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Expression and Biosynthetic Variation of the Epidermal Growth Factor Receptor in Human Hepatocellular Carcinoma-Derived Cell Lines

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Expression of the epidermal growth factor (EGF) was analyzed in six human hepatocellular carcinoma-derived and one human hepatoblastoma-derived cell line, each of which retained the differentiated phenotype and functions of the parenchymal hepatocyte. The level of receptor expression of each hepatoma cell line was similar to that of the normal human fibroblast, approximately 10^5 molecules per cell. However, NPLC/PRF/5, a subline of the PLC/PRF/5 cell line obtained following reestablishment of a xenograft tumor in vitro, was found to express 4×10^6 high-affinity EGF receptor molecules per cell. Proliferation of the NPLC/PRF/5 cell line was inhibited in the presence of nanomolar quantities of ligand. Receptor overexpression was found to result from EGF receptor gene amplification without apparent rearrangement of the EGF receptor coding sequences. Although cell-specific variability in posttranslational processing of EGF receptor N-linked oligosaccharides in the hepatoma cell lines was found, no difference between the receptors in PLC/PRF/5 and NPLC/PRF/5 was observed and no aberrant receptor-related species were detected. EGF receptor gene amplification in the NPLC/PRF/5 cell line is probably a reflection of genome instability and selection of variants with augmented growth potential in limiting concentrations of EGF in vivo. When viewed in this light, EGF receptor overexpression could represent a manifestation of tumor progression in the EGF-responsive hepatocyte.

The adult parenchymal hepatocyte is a long-lived differentiated cell which can be stimulated to proliferate in a compensatory growth model. Although the molecular interactions which lead to liver cell proliferation are unknown, insulin, glucagon, and epidermal growth factor (EGF) are known to be necessary to the regenerative process (41). To determine whether abnormalities in the receptor for one growth factor implicated in the regulation of proliferation of the normal hepatocyte could be a factor in hepatocellular carcinogenesis, we examined the display of the EGF receptor in a series of hepatoma-derived cell lines. Increased expression of the EGF receptor has been observed in primary tumors and cell lines derived from human glioblastomas (31), pancreatic tumors (27), squamous cell carcinomas (22), and breast tumors (44). Hepatocellular carcinoma is a common tumor in areas of the world where hepatitis B virus (HBV) is endemic, but despite the frequent and stable integration of the HBV genome in these tumor cells, no viral transforming gene product and no common site of HBV integration within the host cell genomes have been identified (6, 12, 20a, 43, 49, 52). In the present analysis, we found that EGF receptor expression in the hepatoma-derived cell lines did not differ from that in normal cells, with the exception of one laboratory-derived subline of the PLC/PRF/5 hepatoma-derived cell line which expresses 20-fold more receptors per cell than the parental cell line, the result of EGF receptor gene amplification. Although receptor affinity for EGF was similar in the parental and derivative cell lines, proliferation of the variant was inhibited by EGF, a characteristic of the A431 cell line and others that overexpress the receptor (4, 18, 22). Amplification of the EGF receptor gene in A431 cells is associated with internal rearrangement of the EGF receptor gene, leading to the generation of abnormal receptor-related species. We found no new primary EGF receptor-related products, although cell-specific variability in the extent of posttranslational processing of receptor N-linked oligosaccharides was detected among the hepatoma cell lines. Our results emphasize the plasticity of the genome in the tumor cell and suggest that cells expressing an increased number of high-affinity receptors may have a selective advantage in an environment in which EGF is present in limiting amounts.

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MATERIALS AND METHODS

Cell lines. The cell lines used in this study are listed in Table 1. The hepatoma-derived cell lines, which originated from seven patients, exhibited the epithelial morphology of the parenchymal hepatocytes and expressed either albumin or alpha-fetoprotein. All but the Hep G2 cell line contained the HBV genome integrated into the cellular genome. The NPLC/PRF/5 cell line was derived in this laboratory from a twice-passaged tumor that arose in an athymic (*nu/nu*) mouse injected with the PLC/PRF/5 cell line (2). All cells except A431 were grown in Eagle minimal essential medium (MEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, at 37°C in a humidified atmosphere of 5% CO_2–95% air. A431 cells were grown in the same conditions but in Dulbecco modified MEM (D-MEM) supplemented with 10% FBS and 2 mM glutamine. Cells were subcultured after removal from culture dishes with 0.25% trypsin and 0.1% EDTA in phosphate-buffered saline (PBS) and vigorous pipetting. All cells were routinely tested for mycoplasma contamination, and with the exception of the original PLC/PRF/5 cell line, they were all found to be mycoplasma free.

Immunofluorescence analysis. Cell suspensions (10^5/100

TABLE 1. Analysis of EGF receptor expression on human cell lines

Cell line	Origin (reference)	Mean EGF-R1 binding[a] (SD)
huH-2[b]	Hepatocellular carcinoma (25)	7.1 (7.4)
PLC/PRF/5	Hepatocellular carcinoma (2)	11.5 (7.3)
Hep G2	Hepatoblastoma (23)	11.9 (8.5)
huH-1[b]	Hepatocellular carcinoma (25)	13.1 (7.7)
Hep 10	Hepatocellular carcinoma (51)	14.1 (9.9)
Tong[c]	Hepatocellular carcinoma[d]	14.7 (9.3)
WI-38	Embryonic lung (20)	14.9 (8.5)
Hep 3B-2.1/7	Hepatocellular carcinoma (1)	29.5 (17.6)
NPLC/PRF/5	Hepatocellular carcinoma[e]	142.2 (67.1)
A431	Epithelial carcinoma (17)	190.0 (30.3)

[a] Cells were incubated with either EGF-R1 antibody or P3X63Ag8 antibody followed by fluorescein-conjugated goat IgG anti-mouse IgG. The fluorescence intensity of individual cells was measured by flow cytofluorimetry and recorded on an arbitrary scale of 1 to 200. Mean fluorescence of 1,000 cells (± standard deviation) is shown. In each case, a significant difference was found between the fluorescence reported and that after incubation with P3X63Ag8, which ranged from 3.1 to 7.4.

[b] Obtained from Namho Huh, University of Tokyo, Japan.

[c] Obtained from D. Stevenson, Huntington Memorial Hospital, Pasadena, Calif.

