig. 3C), and addition of growth factor actually retarded cell growth (Fig. 3D), a characteristic that was consistently observed with three independent HER-erbB-ES4-expressing cell lines. Under these low-serum conditions, HER-erbB-ES4 cells exhibited a typical transformed morphology even in the absence of EGF. Acquisition of this morphological phenotype by all the other cell lines required the addition of growth factor (not shown).

Transforming activity of EGF receptor chimeras. The property of contact inhibition and the ability of normally attached, growing cells to form colonies in soft agar are two commonly used criteria to assess the transformed state of a cell. To examine whether the transforming potential of v-*erbB* oncogene sequences was retained in the HER ectodomain fusions and to test whether this activity had become ligand dependent, we subjected all chimeric EGF receptor mutant lines and the corresponding expression constructs to soft agar and focus formation assays, respectively (Table 2 and 3; Fig. 4).

In contrast to the NIH 3T3 and CVN control cell lines, all transfected cell lines formed significant numbers of colonies in soft agar in the presence of EGF, including cells expressing wild-type HER (HERc) and the chimeric control plasmid HER-CER (Table 2). In EGF-free soft agar, however, only HER-erbB-ES4 lines formed colonies (CL1 did so rather weakly but reproducibly). Colony formation by all three HER-erbB-ES4 lines was reproducibly enhanced in the presence of EGF (Table 2).

Similarly, in cotransfection experiments with a TGF-α expression construct (27), transformed phenotype was induced in the stringent focus formation assay for all receptor expression plasmids (Fig. 4). However, whereas HERc, HER-CER, HER-CERΔCT, HER-erbB-H, and HER-erbB-H+CT plasmids induced foci only in conjunction with the TGF-α expression plasmid, the HER-erbB-ES4 expression vector was active in mediating this effect even without TGF-α (not shown), similar to the v-*fms* positive control (Table 3). Interestingly, foci induced by cotransfection of

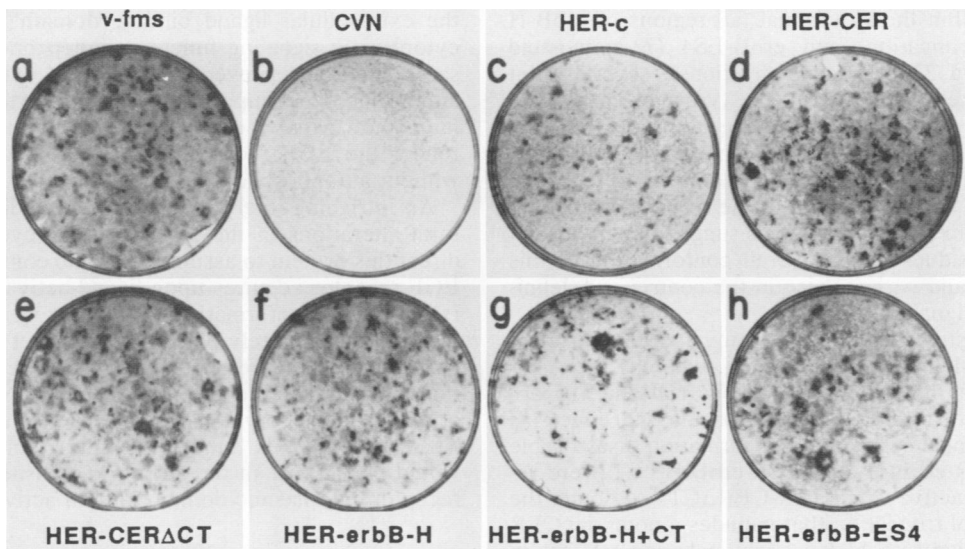

FIG. 4. NIH 3T3 focus formation. Subconfluent NIH 3T3 monolayers were transfected with expression plasmids. In parallel, cells were cotransfected with a TGF-α expression construct. After 21 days in culture, cell foci were stained with crystal violet. Only the TGF-α cotransfection results are shown. No foci were detected in TGF-α controls except for HER-erbB-ES4 and v-*fms*. Several foci of each transfection experiment were picked and found to express both receptor and TGF-α proteins (data not shown).

