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Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines

(c-*erb*B/transforming protein/DNA amplification/RNA blot analysis/immunoprecipitation)

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ABSTRACT **To investigate the possibility that the epidermal growth factor (EGF) receptor functions as an oncogene product, we have determined the levels of EGF receptor protein and RNA in a variety of malignant and normal human cells, using a specific polyclonal antibody to the EGF receptor and a cDNA clone (plasmid pE7) that encodes the EGF receptor, respectively. Besides A431 epidermoid carcinoma cells, which are known to make large amounts of EGF receptor, cell lines from two ovarian cancers, two cervical cancers, and one kidney cancer were found to contain substantial amounts of receptor protein (11–22% of A431). Normal human fibroblasts (Detroit 551), a human lymphocyte line (IM-9), and a leukemic lymphocyte line (CEM) contained low or undetectable levels of EGF receptor. RNA blot analysis showed that among the human cell lines examined the levels of a 10- and a 5.6-kilobase species of pE7-specific RNA generally correlated with the amount of the EGF receptor protein. Genomic DNA blot analysis revealed that except for A431 none of these cell lines expressing high levels of EGF receptor protein possessed amplified receptor gene sequences. A431 cells are known to secrete a truncated form of the EGF receptor. An abundant 2.9-kilobase RNA is found only in A431 cells; it could encode the truncated form of the EGF receptor.**

Epidermal growth factor (EGF) receptor is a 170-kDa glycoprotein found in many cell types (1–6). The receptor has an intrinsic tyrosine-specific protein kinase activity that is stimulated by EGF (6–8). EGF binding is known to cause "down regulation" of the receptor (9–13). Downward *et al.* (14) have recently isolated six separate peptides from the EGF receptor. The peptides were found to be very similar to the deduced amino acid sequence of the oncogene *erb*B protein product [v-*erb*B (15)] of the avian erythroblastosis virus). The *erb*B gene product has been shown to cause erythroblastosis and sarcomas in infected chickens (16, 17). The strong similarity between the v-*erb*B protein and the EGF receptor suggests that the human cellular homolog to v-*erb*B (c-*erb*B) and the EGF receptor gene are closely related or are identical.

We have used a fragment of the v-*erb*B gene to isolate cDNA clones structurally related to the EGF receptor gene (18). One 2.4-kilobase pair (kbp) cDNA clone (pE7) encodes three of the peptides sequenced by Downward *et al.* (14) and is highly homologous to a large portion of the v-*erb*B oncogene. It seems likely that clone pE7 encodes a portion of the EGF receptor because such clones were easily isolated from a cDNA library made from A431 epidermoid carcinoma cells, which have an unusually large number of EGF receptors (19, 20), and could not be detected in a cDNA library from WI38 human fibroblasts, which have a relatively low number of EGF receptors (unpublished data). Clone pE7 hybridizes to three major A431 RNA species, of 10, 5.6, and 2.9 kilobases (kb), as well as minor RNAs of 6.3, 4.6, and 3.3 kb (18). Other groups have identified similar-sized EGF receptor RNAs in A431 cells (21, 22). In A498 kidney carcinoma cells the only major RNAs that were found to hybridize to this probe were the 10- and 5.6-kb species (18).

To gain further information about the EGF receptor gene and its expression, a variety of cell lines were examined to determine the quality and quantity of pE7-hybridizable RNAs. EGF receptor levels were measured by using a specific rabbit polyclonal antibody to the EGF receptor (anti-EGFR) and RNA levels were quantified, utilizing the pE7 cloned EGF receptor cDNA (18) as a probe. The copy number of the EGF receptor gene in these cell lines was determined by using the same pE7 probe.