[d] D. Stevenson, J.-H. Lin, G. J. Marshall, and M. J. Tong, Hepatology, in press.

[e] Derived from a PLC/PRF/5-induced tumor in nude mice.

μl), prepared by trypsinization as above, were incubated (30 min at 4°C with shaking) in a dilution of EGF receptor-specific monoclonal antibody EGF-R1 (56), previously determined to give maximal cell surface binding (1:400 ascites fluid in MEM and 10% FBS). Control incubations were performed with an equivalent concentration of the P3X 63Ag8 antibody of the same isotype (24). Following three rinses with MEM supplemented with 10% FBS and 0.02% sodium azide, the cells were suspended in 100 μl of diluted fluoresceinated goat anti-mouse immunoglobulin G (IgG) antiserum (Cooper Biomedical, West Chester, Pa.), incubated for 30 min at 4°C with shaking, rinsed three times as above, and suspended in Dulbecco modified PBS supplemented with 5% FBS. Cell immunofluorescence was analyzed by flow cytometry with an Ortho Instruments Cytofluorograf.

Scatchard analysis. Recombinant human EGF (AmGen) was iodinated as described previously (21). Cells were seeded in 24-well Linbro dishes, refed at confluence with serum-free D-MEM, incubated for 18 h, and rinsed twice with binding buffer (serum-free D-MEM supplemented with 0.01 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], and 0.2% bovine serum albumin) at room temperature and twice at 4°C. Cells were incubated with [^{125}I]EGF (10 ng/ml) supplemented with from 1 to 5,000 ng of unlabeled murine EGF (Collaborative Research, receptor grade) per ml for 3 h at 4°C, rinsed four times, twice with binding buffer at 4°C and twice with PBS at 4°C, and solubilized in 1 M NaOH, and radioactivity was measured in a gamma counter. All determinations were performed in triplicate and adjusted to represent binding by 10^5 cells. Analysis of data was done by the standard method of Scatchard (46).

Cell proliferation assay. Cells were seeded in growth medium supplemented with 5% FBS at a density of 10^5 cells per 35-mm dish. Cells were refed 24 h later and every other day thereafter with fresh medium containing 0 to 100 ng of EGF (Collaborative Research, culture grade) per ml. Cells were harvested by trypsinization 8 days after seeding and counted with a Coulter counter.

Analysis of genomic DNA. High-molecular-weight DNA was isolated, digested with restriction enzymes, separated by agarose gel electrophoresis, transferred to Nytran (Schleicher & Schuell), and hybridized to nick-translated probe by standard protocols (33). Relative gene copy number was determined by densitometric analyses of autoradiograms. Hybridization was performed with the 2.4-kilobase *Cla*I insert of plasmid pE7 (59) as probe for the human EGF receptor; Jurkat-2, a pBR322 plasmid (61), for human T-cell receptor beta-chain; or the *Eco*RI insert purified from pHBV (54) for HBV.

Chromosome analysis. Metaphase spreads were obtained from the PLC/PRF/5 and NPLC/PRF/5 cell lines by standard techniques, stained with Giemsa stain (Gurr), scanned to detect homogeneous staining regions or double minutes, destained, and then treated to develop C or G bands (48, 53). Chromosomes from 50 well-spread metaphases at several widely separated passage levels were enumerated. Karyotypes were prepared and compared with those of PLC/PRF/5.

Cell labeling and extraction. Cells to be metabolically labeled were rinsed twice and preincubated for 1 to 2 h with methionine- or phosphate-free MEM and exposed, respectively, to 0.5 mCi of L-[^{35}S]methionine (>800 Ci/mmol; Amersham) or $^{32}P_i$ (900 to 1,100 mCi/mmol; Amersham) per ml for 4 to 5 h. In some experiments, cells were preincubated for 3 h and labeled for 3 h with the appropriate labeling MEM supplemented with 10 μg of tunicamycin (Calbiochem) per ml or 10 μM monensin (Calbiochem). For pulse-chase experiments, cells were labeled with 0.5 mCi of L-[^{35}S]methionine per ml for 10 min and chased with MEM supplemented with 40 mM methionine for the times indicated in the legend to Fig. 7. Cell surface labeling by the lactoperoxidase method was performed as described (8), and the cells were removed from the plate with a rubber policeman in 4°C Mg^{2+}- and Ca^{2+}-free PBS supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM benzamidine. In experiments to determine the effect of activating endogenous Ca^{2+}-dependent proteases, cells were harvested and incubated in 1 M Tris hydrochloride, pH 7.4, supplemented with 9 mM $CaCl_2$ for 5 to 10 min at room temperature (36). Cell pellets were suspended in a small volume of lysis buffer (0.1 M Tris hydrochloride, pH 6.8, 2 mM EDTA, 15% [wt/vol] glycerol) supplemented with protease inhibitors as above and extracted with 0.5 to 1.0% (wt/vol) Nonidet P-40 (NP-40) for 1 h at 4°C. Cell extracts were clarified by centrifugation at 10,000 × *g* for 15 min at 4°C and stored at −70°C if not analyzed immediately.

Immunoprecipitation and SDS-PAGE. P3X63Ag8 or EGF-R1 monoclonal antibody was absorbed onto protein A-bearing *Staphylococcus aureus* Cowan I (10 μl of ascites per 50 μl of a 10% suspension of bacteria) for 1 h at 4°C to produce a solid-phase immunoabsorbant. Radiolabeled cell extracts extensively precleared of material binding to *S. aureus* were immunoprecipitated for 1 h at 4°C, and immune complexes were washed in a 0.01 M Tris, pH 7.4, buffer containing 0.25% NP-40 and 0.15 M NaCl. For experiments to assay receptor-catalyzed tyrosine autophosphorylation, immune complexes were formed from freshly prepared extracts of unlabeled cells and incubated with [γ-^{32}P]ATP as described previously (9). Proteins to be analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique of Laemmli (28) were removed from bacteria by boiling for 3 min in a small volume of lysis buffer (see above) supplemented with 2% (wt/vol) SDS and 0.1 M dithiothreitol. Proteins to be analyzed by the two-dimen-

