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ISOLATION OF AN EVOLUTIONARILY CONSERVED EPIDERMAL GROWTH FACTOR RECEPTOR cDNA FROM HUMAN A431 CARCINOMA CELLS

Frank A. Simmen, Mohan L. Gope, Tanya Z. Schulz, David A. Wright*, Graham Carpenter** and Bert W. O'Malley

Department of Cell Biology
Baylor College of Medicine
1 Baylor Plaza
Houston, Texas 77030

*Department of Genetics
M.D. Anderson Hospital and Tumor Institute
Houston, Texas 77030

**Department of Biochemistry
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

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SUMMARY: Complementary DNA corresponding to total poly(A)$^+$-RNA from the human A431 epidermoid carcinoma cell line was cloned in the phage expression vector λgt11. An epidermal growth factor (EGF) receptor cDNA clone was obtained by screening of the expression library with a rabbit polyclonal antibody (IgG), raised to the purified A431 EGF receptor, in combination with [^{125}I]protein A of S. aureus. The cloned cDNA was able to select, by hybridization, messenger RNA which was translated in Xenopus oocytes and yielded an immunoprecipitable EGF receptor protein of M_r=160,000. The insert of this cDNA (phEGFR-1), is approximately 880 base pairs in length and encodes the carboxyterminal portion of the EGF receptor protein. Its sequence is evolutionarily conserved among vertebrates as shown by hybridization to unique chromosomal DNA sequences from human, baboon, dog, rat, mouse and frog.

The receptor for epidermal growth factor (EGF) is a plasma membrane bound glycoprotein, possessing an intrinsic protein kinase activity (1,2). The EGF receptor is an interesting protein from the standpoint of its major role in mediating the multiple biological effects that binding of EGF elicits in cells (3). Furthermore the erb B transforming protein of avian erythroblastosis virus has been shown to be a truncated EGF receptor protein, lacking the extracellular EGF binding domain (4,5). This suggests that abnormal expression of EGF receptors could contribute to uncontrolled growth of cells as for example in tumorigenesis.

Correspondence should be addressed to Dr. B.W. O'Malley

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We are interested in studying how the gene for EGF receptor is regulated in cells during different physiological, hormonal and developmental states. In order to obtain a suitable probe for these studies, a cDNA library was prepared from messenger RNA of the human epidermoid carcinoma cell line, A431, and cloned in the phage expression vector λgt11 (6,7). Screening of this library with an antibody to the EGF receptor yielded one EGF receptor cDNA clone. This DNA sequence is evolutionarily conserved among vertebrates, indicating the very essential function of this protein in diverse cell types.

MATERIALS AND METHODS

Construction and immunological screening of an A431 cDNA expression library

Total poly(A)$^+$-RNA was isolated from human A431 epidermoid carcinoma cells as described previously (8). Double stranded cDNA was synthesized from the poly(A)$^+$-RNA by conventional procedures (9). This material was methylated by incubation with Eco RI methylase & S-adenosylmethionine, and made blunt ended by incubation with the Klenow fragment of E. coli DNA polymerase I and all four deoxynucleotide triphosphates. Synthetic Eco RI linkers were added to the cDNA (9), which was then digested with Eco RI. Following an extraction with phenol, cDNA was size fractionated on a column of Sepharose CL-4B. cDNA of 500 base pairs and larger was pooled, ligated into the Eco RI site of λgt11 (6,7), and the mixture packaged in vitro (9). The resultant library (containing approximately 450,000 recombinants) was screened with a rabbit polyclonal antibody (IgG; #986) raised to purified EGF receptor protein from A431 cells (10,11), in combination with [^{125}I]labelled S. aureus protein A prepared by the IODO-BEAD method (12). The protocol used for screening was a modification of that of Young and Davis (7). The IgG was used at a dilution of 1 in 5,000. [^{125}I]protein A was used at 1 x 10^6 cpm/ml.