MATERIALS AND METHODS

Cell Cultures. Cell lines were maintained in medium supplemented with 10% fetal bovine serum (GIBCO) unless stated otherwise. The human epidermoid carcinoma cell lines A431 (George Todaro, National Institutes of Health) and KB (American Type Culture Collection), and the human kidney carcinoma cell line A498 (G. Todaro) were maintained in Dulbecco's modified Eagle's medium (DME medium). HTB32 (a cervical carcinoma cell line from the ATCC) and IM-9 lymphocytes (J. Roth, National Institutes of Health) were grown in RPMI 1640 medium. The ovarian cell lines were 1847 from S. Aaronson (National Institutes of Health), and OVCAR2 and OVCAR3 (referred to in this paper as OVCA2 and OVCA3, respectively) from T. Hamilton and R. Ozols (National Institutes of Health). The ovarian lines were maintained in RPMI 1640 medium with insulin at 10 μg/ml. MCF-7 breast cancer cells (M. Lippmann, National Institutes of Health) were grown in improved minimal Eagle's medium (National Institutes of Health Media Unit). Detroit 551 normal human fibroblasts (ATCC no. CCL10) were grown in DME medium, 1 mM sodium pyruvate, and the nonessential amino acids of minimal Eagle's medium at 0.1 mM each. Human leukemic lymphocytes (CEM) were maintained in Eagle's minimal spinner medium (HEM Research, Rockville, MD).

Toxicity Assay. A conjugate of EGF and *Pseudomonas* exotoxin (PE) was constructed by the disulfide exchange reaction previously described (23). The conjugate has 2 mol of EGF per mol of PE (M_r 66,000). Therefore the EGF concentration in EGF-PE at 1 μg/ml is 0.2 μg/ml.

For toxicity assays, various cell lines were plated in 24-well Costar dishes at 5×10^4 cells per well. The following day 1:10 serial dilutions of EGF-PE were added to the wells at concentrations ranging from 100 to 0.01 ng/ml. The cells were incubated with the toxin conjugate at 37°C for 48 hr. The dishes were stained with 0.5% methylene blue in etha-

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Abbreviations: EGF, epidermal growth factor; anti-EGFR, antibody to EGF receptor; kbp, kilobase pair; kb, kilobase(s); PE, *Pseudomonas* exotoxin.

nol/phosphate-buffered saline (P_i/NaCl) (1:1, vol/vol) to assess cell killing.

Isolation of RNA and RNA Blotting. For RNA isolation cells were plated so that they were just confluent 24 hr later. Total RNA was isolated from various cell lines by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion (24). Poly(A)$^+$ RNA, purified by passage over oligo(dT)-cellulose, was fractionated on 1% agarose/formaldehyde gels (5 μg per well) and transferred to nitrocellulose (25). The resulting filter-bound RNA was prehybridized and hybridized (18, 25) for 36 hr with nick-translated (26) ^{32}P-labeled cDNA inserts. Washing included 1 hr in 30 mM NaCl/3 mM sodium citrate/0.1% $NaDodSO_4$ at 43°C. Filters were subjected to autoradiographic analysis, which was quantitated by microddensitometry.

Isolation of DNA and Southern Blotting. High molecular weight genomic DNA was isolated by using $NaDodSO_4$/proteinase K lysis, organic extraction, and extensive dialysis (27, 28). Routinely, 15 μg of genomic DNA was digested with *Eco*RI, electrophoresed on 1% agarose, transferred to nitrocellulose (27, 29), and probed with ^{32}P-labeled pE7 cDNA insert. Prehybridization and hybridization were as described (27–29). To reduce probe-related nonspecific background, the pE7 probe was first exposed to a blank nitrocellulose filter in complete hybridization buffer containing 10% dextran sulfate for 2–3 hr before hybridization to the DNA filter. Some variation in the amount of DNA loaded into each well is due to difficulty in pipetting viscous high molecular weight DNA preparations but should not exceed a factor of 2.