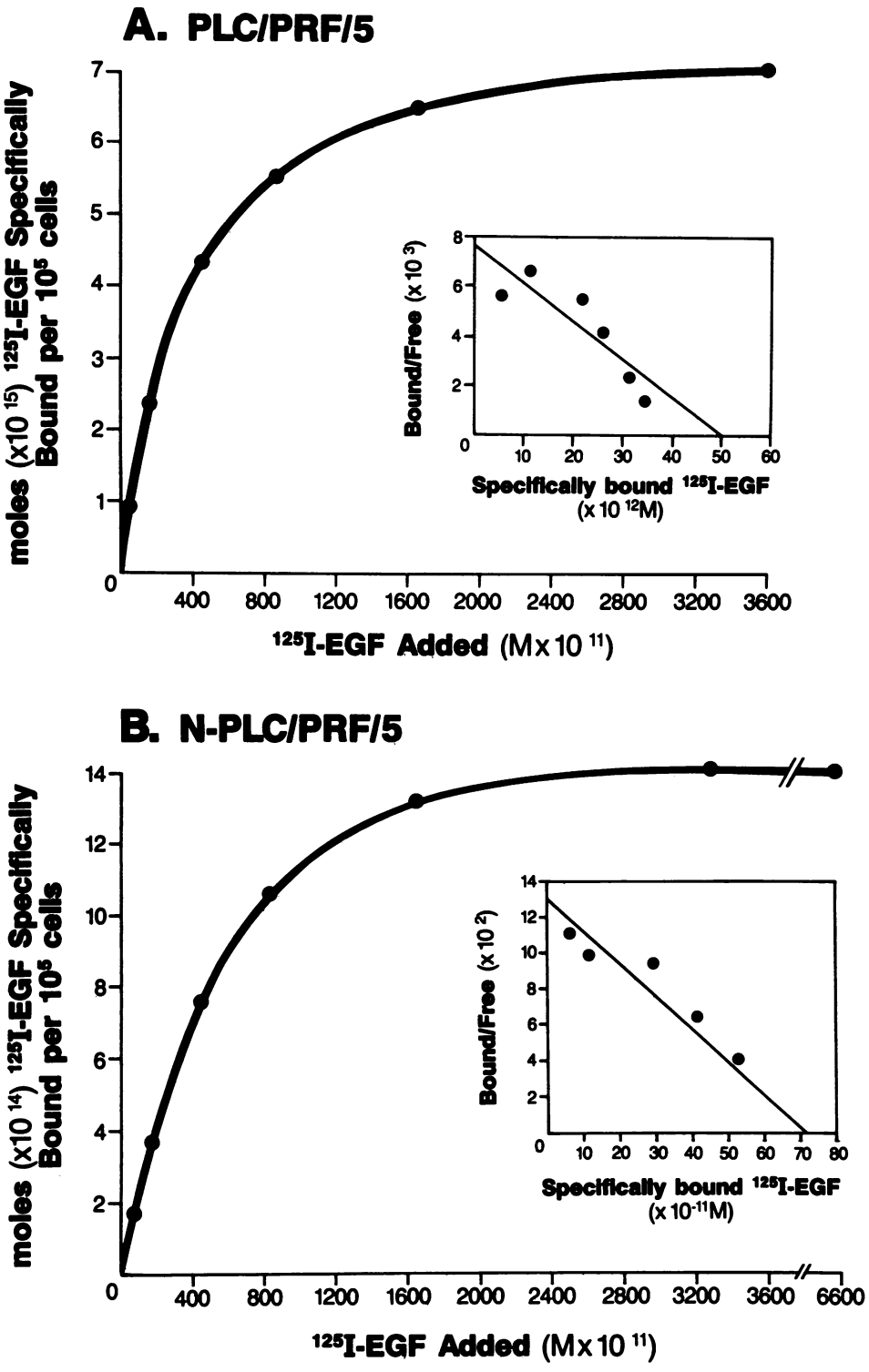

FIG. 1. Binding of increasing concentrations of [^{125}I]EGF by PLC/PRF/5 (A) and NPLC/PRF/5 (B) cells. Data points represent triplicate determinations, and standard deviations were 10% or less. Analyses of these data by the method of Scatchard are presented in the insets. Linear regression analysis was performed; r^2 = 0.87 for PLC/PRF/5, r^2 = 0.95 for NPLC/PRF/5.

sional gel electrophoretic technique (38) were removed from bacteria by incubation at 42°C for 20 min in a small volume of a solution containing 9.5 M urea, 2% (wt/vol) NP-40, 5% (vol/vol) 2-mercaptoethanol, and 2% (wt/vol) pH 6 to 8 ampholytes. SDS-polyacrylamide gels were dried onto filter paper under vacuum and exposed for autoradiography. Gels containing proteins labeled with L-[^{35}S]methionine were prepared for fluorography by established procedures.

Digestion with endoglycosidases. For digestion with endoglycosidase F (endo F; New England Nuclear Corp.), radiolabeled cell extracts were immunoprecipitated with EGF-R1 ascites absorbed onto protein A-bearing Sepharose beads (10 μl of ascites per 25 μl of a 50% suspension of beads; Pharmacia). Immune complexes were washed three times in the buffer described above and twice with 0.1 M phosphate buffer, pH 6.1, and suspended in a small volume of the phosphate buffer supplemented with 50 mM EDTA, 0.5% (wt/vol) NP-40, 0.1% (wt/vol) SDS, 2 mM PMSF, 50 mM benzamidine, and 40 U of endo F. Digestion with recombinant endoglycosidase H (endo H; Miles Biochemicals) was carried out in a similar manner, except that immune complexes were washed with 0.25 M sodium citrate, pH 6.0, and suspended in a small volume of the sodium citrate buffer supplemented with PMSF, benzamidine, and endo H (30 μg/ml). Following incubation for 18 h at 37°C, immune complexes were washed five times and prepared for analysis by SDS-PAGE as described above.

RESULTS

EGF receptor expression. EGF receptor expression of the seven human hepatoma-derived cell lines was compared with that of the normal human fibroblast cell line WI-38 and of the A431 cell line by quantitating their ability to bind EGF receptor-specific monoclonal antibody. Receptor expression on the surface of the hepatoma cell lines fell within the normal range, while the NPLC/PRF/5 cell line appeared to express the same level of receptor as the A431 cell line (Table 1). Scatchard analysis of EGF binding (Fig. 1) indicated that the parental PLC/PRF/5 cells expressed approximately 3×10^5 receptors per cell, while the NPLC/PRF/5 cells expressed approximately 4×10^6 receptors per cell (insets, Fig. 1). Receptor affinity for ligand did not differ significantly in PLC/PRF/5 and NPLC/PRF/5 cells ($K_d = 1.5 \times 10^{-8}$ and 1.8×10^{-8}, respectively) and was equivalent to that reported for other cells and hepatocytes (3, 11, 40).