HER-erbB-ES4 with TGF-α plasmids developed faster and could be scored after 2 weeks, whereas TGF-α-independent foci were evident 1 week later. Focus formation efficiency in this assay was similar for all constructs; HER-erbB-H and HER-erbB-H+CT were consistently the most efficient, whereas HER-erbB-ES4 scored the lowest of all chimeric receptor constructs (Table 3).

DISCUSSION

We constructed a set of chimeric EGF receptors consisting of identical ligand-binding and transmembrane sequences combined with cytoplasmic sequences of either cellular or retroviral origin to investigate the significance of structural alterations on the mitogenic and transforming activities and the biochemical properties of these proteins. After introduction into mouse fibroblasts, all of the EGF receptor chimeras were synthesized and transported to the cell surface, where they formed high- and low-affinity binding sites for EGF and TGF-α with properties comparable to those of the HER control (24). Apparently, the extensive sequence differences found between HER and CER cytoplasmic sequences (19, 30) had little or no influence on ligand-binding characteristics. Most of the sequence divergence between the EGF receptors of these two evolutionarily distant species is found in the carboxy-terminal-most tail region (33.5% divergence), whereas juxtamembrane and tyrosine kinase core regions are highly conserved (only two and seven differences, respectively; 4 and 2% divergence; 19). In addition, these differences in receptor primary structure did not alter the normal biochemical characteristics or mitogenic and transforming signaling potential, as demonstrated by a comparison of HERc and HER-CER expressed in NIH 3T3 cells. These findings suggest that cellular components within mouse fibroblasts are sufficiently conserved in structure among human, chicken, and mouse cells to interact directly with these foreign receptors and mediate cellular responses.

The chimeric molecules, HER-CERΔCT, HER-erbB-H, HER-erbB-H+CT, and HER-erbB-ES4, contained mutated CER cytoplasmic domains that led to the generation of altered receptor signals and, consequently, altered cellular responses. In addition to previously identified truncations and deletions within the C-terminal tail regions of erbB-H (34-amino-acid truncation) and erbB-ES4 (72-amino-acid truncation; 1- and 22-amino-acid deletions), several point mutations have been identified in the tyrosine kinase core regions of these receptor-derived oncogene products (6, 19, 22, 34). All of the point mutations in erbB-H and -ES4 sequences are in positions that are conserved between normal CER and HER, and even in the related but distinct HER2/neu receptor homolog (7). This suggests an important role for these residues in the overall conformation of this receptor domain and perhaps also in the control and definition of receptor signals.

In the assay systems used, the C-terminal erbB-H truncation had only a slight enhancing effect, whereas the ATP-binding site point mutations at positions 699 and 705 appeared to have a more significantly stimulating effect, as evidenced in the most stringent focus formation assay (Table 3), in which HER-erbB-H and HER-erbB-H+CT were reproducibly more active than HER-CERΔCT. Although the erbB-H C-terminal truncation that includes a potential CER autophosphorylation site had a slight enhancing effect in several growth and transformation assays, it appears that point mutations in the tyrosine kinase domain can have even more dramatic effects on receptor-mediated proliferation and transformation signals.

The most surprising aspect of our findings was the dramatic difference between the erbB-ES4 mutations compared with erbB-H sequences or variants thereof. At first, it appeared that faithful reconstitution of a ligand-binding function, and thus elimination of the extracellular domain deletion, resulted in fully ligand-responsive chimeric receptors. Autophosphorylation experiments in detergent lysates of transfected cells indicated that all receptors showed various degrees of basal activity in vitro, and in intact cells, basal autophosphorylation was either barely detectable or not detected for HER-erbB-H and HER-erbB-ES4. In all cases of autophosphorylation, induction was more than 10-fold in vivo.