Rabbit Anti-EGFR. Antiserum no. 2913 was generated by immunizing a rabbit with EGF receptor purified from A431 cells by affinity chromatography on EGF-agarose followed by $NaDodSO_4$ gel electrophoresis. For receptor purification, plasma membranes were isolated by the method of Thom *et al.* (30), using calcium- and magnesium-free medium (31). The membranes were solubilized with 5% Triton X-100/10% glycerol/20 mM Hepes, pH 7.4, clarified by centrifugation at 100,000 × *g*, and applied to a 1-ml column of EGF-Affi-Gel 10 (2 mg of EGF per ml of gel) prepared as described (32). The column was washed extensively with 20 vol of 20 mM Hepes, pH 7.4/0.2% Triton X-100/10% glycerol/0.5 mM EGTA/0.5 mM EDTA to remove unbound material. EGF receptor was eluted with 0.5% $NaDodSO_4$ in 20 mM NH_4HCO_3 and electrophoresed on a $NaDodSO_4$/8.5% polyacrylamide gel. The EGF receptor protein band (170 kDa) was excised after staining the gel with 0.2% Coomassie brilliant blue in water. The yield of EGF receptor from 10 roller bottles was 200–300 μg. For injection, gel slices (containing 100 μg of receptor) were lyophilized, pulverized with a mortar and pestle, and resuspended in P_i/NaCl. The receptor preparation was emulsified with complete Freund's adjuvant and injected at multiple sites intradermally. Subsequent injections (using 100 μg of receptor in incomplete Freund's adjuvant) were performed twice at 3-week intervals, then once a week for 5 weeks. The properties of this antibody, which recognizes intracellular but not extracellular determinants present in the EGF receptor and the v-*erb*B gene product, will be described elsewhere.

Immunoprecipitation of Radiolabeled Human Cell Extracts. Monolayer cultures plated 6 hr prior to labeling at 6 × 10^6 cells per 100-mm dish were incubated with [^{35}S]methionine (0.25 mCi/ml) in 4 ml of methionine-free minimal essential medium and 5% fetal bovine serum for 16 hr. Suspension cells (IM-9 and CEM) were labeled in 10 ml of the same medium at 2 × 10^6 cells per ml for 16 hr. Cell extracts were prepared as described (33), using 1 ml per dish of lysis buffer [20 mM Hepes, pH 7.4/10% glycerol/1% Triton X-100/0.17 unit of trypsin inhibitor Aprotinin (Sigma) per ml]. The cell extracts were clarified by centrifugation at 100,000 × *g* for 30 min and adsorbed with 0.2 ml of Formalin-fixed *Staphylococcus aureus* (10%, wt/vol) at 4°C for 15 min prior to immunoprecipitation. The protein concentration of the cell lysates was 0.5–1.0 mg/ml.

To ensure that the precipitation was linear with extract concentration, three aliquots of each cell lysate (50, 100, and 250 μl) were immunoprecipitated with 20 μg of rabbit antiserum and 50 μl of *S. aureus*, using the procedure previously described (33). The immunoprecipitates were analyzed by $NaDodSO_4$/PAGE, using 5–15% linear gradient gels and the Laemmli buffer system (34). After fluorography (35) and autoradiography, the gels were quantitated by excising the EGF receptor protein band immunoprecipitated from each cell extract. The gel slices were extracted with 2 ml of 30% (wt/vol) H_2O_2/1% NH_4OH in scintillation vials at 39°C for 24 hr, then 20 ml of Aquasol (New England Nuclear) was added for counting. Background radioactivity was determined for each lane by excising another region of the gel of comparable size.

[^{35}S]Methionine incorporation into total cell protein was determined by precipitating duplicated aliquots of each cell extract with 1 ml of 20% (wt/vol) trichloroacetic acid, using 200 μg ovalbumin as a carrier. Protein concentrations were determined by the Bradford method (36), using bovine gamma globulin as a standard.