The effect of EGF binding on the proliferation of PLC/PRF/5 and NPLC/PRF/5 cells was assessed by adding various concentrations of EGF to logarithmically growing cells (Fig. 2). When maintained in growth medium supplemented with 5% FBS alone, PLC/PRF/5 and NPLC/PRF/5 cells underwent 3.7 and 4.7 population doublings, respectively, during the course of the experiment. Addition of EGF slightly enhanced the growth response of PLC/PRF/5 cells, but proliferation of NPLC/PRF/5 cells was inhibited, with a half-maximal effective concentration of approximately 0.5 nM. A431 cells were included as a control in this experiment, since nanomolar concentrations of EGF are known to block the proliferative response of these cells (18).

Analysis of EGF receptor genomic DNA. Specific amplification of the gene encoding the EGF receptor in the NPLC/PRF/5 cells was detected by Southern blot analysis of restriction enzyme-digested cellular DNA (Fig. 3A). Densitometry of the autoradiograms revealed 20- to 30-fold greater intensity of the bands in the NPLC/PRF/5 lane relative to that of bands in the PLC/PRF/5 and WI-38 lanes. The EGF receptor gene has been localized to the short arm, 7p12 (8, 26), and the T-cell receptor beta-chain gene has been localized to the long arm, 7q32 (37), of chromosome 7. To determine whether amplification of the EGF receptor could be attributed to polysomy 7, the filters were probed with the T-cell receptor beta-chain probe Jurkat-2. Since the DNA from PLC/PRF/5 and NPLC/PRF/5 hybridized to an equivalent extent with this probe (Fig. 3A, right panel), amplification of the EGF receptor genes, or genes on the short arm of chromosome 7, appears probable. The patterns of hybridization of PLC/PRF/5 and NPLC/PRF/5 genomic DNA to the EGF receptor probe, after digestion with several restriction enzymes, were essentially identical (Fig. 3B), suggesting that the amplified EGF receptor gene in NPLC/PRF/5 cells was not grossly rearranged. EGF receptor amplification was also not accompanied by any obvious change in HBV integration; both cell lines displayed identical hybridization with the HBV probe (Fig. 3C).

Chromosome analysis. Comparison of the karyotypes of PLC/PRF/5 and its derivative NPLC/PRF/5 was performed to determine whether EGF receptor gene amplification was associated with specific chromosome aberrations. Neither double minutes (35) nor homogeneous staining regions (5),

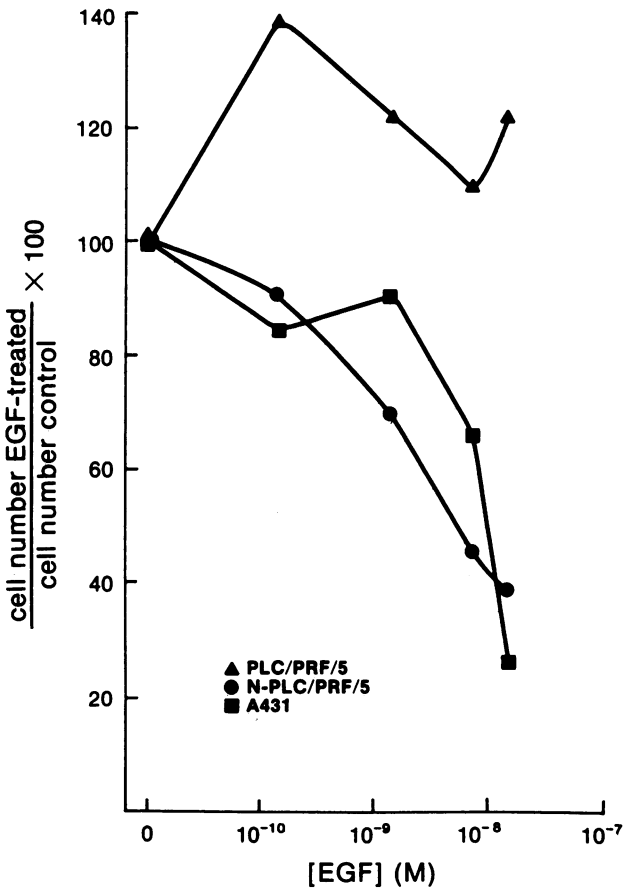

FIG. 2. Effect of EGF on growth of PLC/PRF/5, NPLC/PRF/5, and A431 cells. Cells that had been allowed to attach were refed with medium containing from 0 to 100 ng of EGF per ml and counted 6 days later. Data are expressed as (the number of cells in EGF-treated cultures/the number of cells in control cultures) × 100. All points represent means of triplicate cell counts. Standard deviations were always ≤10%.