Although all chimeric constructs showed EGF-inducible kinase activity, the structural alterations in the erbB-ES4 cytoplasmic domain generated proliferation signals that could not be controlled by a human EGF-binding domain. In contrast to the HER-erbB-H receptor, expression of the HER-erbB-ES4 receptor consistently rendered cells ligand independent in proliferation and transformation assays. When compared with the ligand-induced cell responses mediated by the other receptors, the EGF-independent proliferation signals generated by HER-erbB-ES4 were more effective under serum-free or low-serum conditions, whereas thymidine incorporation was constitutive and did not reach the maximal values of EGF-stimulated, [^{3}H]thymidine incorporation in other cell lines. Whether the lower HER-erbB-ES4 effects in this assay correlate with the lower expression levels of the two lines tested is not known with certainty. Nevertheless, ligand activation and autophosphorylation of HER-erbB-ES4 appear to modulate the receptor signal, as evidenced by suppressed growth of expressing cells under low-serum conditions and an enhanced rate of focus formation in monolayers.

These findings add a novel alternative to the currently known possibilities of growth factor receptor activation in mitogenesis and transformation. Most relevant for our studies on the biological effects of *erbB* mutations is a comparison with the tumorigenic *neu* and v-*fms* genes (1, 2, 21, 28, 32). While a single point mutation in the transmembrane domain of the *neu* oncogene product and point mutations in the extracellular ligand binding domain of v-*fms* activate cytoplasmic signaling functions independent of ligand, the same effect is achieved by erbB-ES4 cytoplasmic domain alterations. Most importantly, in contrast to *neu* and *fms*, we have shown that these cytoplasmic domain mutations can render the EGF receptor active in the absence of ligand without autophosphorylation.

An intriguing interpretation of our findings is that structural alterations in the EGF receptor cytoplasmic domain direct this protein to assume the same conformation that the EGF receptor acquires upon ligand-activated autophosphorylation; this conformation would represent an active state. Such an incompletely phosphorylated yet constitutively active kinase would be expected to catalyze the phosphorylation of the same repertoire of cellular substrates that is normally phosphorylated in response to ligand stimulation. According to this model, receptor autophosphorylation would play a role in the allosteric transition of an inactive receptor cytoplasmic domain into an active conformation.

ACKNOWLEDGMENTS

We thank H. Riedel, who was involved in the early stages of this work, for his contributions. We are grateful to S. Pfeffer for expert editing advice and to J. Arch for preparation of the manuscript.