RESULTS

Initially a large number of human cell lines were screened for their ability to bind and internalize EGF. This was accomplished by exposing cells for 48 hr to a conjugate of EGF and PE (EGF-PE). This toxin conjugate has previously been shown to bind to cells via the EGF receptor and to kill cells possessing this receptor (23). As shown in Table 1, A431 cells, which each have 2–3 × 10^6 receptors for EGF (19, 20), were killed by EGF-PE at 0.01 ng/ml, and KB cells which have 1–2 × 10^5 EGF receptors per cell (37) were killed by EGF-PE at 10 ng/ml. The MCF-7 breast cancer cell line has a very low number of EGF receptors [<1000 per cell (B. Dickson, National Institutes of Health, personal communication)] and was killed by EGF-PE at 100 ng/ml. Cell killing by this concentration of EGF-PE probably represents background nonspecific killing. Cell lines that were killed by EGF-PE at 100 ng/ml or more included five melanoma lines (HTB63, HTB66, HTB70, HTB72, and H234A), two bladder cancer lines (HTB2 and HTB3), and one Wilms tumor (HTB-50).

To determine more precisely the amount of EGF receptor present in the various cell lines, the cells were labeled for 16 hr with [^{35}S]methionine and the cell lysates were immunoprecipitated with rabbit anti-EGFR. As shown in Fig. 1, the highest amounts of EGF receptor were found in A431 cells. Readily detectable amounts were found in two ovarian cell lines (1847 and OVCA2), a cervical carcinoma cell line (HTB32), and a kidney carcinoma cell line (A498). Very low amounts were found in normal human fibroblasts (D551). Under the conditions used, EGF receptor levels were below the limits of detection in MCF-7, CEM (leukemic lymphoblasts), and IM-9 (lymphocytes). Two EGF receptor protein bands of 170 and 150 kDa are precipitated by anti-EGFR in many of the cell extracts (Fig. 1). The lower band could represent either a precursor (38) or a proteolytic breakdown product (31).

The radioactivity in the immunoprecipitated EGF receptor protein band was determined in at least two separate labeling experiments for each cell line. From the radioactivity precipitated and the specific activity of the cell extract (^{35}S cpm/mg of total cell protein), the amount of EGF receptor protein in each cell line was estimated (Table 1). In the experiment shown, A431 cells had 10.7 μg of EGF receptor per mg of total cell protein. Assuming a molecular weight of

Table 1. Levels of EGF receptor in various human cell lines

Cell line	Tissue of origin	Toxic concentration* of EGF-PE, ng/ml	EGF receptor per ml,† ^{35}S cpm $\times 10^{-3}$	Specific activity,‡ ^{35}S cpm $\times 10^{-6}$/mg	EGF receptor,§ μg/mg	EGF binding sites per cell
A431	Vulva	0.01	310	28.9	10.7	3,000,000¶
A498	Kidney	1–10	19	8.5	2.3	
KB (HeLa)	Cervix	10	49	36.7	1.3	200,000‖
MCF-7	Breast	100	1.7	13.5	0.1	10,000**
HTB32	Cervix	0.1	9.5	6.2	1.5	
OVCA2	Ovary	1	41	17.0	2.4	
1847	Ovary	0.1–1	22	17.8	1.2	

*Toxic concentration represents the minimal dose of EGF-PE required to kill >90% of the cells.
†EGF receptor per ml was determined by quantitating the radioactivity in the 170-kDa protein band precipitated with the rabbit antisera from three concentrations of each cell extract. The cell lines were metabolically labeled and immunoprecipitated simultaneously. The results were confirmed by immunoprecipitating cell extracts with a monoclonal antibody to the EGF receptor [EGF-R1, kindly provided by M. D. Waterfield (Imperial Cancer Research Fund)].
‡The specific activity of the cell lysate represents total trichloroacetic acid-precipitable material expressed per mg of protein in each cell lysate.
§The amount of EGF receptor (μg/mg of total cell protein) is intended to represent relative rather than absolute amounts of EGF receptor in various cell lines. We have found that the absolute amount of methionine-labeled EGF receptor varies with different labeling conditions. However, when compared to A431, the relative amounts of receptor in these cell lines are constant.
¶Haigler *et al.* (20), Fabricant *et al.* (19).
‖Dickson *et al.* (37).
**R. B. Dickson, personal communication.