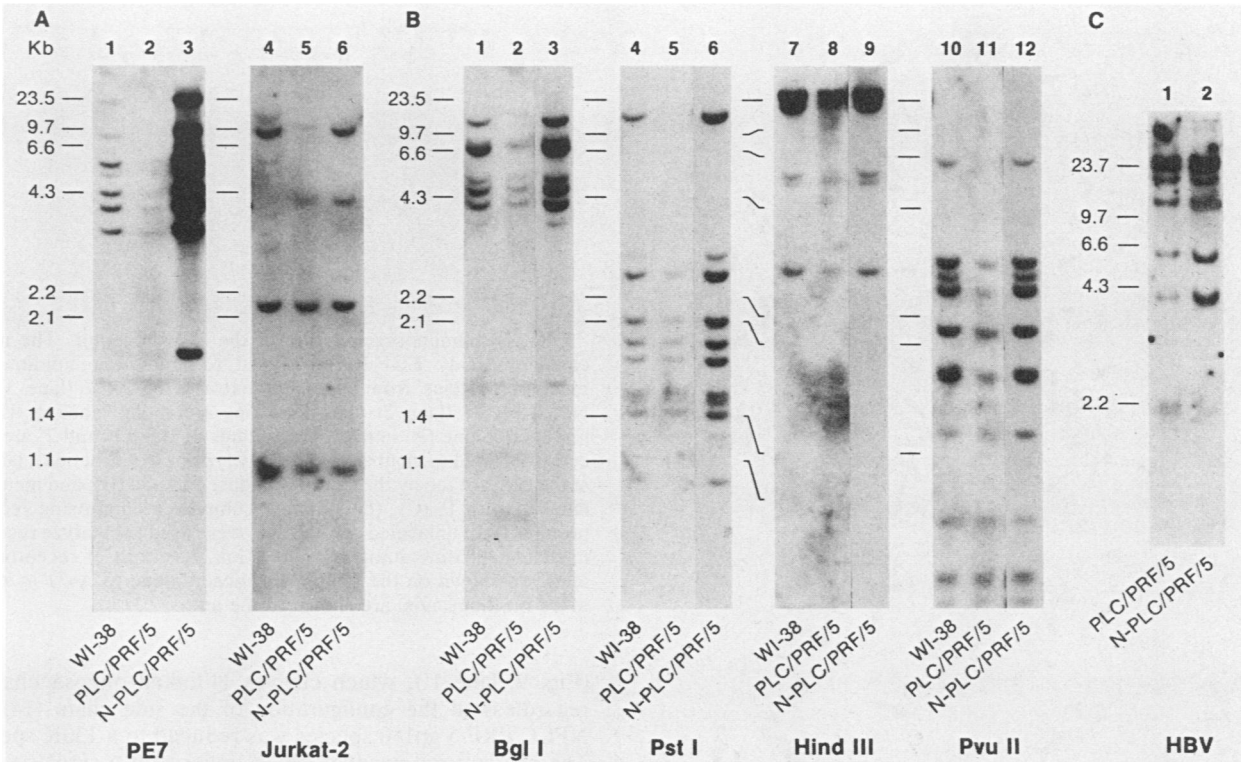

FIG. 3. Amplification of the EGF receptor gene. Genomic DNA was isolated from WI-38, A431, PLC/PRF/5, and NPLC/PRF/5 cells. (A) *Eco*RI restriction fragments of high-MW DNA (20 μg) were separated by gel electrophoresis, transferred to Nytran, and then hybridized to nick-translated, ^{32}P-labeled human EGF receptor-specific sequences (*Cla*I insert purified from pE7) (left) or human T-cell receptor beta-chain-specific sequences (Jurkat-2) (right). (B) Restriction fragments were generated with the enzymes indicated and hybridized with ^{32}P-labeled EGF-specific sequences (*Cla*I insert purified from pE7). Autoradiograms were exposed for 4 h (NPLC/PRF/5) or 5 days (WI-38 and PLC/PRF/5). (C) Restriction fragments were generated by digestion with *Hin*dIII and hybridization with ^{32}P-labeled HBV sequences (*Eco*RI insert purified from pHBV).

chromosome changes often associated with specific gene amplification (47), were found (Fig. 4). The marker chromosomes, previously identified in the PLC/PRF/5 cell line (50), were present in the NPLC/PRF/5 cell line. However, there were significantly more chromosomes in the NPLC/PRF/5 cells (range, 75 to 85) than in the original PLC/PRF/5 cell line (range, 55 to 60). Furthermore, a unique marker chromosome, whose origin could not be determined by analysis of its banding pattern, was detected in each NPLC/PRF/5 metaphase (Fig. 4).

Characterization of the EGF receptor protein expressed by PLC/PRF/5 and NPLC/PRF/5 cells. To determine whether the elevated expression of the EGF receptor in NPLC/PRF/5 cells compared with that in the parental and other cell lines rested in differences in the EGF receptor itself, we examined detergent extracts prepared from cells metabolically labeled with L-[^{35}S]methionine and immunoprecipitated with the monoclonal EGF-R1 antibody (Fig. 5). Receptor molecules detected in A431, Hep 3B, and Hep G2 cells were of slightly higher molecular weight (MW) than those from both NPLC/PRF/5 cells and the parental cells. The mature form of the EGF receptor from A431 cells was of 165,000 apparent MW (gp 165), while that of the PLC/PRF/5 cell lines was 150,000 (gp 150) (Fig. 5 and 6). The gp150 could be cleaved by an endogenous Ca^{2+}-mediated protease to a species denoted gp138; the abundance of gp138 in NPLC/PRF/5 cells, like that of gp140 in A431 cells (16, 60), increased after activation of this protease during cell harvesting (Fig. 6A). Differential migration of the EGF receptor from the NPLC/PRF/5 and A431 cell lines was also detectable when immunoprecipitates from surface-radiolabeled cells (Fig. 6B), cells metabolically labeled with $^{32}P_i$ (Fig. 6C), and cells labeled with ^{32}P by receptor-mediated autophosphorylation in an immune complex assay (Fig. 6D) were comparatively analyzed by SDS-PAGE.

Biosynthesis and posttranslational processing of the gp150 receptor species. To determine whether the aberrant apparent MWs of the EGF receptor from the PLC/PRF/5 cell lines reflected primary changes or differences in posttranslational modification of the primary gene product, we examined EGF receptor biosynthesis. Pulse-chase labeling analysis of the EGF receptor in NPLC/PRF/5 cells revealed a single receptor protein at early time points in the chase period (Fig. 7) that became more diffuse at the late time point. When the cotranslational addition of N-linked oligosaccharides is inhibited by metabolic labeling in the presence of tunicamycin (30), or when Golgi-mediated processing is disrupted by monensin, biosynthetic precursors of the mature form of the EGF receptor can be detected (19). Under these conditions, NPLC/PRF/5 cells displayed a species denoted p135 (Fig. 8B, lane 2), which represented the aglycosyl form of gp150, and gp145 (Fig. 8C, lane 2), its Golgi-associated biosynthetic precursor. In both cases, these receptor species comigrated with those immunodetectable in A431 (Fig. 8B and C, lanes 1) and Hep 3B (7) cells, suggesting that the differences in mobility between the mature form of the receptor in both PLC/PRF/5 cell lines was the result of posttranslational processing. Under the same conditions, the aberrant species of the EGF receptor expressed by A431 cells, such as gp95, the precursor of the receptor-related species detected in the