LITERATURE CITED

1. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature (London) **319**:226–229.
2. **Bargmann, C. I., and R. A. Weinberg.** 1988. Increased tyrosine kinase activity associated with the protein encoded by the activated *neu* oncogene. Proc. Natl. Acad. Sci. USA **85**:5394–5398.
3. **Beug, H., M. J. Hayman, M. A. Raines, H.-J. Kung, and B. Vennstrom.** 1986. Rous-associated virus 1-induced erythroleukemia cells exhibit a weakly transformed phenotype in vitro and release c-*erbB*-containing retroviruses unable to transform fibroblasts. J. Virol. **57**:1127–1133.
4. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
5. **Chen, S. W., S. C. Lazar, M. Poenie, R. Y. Tsien, G. Gill, and G. M. Rosenfeld.** 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature (London) **328**:820–823.
6. **Choi, O.-R., C. Trainor, T. Graf, H. Beug, and J. D. Engel.** 1986. A single amino acid substitution in v-*erbB* confers a thermolabile phenotype to *ts*167 avian erythroblastosis virus-transformed erythroid cells. Mol. Cell. Biol. **6**:1751–1759.
7. **Coussens, L., T. L. Yang-Feng, Y.-C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science **230**:1132–1139.
8. **Crowley, C. W., C.-C. Liu, and A. D. Levinson.** 1983. Plasmid-directed synthesis of hepatitis B surface antigen in monkey cells. Mol. Cell. Biol. **3**:44–55.
9. **Damm, K., H. Beug, T. Graf, and B. Vennstrom.** 1987. A single point mutation *erbA* restores the erythroid transforming potential of a mutant AEV defective in both *erbA* and *erb*B oncogenes. EMBO J. **6**:375–382.
10. **Downward, J., P. Parker, and M. D. Waterfield.** 1984. Autophosphorylation sites on the epidermal growth factor receptor. Nature (London) **311**:483–485.
11. **Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield.** 1984. Close similarity of epidermal growth factor receptor and v-*erbB* oncogene protein sequences. Nature (London) **307**:521–527.
12. **Gammett, D. C., S. E. Tracy, and H. L. Robinson.** 1986. Differences in sequences encoding the carboxyl-terminal domain of the epidermal growth factor receptor correlate with differences in the disease potential of viral *erbB* genes. Proc. Natl. Acad. Sci. USA **83**:6053–6057.
13. **Graf, T., B. Royer-Pokora, G. E. Schubert, and H. Beug.** 1976. Evidence for the multiple oncogenic potential of cloned leukemia virus: in vitro and in vivo studies with avian erythroblastosis virus. Virology **71**:423–433.
14. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
15. **Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger.** 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell **51**:199–209.
16. **Hudziak, R. M., J. Schlessinger, and A. Ullrich.** 1987. Increased expression of the putative growth factor receptor p185^{HER2} causes transformation and tumorigenesis of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA **84**:7159–7163.
17. **Kris, R., I. Lax, M. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin, and J. Schlessinger.** 1985. Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF-R and v-erbB protein. Cell **40**:619–625.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lax, I., A. Johnson, R. Howk, J. Sap, F. Bellot, M. Winkler, A. Ullrich, B. Vennstrom, J. Schlessinger, and D. Givol.** 1988. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol. Cell. Biol. **8**:1970–1978.
20. **Moolenaar, W. H., A. J. Bierman, B. C. Tilly, I. Verlaan, L. H. K. Defize, A. M. Honegger, A. Ullrich, and J. Schlessinger.** 1988. A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. **7**:707–710.
21. **Muller, W. J., E. Sinn, P. K. Pattengale, R. Wallace, and P. Leder.** 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell **54**:105–115.
22. **Nilsen, T. W., P. A. Maroney, R. G. Goodwin, F. M. Rottman, L. B. Crittenden, M. A. Raines, and H.-J. Kung.** 1985. c-erbB activation in ALV-induced erythroblastosis: novel RNA processing and promoter insertion result in expression of an amino-truncated EGF receptor. Cell **41**:719–726.
23. **Pelley, R. J., C. Moscovici, S. Hughes, and H. J. Kung.** 1988. Proviral-activated c-*erbB* is leukemogenic but not sarcomagenic: characterization of a replication-competent retrovirus containing the activated c-*erbB*. J. Virol. **62**:1840–1844.
24. **Prywes, R., E. Livneh, A. Ullrich, and J. Schlessinger.** 1986. Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. EMBO J. **5**:2179–2190.
25. **Raines, M. A., N. J. Maihle, C. Moscovici, M. G. Moscovici, and H. J. Kung.** 1988. Molecular characterization of three *erbB* transducing viruses generated during avian leukosis virus-induced erythroleukemia: extensive internal deletion near the kinase domain activates the fibrosarcoma and hemangioma-inducing potential of *erbB*. J. Virol. **62**:2444–2452.
26. **Riedel, H., J. Schlessinger, and A. Ullrich.** 1987. A chimeric, ligand-binding v-*erb*B/EGF receptor retains transforming potential. Science **236**:197–200.
27. **Rosenthal, A., P. B. Lindquist, T. S. Bringman, D. V. Goeddel, and R. Derynck.** 1986. Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. Cell **46**:301–309.
28. **Roussel, M. F., J. R. Downing, C. W. Rettenmier, and C. J. Sherr.** 1988. A point mutation in the extracellular domain of the human CSF-1 receptor (c-*fms* proto-oncogene product) activates its transforming potential. Cell **55**:979–988.
29. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80**:2495–2499.
30. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg,** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature (London) **309**:418–425.
31. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from prokaryocytes and leukocytes. Cell **16**:777–785.
32. **Woolford, J., A. McAuliffe, and L. R. Rohrschneider.** 1988. Activation of the feline c-*fms* proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. Cell **55**:965–977.,
33. **Yamamoto, T., H. Hihara, T. Nishida, S. Kawai, and K. Toyoshima.** 1983. A new avian erythroblastosis virus, AEV-H carries *erbB* gene responsible for the induction of both erythroblastosis and sarcomas. Cell **34**:225–234.
34. **Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Ooi, and K. Toyoshima.** 1983. The *erbB* gene of avian erythroblastosis virus is a member of the *src* family. **35**:71–78.
35. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. **57**:443–478.