170,000 for the receptor, and 10^7 cells per mg of protein, this would calculate to 63 pmol of EGF receptor per mg or 3.8×10^6 EGF receptor molecules per cell, which is in good agreement with ^{125}I-labeled EGF binding data (Table 1, refs. 19 and 20).

It has been reported that there is a very low and constant level of c-*erb*B specific RNAs in a variety of lymphoma and leukemia cells as determined by dot blot analysis (39). We decided to analyze in detail poly(A)$^+$ RNA from a selected group of cell lines, many of which expressed readily detectable levels of EGF receptor (Fig. 1). The RNA was fractionated on an agarose gel and analyzed by blotting using ^{32}P-labeled pE7 as a probe. Fig. 2 demonstrates that A431 cells contain approximately six species of RNA, of which three were prominent (10, 5.6, and 2.9 kb), and three were minor (6.3, 4.6, and 3.3 kb). In the other cell lines expressing moderate levels of the EGF receptor (A498, KB, OVCA2, HTB32, and 1847) the 2.9-kb RNA was not found. Only the 10-kb and the 5.6-kb major RNA species were readily detected. They were present at lower levels than in A431 cells; the ratio between the two RNAs varied slightly from cell line to cell line (Fig. 2). RNA from MCF-7, D551, IM-9, and CEM cells, which have very low or undetectable levels of EGF receptor, did not give a detectable signal when pE7 was used as probe.

The amount of the 10-kb RNA and the 5.6-kb RNA in the various cell lines was determined by scanning appropriate autoradiographs with a microdensitometer. The levels of EGF receptor RNA and protein were normalized relative to A431 cells and the resulting values are plotted in Fig. 3. Both RNA species correlated fairly well with the amount of EGF receptor protein.

We and others have recently reported that EGF receptor gene sequences are ≈30-fold amplified in A431 cells (refs. 21, 22, and 27; Fig. 4, lane a). To determine if other cell lines expressing elevated levels of EGF receptor contain amplified receptor DNA, we isolated genomic DNA from primary epithelial cells, KB cells, several ovarian cell lines (OVCA2, OVCA3, 1847, and 2780), a cervical carcinoma (HTB32),

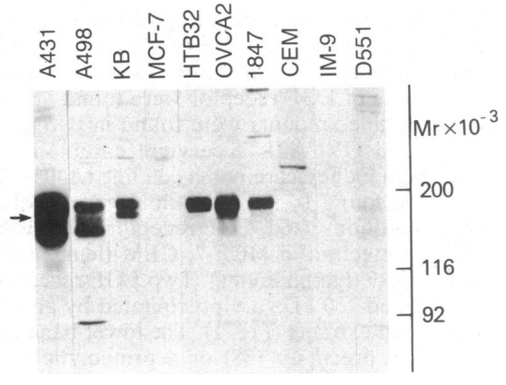

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled cell lysates from various human cell lines. Radiolabeled cell lysates were immunoprecipitated by incubating 250 μl of each lysate with 20 μg of rabbit anti-EGFR and 50 μl of *S. aureus*. The immunoprecipitated proteins were analyzed on a 5–15% linear gradient gel. The radioactivity in the EGF receptor protein band (arrow) was quantitated and is expressed as ^{35}S cpm in parentheses: A431 (53,676); A498 (8013); KB (5771); HTB32 (3958); OVCA2 (7927); 1847 (5834); MCF-7, CEM, IM-9, and D551 (<500).