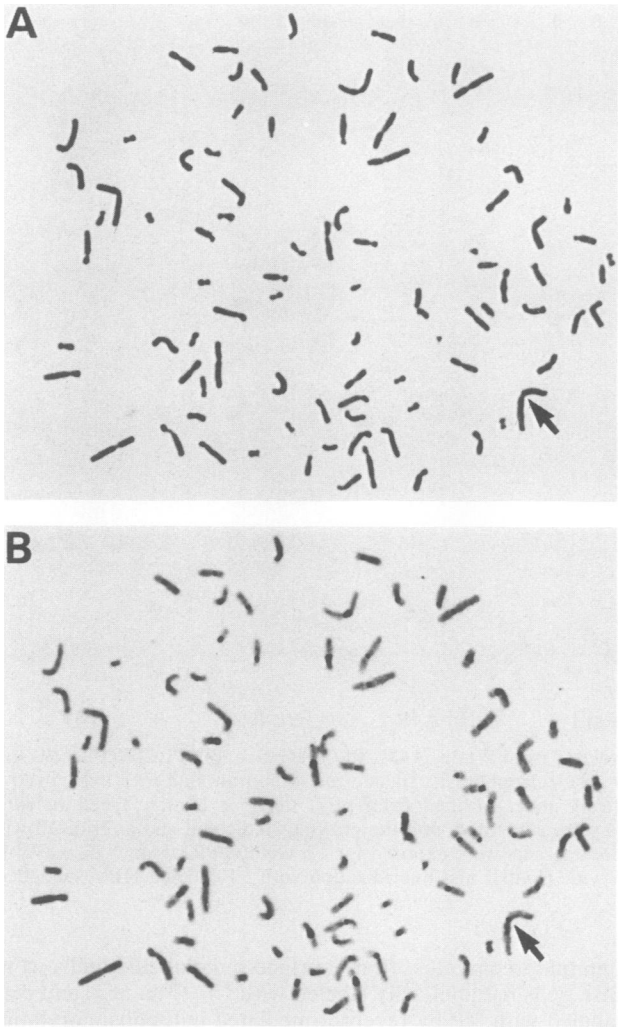

FIG. 4. Chromosome spread from NPLC/PRF/5. (A) Giemsa banding (B) C banding. Arrows indicate the chromosome unique to NPLC/PRF/5.

culture supernatants (7, 36), the p135, p115, and p70 species (7), were clearly detectable.

To determine whether differential glycosylation might underlie the differing mobilities of the EGF receptor in the PLC/PRF/5 cell lines, we examined the effect of endoglycosidase treatment on MW. Following digestion with endo F

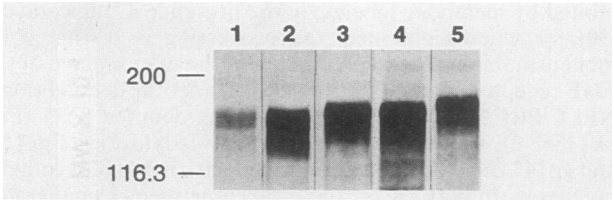

FIG. 5. Immunoprecipitation of EGF receptor proteins from NP-40 extracts of L-[^{35}S]methionine-labeled cells with EGF-R1. Analysis was by SDS-PAGE (7.5% acrylamide gel) and autoradiography. Lane 1, PLC/PRF/5 cells; lane 2, NPLC/PRF/5 cells; lane 3, A431 cells; lane 4, Hep 3B cells; lane 5, Hep G2 cells. MW markers were myosin, 200,000; β-galactosidase, 116,300; phosphorylase b, 92,500; bovine serum albumin, 66,200; and ovalbumin, 45,000. MWs are indicated (in thousands).

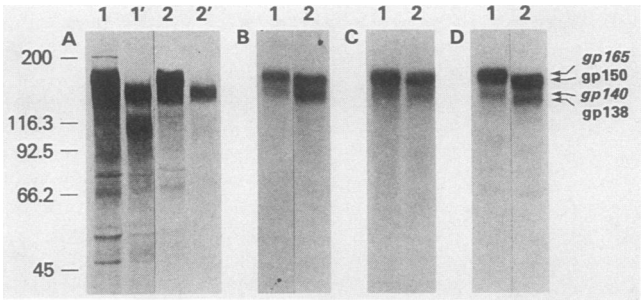

FIG. 6. Immunoprecipitation of the EGF receptor. The monoclonal antibody EGF-R1 was used to immunoprecipitate EGF receptor proteins from detergent extracts of A431 (lane 1) and NPLC/PRF/5 (lane 2) cells following metabolic labeling with L-[^{35}S]methionine (A; immunoprecipitates in lanes 1′ and 2′ are from cells subjected to incubation with $CaCl_2$ prior to extraction); labeling at the cell surface by the lactoperoxidase-method (B); and metabolic labeling with P_i (C). (D) Immune complexes comprising receptor proteins from unlabeled cell extracts were used to catalyze receptor-mediated tyrosine autophosphorylation. Positions of receptor proteins are shown on the right, with those unique to A431 in italics. MWs (in thousands) are shown to the left.

(Fig. 9, lane 1′), which cleaves N-linked oligosaccharides regardless of the configuration of the side chain (14), the NPLC/PRF/5 gp150 species was reduced to a 130K species. The proteolyzed product gp138 underwent a similar reduction. gp150 was also diminished in size, although to a lesser extent, following digestion with endo H (Fig. 9, lane 2′). This endoglycosidase specifically cleaves the cotranslationally acquired N-linked oligosaccharides in the "high-mannose" configuration (32) that are associated with glycoproteins undergoing Golgi-mediated processing (42). Although all species detected during pulse-chase labeling served as substrates for endo H, the MW of the gp145 biosynthetic intermediate was reduced to a greater extent than that of gp150 (data not shown), suggesting that only a portion of the N-linked oligosaccharides of the mature gp150 receptor molecule were converted from the high-mannose to the complex carbohydrate configuration. Processing of N-linked oligosaccharides may occur to a lesser or greater degree, giving rise to subtle differences in MWs in other cells as well

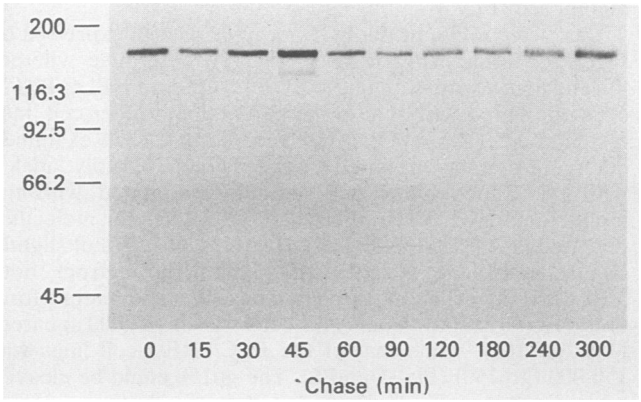

FIG. 7. Pulse-chase labeling of NPLC/PRF/5 cell EGF receptor. NPLC/PRF/5 cells were preincubated with methionine-free MEM for 3 h and pulse-labeled with L-[^{35}S]methionine for 10 min, followed by incubation with 40 mM unlabeled methionine for the lengths of time indicated. NP-40 cell extracts were immunoprecipitated with EGF-R1. MWs (in thousands) are indicated to the left.

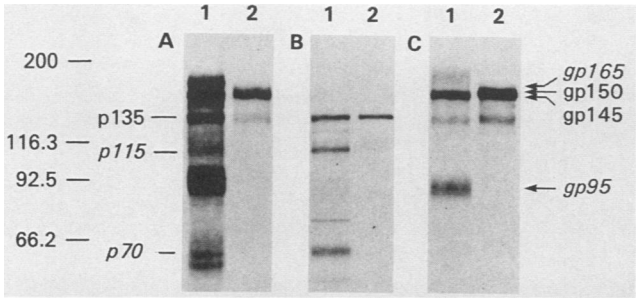

FIG. 8. Biosynthesis of the EGF receptor in the presence of drugs that disrupt co- and posttranslational processing. A431 (lane 1) and NPLC/PRF/5 (lane 2) cells were preincubated for 3 h with methionine-free MEM (A) or with methionine-free MEM supplemented with 10 μg of tunicamycin per ml (B) or 10 μM monensin (C). Cells were labeled with L-[^{35}S]methionine for 3 h, and NP-40 extracts were immunoprecipitated with EGF-R1. Aglycosyl species are indicated on the left, and glycosylated species on the right; species detected in A431 cells only are shown in italics. MWs (in thousands) are shown to the far left.

(see lanes 3 to 5 in Fig. 5; note that the apparent MW of Hep G2 cells was comparatively greater). The gp150 species was also subject to posttranslational phosphorylation by one or more kinases in both the absence and presence of newly synthesized glycoprotein.

Two-dimensional gel electrophoresis of EGF receptor proteins expressed by NPLC/PRF/5 cells. The isoelectric points of receptor proteins from NPLC/PRF/5 were between pI 7.2 and 7.4 (Fig. 10A to D), the same range previously reported for receptor molecules expressed by WI-38 (9) and A431 cells (8). The gp145 biosynthetic precursor and the mature gp150 species (Fig. 10A), as well as the two species derived by Ca^{2+}-mediated proteolysis (Fig. 10D), were all clearly discriminated. The species detected in cells labeled in the presence of monensin (Fig. 10C) comigrated with the gp145 species in untreated cells (cf. Fig. 10A), and the prominent phosphoprotein (Fig. 10E) comigrated with the mature gp150 species (cf. Fig. 10A). The pI of gp150 was higher than that of gp145, suggesting the presence of a phosphate moiety in the former.

DISCUSSION

Normal hepatocytes are long-lived cells which rarely divide yet retain the ability to regenerate in response to physiologic stimuli. The hepatoma cell lines investigated herein can be considered functional equivalents of the parenchymal hepatocytes (23), which continuously proliferate due to a defect in their ability to enter a resting state. From these studies, we conclude that inappropriate expression of the EGF receptor on the hepatoma cell surface does not appear to be among the factors responsible for the aberrant proliferation of these cells. EGF receptor expression of each of the seven independently derived human hepatoma cells was within the range of that previously detected on the hepatocyte, a result at variance with those of others whose data suggest that EGF receptor expression is decreased on hepatoma cells (11). We found that (i) the EGF receptor was substantially overexpressed in our laboratory derivative (NPLC/PRF/5) of the original PLC/PRF/5 cell line, (ii) the affinity of the receptor for ligand was equivalent in both of these cell lines, and (iii) some concentrations of EGF that elicited mitogenesis in PLC/PRF/5 cells inhibited NPLC/PRF/5 proliferation.

EGF receptor overexpression and concomitant inhibition of proliferation in the presence of similar concentrations of ligand also characterize the A431 cell line. In those cells, EGF receptor is not only overexpressed, but complex chromosome rearrangements involving the p arm of chromosome 7 and internal rearrangement of the EGF receptor gene result in production of aberrant truncated receptor-related molecules (55, 57). In contrast to the findings with the A431 cells, no evidence for internal rearrangement within the EGF receptor gene was found by analysis of NPLC/PRF/5-derived genomic DNA, and although the apparent MW of the EGF receptor from both PLC/PRF/5 cell lines was decreased, no aberrant forms of the receptor were detected. Fewer high-mannose moieties of the EGF receptor N-linked oligosaccharides in the PLC/PRF/5 cell lines appeared to undergo posttranslational processing to complex carbohydrate than in the A431 and Hep 3B cell lines (7), an alteration which did not appear to generally affect other glycoproteins synthesized by PLC/PRF/5 cells (data not shown). Variable processing of the EGF receptor oligosaccharide did not affect the ability of the receptor to bind ligand or to catalyze autophosphorylation, an observation compatible with the information available from analysis of other receptors. For example, the normal glycosylation pattern of the receptors for insulin and insulinlike growth factor I have been experimentally manipulated without effect on receptor function (13). Furthermore, naturally occurring variation in the processing of N-linked oligosaccharides of the C3b/C4b receptor in granulocytes versus monocytes does not alter the ability of these cells to bind ligand (58). Thus, oligosaccharide side chain heterogeneity is probably a reflection of cell or host genetic variation without effect on the function of the EGF receptor. Clarification of the basis of inhibition of proliferation in response to ligands awaits further investigation of the intracellular events following receptor-ligand interaction.