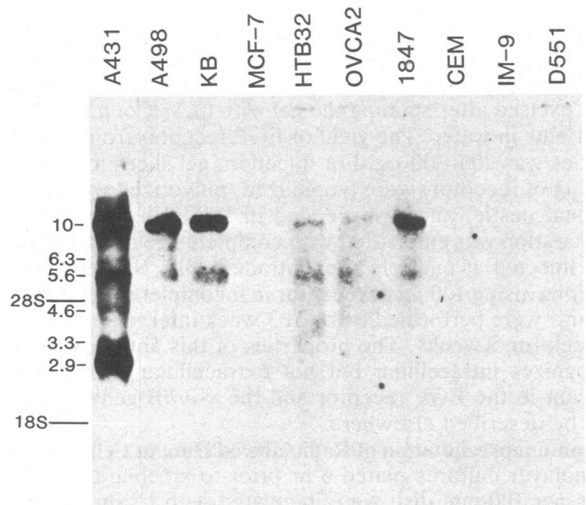

FIG. 2. Analysis of EGF receptor RNA by blotting. Poly(A)$^+$ RNA was isolated from a variety of cell types, fractionated on 1.1% agarose/formaldehyde, transferred to nitrocellulose, and hybridized to the ^{32}P-labeled pE7 plasmid 2.4-kbp cDNA insert. The filter was washed and autoradiography was performed. The autoradiogram shown is overexposed to reveal less abundant RNA species. Markers included human 28S (4.7 kb) and 18S (1.9 kb) rRNAs. Cell lines are described in the text.