By comparative karyology of PLC/PRF/5 and NPLC/PRF/5 and Southern blot hybridization with the probe for the chromosome 7q encoded T-cell receptor β-chain, we found that EGF amplification was the result neither of the formation of double minutes or obvious homogeneous staining regions nor of simple polysomy of chromosome 7. Rather, we suggest that the unique chromosome present in

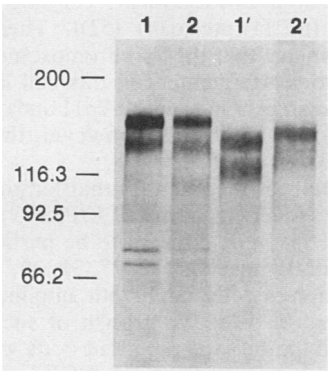

FIG. 9. Digestion of EGF receptor proteins with endoglycosidases. NPLC/PRF/5 cells were metabolically labeled with L-[^{35}S]methionine, and cell extracts were immunoprecipitated with EGF-R1 absorbed onto protein A-bearing Sepharose beads. Digestion with endoglycosidases was for 24 h at 37°C. Incubation was done in the absence (lane 1) or presence (lane 1') of endo F and the absence (lane 2) or presence (lane 2') of endo H. MWs (in thousands) are indicated.

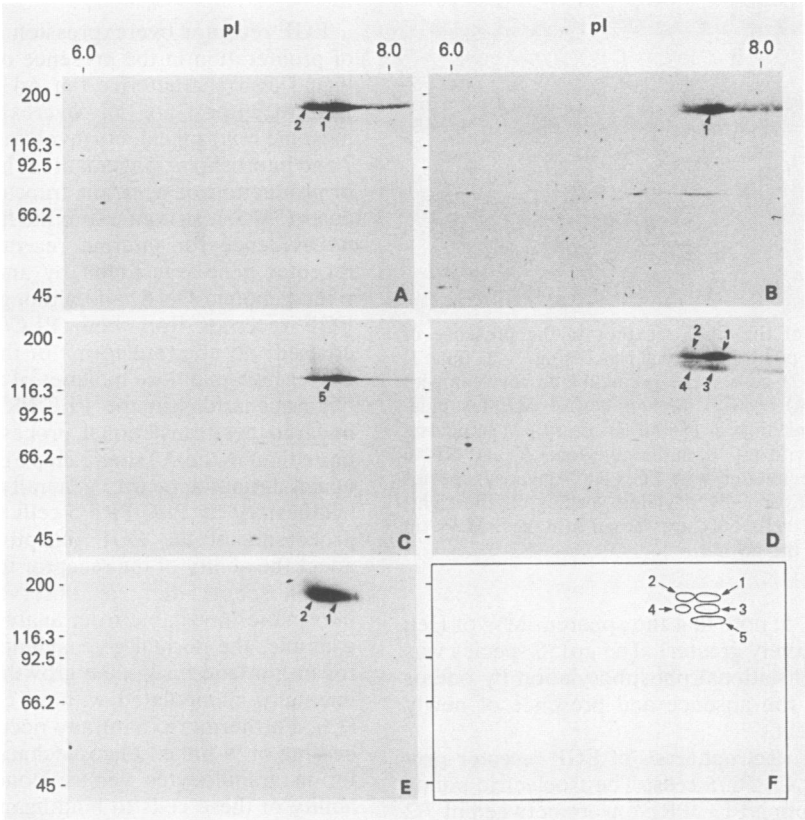

FIG. 10. Two-dimensional gel electrophoresis of the EGF receptor expressed by NPLC/PRF/5 cells. NP-40 cell extracts were immunoprecipitated with EGF-R1 and separated by isoelectric focusing in a pH 6.0 to 8.0 gradient in the first dimension and by SDS-PAGE (7.5% acrylamide gel) in the second dimension. Receptor proteins were immunoprecipitated from cells that had been metabolically labeled with L-[^{35}S]methionine (A) or metabolically labeled with L-[^{35}S]methionine in the presence of monensin (B) or tunicamycin (C); from cells metabolically labeled with L-[^{35}S]methionine and subjected to processing by endogenous Ca^{2+}-dependent proteases (D); or from cells metabolically labeled with $^{32}P_i$ (E). Results are summarized in panel F. The EGF receptor species are indicated as follows: 1, gp145; 2, gp150; 3 and 4, forms derived by Ca^{2+}-mediated proteolysis of gp145 and gp150, respectively; 5, p135. MWs (in thousands) are shown to the left.

NPLC/PRF/5 but not PLC/PRF/5 cells may carry multiple copies of portions of the p arm of chromosome 7 containing the intact EGF receptor gene, a hypothesis that warrants substantiation by in situ hybridization of the EGF receptor probe to NPLC/PRF/5 metaphase spreads. It is perhaps worthwhile to note that the p arm of one copy of chromosome 7 in the original PLC/PRF/5 cell line is involved in a translocation [t(7;11)(pter;q14) (52)]. These observations raise the possibility that the 7p chromosome may be particularly prone to rearrangement in this cell line. Two fragile sites in the human genome, one at 7p11 and one at 7p13, map to this area of the genome (29); however, the significance of fragile sites awaits clarification.

Reconstruction of the event(s) that led to amplified EGF receptor gene sequences in the PLC/PRF/5 cell is not possible; however, this gene appears to be particularly prone to amplification in tumor cells (22, 27, 31, 39, 44). To obtain a stable population of cells, each with amplified expression of the EGF receptor, selective growth of such cells must be postulated, a hypothesis at apparent odds with the observation that the concentrations of EGF required to elicit mitogenesis in vitro were inhibitory to NPLC/PRF/5 proliferation. However, the actual concentration of EGF in serum-containing culture medium or encountered during growth in the nude mouse is probably at least 10-fold less than that required to induce growth inhibition (10). Under these conditions, proliferation of those cells with an increased number of high-affinity receptors might be favored, a hypothesis consistent with the data obtained from analyses of the tumorigenic potential in nude mice of A431 clonal lines expressing different levels of the EGF receptor (45). Analogous results come from the study of the MDA-468 cell line and its variants, in which a positive correlation between amplified EGF receptor expression and tumorigenicity was also found (15). When viewed in this light, amplification of EGF receptor in tumor cells might be considered a manifestation of tumor progression, conferring a relative growth advantage to tumor cells in their natural host or under experimental conditions.

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