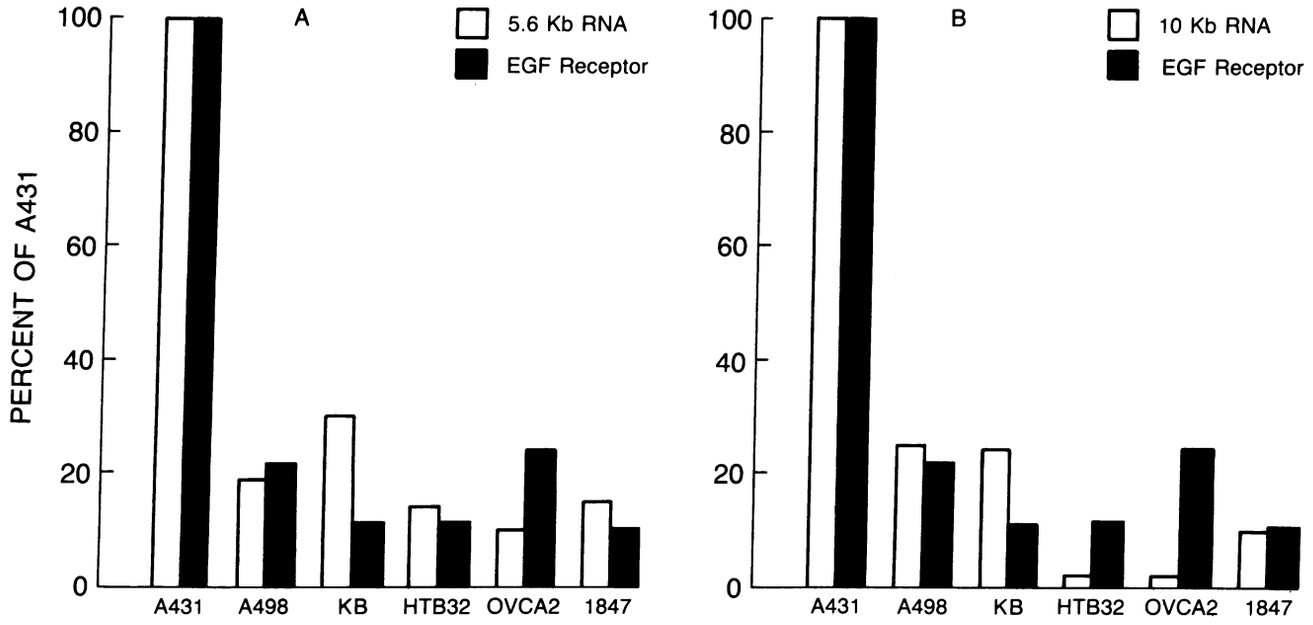

FIG. 3. Comparison between the amount of the EGF receptor protein and the level of the 5.6-kb (*A*) or 10-kb (*B*) pE7-hybridizable RNA in EGF receptor-positive cell types. EGF receptor levels were obtained by immunoprecipitation with anti-EGFR (Table 1) and normalized relative to A431 cells (A431 = 100%). Bands from RNA blots representing 5.6- and 10-kb RNA were quantified by microdensitometric scanning and determination of area under curves. These values were also normalized to A431 levels.

and a kidney carcinoma (A498). OVCA3 cells make relatively low amounts of receptor and serve as a negative control. The DNA was cleaved with *Eco*RI, electrophoresed on 1% agarose, transferred to nitrocellulose, and probed with ^{32}P-labeled pE7 cDNA. Fig. 4 shows that within the range of technical error these cellular DNAs all contain about the same EGF receptor gene copy number (lanes b–h, and unpublished data). This result indicates that DNA amplification is not the sole mechanism whereby cell types generate enhanced levels of EGF receptor protein.

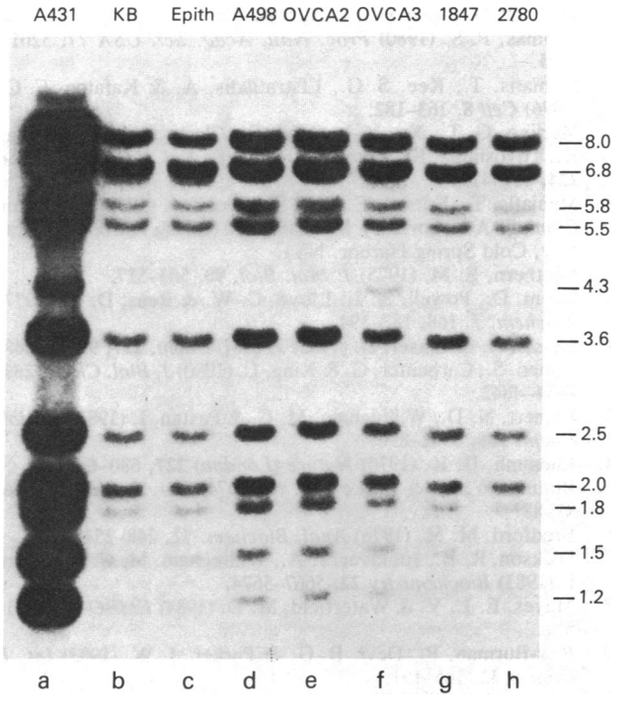

FIG. 4. Analysis of EGF receptor gene sequences by using Southern blotting. Genomic DNA (15 μg) was digested with *Eco*RI before being fractionated on agarose and transferred to nitrocellulose. The blot was prehybridized and hybridized to ^{32}P-labeled pE7 (36 hr). Slight differences in hybridization signals can be accounted for by variation caused when pipetting viscous DNA samples. Lanes d–f were somewhat more heavily stained by ethidium bromide. Cell lines are described in the text. Sizes are in kb.

A431 cells secrete a 95- to 105-kDa truncated form of the EGF receptor (39, 40). To determine if the other EGF receptor-containing cell lines secrete a modified form of the EGF receptor, cells were labeled for 3 hr with [^{35}S]methionine and the medium was collected and immunoprecipitated with a goat affinity-purified polyclonal antibody that detects only external determinants of the receptor (unpublished results). Whereas A431 cells were found to secrete protein reacting with the antibody, a secreted form was not detected in KB, 1847, OVCA2, or D551 cells (data not shown).

DISCUSSION

We have found that there is a good correlation in a variety of cell types between the presence of the EGF receptor and the presence of two RNA species of 10 and 5.6 kb that hybridize to a human cDNA clone (pE7) that is homologous to the avian v-*erb*B oncogene and appears to encode the human EGF receptor (18). The correlation between the EGF receptor protein and pE7-hybridizable RNAs indicates that either RNA species could code for the EGF receptor. The unglycosylated form of the EGF receptor has a molecular mass of about 138 kDa and contains about 1200 amino acids (39). Therefore, the 5.6-kb RNA is large enough to encode the receptor. Another possibility is that one mRNA codes for the receptor and the other for a closely related protein.

Besides the 10- and 5.6-kb RNA species, A431 cells contain at least four other detectable RNA species, one of which probably encodes the truncated form of the EGF receptor (39, 40) found in A431 cells. We examined other cell lines previously found to contain detectable 10- and 5.6-kb RNAs and elevated EGF receptor levels to see if they also secreted a modified form of the receptor; as yet a secreted form has not been detected in other cell types. The fact that the 2.9-kb species is very prevalent in A431 cells and absent from other cell lines (Fig. 2) makes it a good candidate for the RNA coding for the secreted protein. cDNA clones specifically hybridizing with the 2.9-kb RNA have been isolated from an

A431 cDNA library (unpublished data). The 3′ end of such clones contains nucleotide sequences unrelated to pE7 or other clones coding for the EGF receptor (18, 21, 22). Definitive identification of the RNA species that encodes the secreted form of the receptor will require *in vitro* translation of the various RNAs and comparison of the sequences of these RNAs with the sequence of the secreted form of the receptor.

We examined a large number of human cell lines, mostly derived from human cancers, for the presence of the EGF receptor. In addition to A431 cells a few other lines were found to have high EGF receptor levels, including two ovarian cancer lines, one kidney cancer cell line, KB cells [probably derived from HeLa cells (41, 42)], and another cervical carcinoma cell line. It is interesting that many of the EGF receptor-positive tumors originated from tissue of the female urogenital system.

Because of its participation in growth control, and because of its apparent relationship to the *erb*B oncogene, the expression of the EGF receptor gene is undoubtably carefully regulated in normal cells. One question raised by these findings is whether or not elevated EGF receptor or EGF receptor-related protein levels have a causative role in the malignant nature of these cell lines. To study this possibility, A431 cells would seem to represent a model system. However, several observations suggest that it would be unjustified to generalize conclusions reached concerning A431 carcinoma cells to include other malignant cell lines. First, it appears that A431 cells alone secrete an EGF receptor-like protein of unknown function (data not shown). In addition, when several of the cell lines expressing readily detectable levels of EGF receptor were examined to see if their gene copy number was increased, so far only A431 cells were found to have the EGF receptor gene amplified (Fig. 4). Because the EGF receptor gene copy number has been shown to correlate extremely well with EGF receptor levels in a variety of A431-related cells (22), other cell lines found to contain a substantial amount of EGF receptor protein probably utilize regulatory mechanisms other than gene amplification to overproduce EGF receptor RNA and protein.

Shimizu *et al.* (43) have reported that a translocation has occurred in two out of four chromosomes 7s found in A431 cells, resulting in two marker chromosomes. Chromosome 7 is known to contain the human EGF receptor gene (44). Recently, *in situ* hybridization studies have shown that pE7 hybridized with the short arm of chromosome 7. Further, at least one of the marker chromosomes hybridizes strongly with pE7, suggesting that it contains amplified EGF receptor sequences (unpublished data). It remains to be established whether or not these A431-specific translocations are responsible for perturbation of normal EGF receptor gene expression, subsequent production of altered RNAs (i.e., 2.9-kb species) or modified receptor protein, and/or the appearance of the transformed phenotype in A431 cells.

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