Hybrid-selected mRNA translation in Xenopus oocytes

phEGFR-1 and pBR322 plasmid DNAs were linearized by digestion with Bam HI, denatured in 0.5M NaOH and applied to nitrocellulose filters (13). DNA filters were hybridized with 250 µg of A431 poly (A)$^+$-RNA under standard conditions (13). After extensive washing of the filters, bound RNA was eluted by boiling in 1mM EDTA for 1 minute. After addition of 5µg of E. coli tRNA as carrier, the RNA was extracted with phenol and precipitated with ethanol. Each RNA precipitate was dissolved in 5 µl of oocyte injection buffer[1] and microinjected into oocytes, obtained from Xenopus laevis. Injection of total A431 poly(A)$^+$-RNA, labelling and solubilization of oocytes and immunoprecipitation of EGF receptor proteins were as described[1].

DNA sequencing

The sequence of the phEGFR-1 insert was determined by dideoxy sequencing (14) of appropriate restriction fragments after subcloning into M13mp18.

Southern blot hybridization

Genomic DNAs were purified from cultured A431 cells, human blood cells, baboon liver, dog liver, rat liver, mouse liver, chicken spleen and Xenopus laevis liver by standard procedures (9). 10 µg of each DNA was digested with

Bam HI, electrophoresed in agarose gels and transferred to nitrocellulose (15). Nitrocellulose blots were hybridized with [^{32}P]labeled probes corresponding to the avian erythroblastosis viral oncogene erb B (16) or phEGFR-1. Probes were prepared by nick translation of gel purified restriction fragments. Hybridization was carried out at 63°C in 6XSSC (9) containing 0.5% SDS, 5x Denhardts solution (9), 250 µg/ml salmon testes DNA, 50mM Na phosphate and the labelled probe at $2x10^6$ cpm/ml. Post-hybridization washes consisted of several changes of 2xSSC/0.1% SDS (at room temperature) followed by 6xSSC/0.1% SDS at 63°C. After a final rinse in 2xSSC, the filters were air dried and exposed to X-ray film.

RESULTS AND DISCUSSION

The aim of the present work was to obtain, by molecular cloning of cDNA, a probe for studies of EGF receptor gene expression. Total polyadenylated RNA was isolated from human A431 epidermoid carcinoma cells, a cell line that produces relatively high amounts of EGF receptor protein (~0.15% of total cellular protein) (17,18). These cells would therefore be expected to contain a correspondingly higher amount of EGF receptor messenger RNA when compared to other cells. Double stranded cDNA was synthesized from this mRNA (Materials and Methods) and cloned in the phage expression vector λgt11 (6,7). This vector was constructed such that cDNA is inserted into the unique Eco RI site within the 3' end of the lac Z gene. Foreign proteins, encoded by the cDNA, are expressed in hybrid form with the β-galactosidase protein. Approximately 450,000 recombinant phage were screened (as plaques) for production of EGF receptor antigen by solid phase immunoassay with a polyclonal antibody (IgG, #986) raised in rabbits to purified EGF receptor protein from A431 cells (10,11) . Antigen-antibody complexes were identified by secondary reaction with [^{125}I]labelled S. aureus protein A, followed by autoradiography. Three antigen positive recombinants were obtained after the initial screening experiment. These were then rescreened at lower plating density under the same conditions used for the primary screening. One recombinant phage, λhEGFR-1 , remained antigen positive after two cycles of rescreening and after plaque purification. The insert of λhEGFR-1 consisted of a single Eco RI fragment, approximately 880 base pairs in length. This fragment was subcloned into pBR322 and the subclone was designated phEGFR-1.

Purified phEGFR-1 DNA was used to select, by hybridization, complementary messenger RNA which was then translated in vivo in Xenopus oocytes (Materials

and Methods). To assay for synthesis of EGF receptor proteins, the oocytes were homogenized in a detergent buffer and the supernatants incubated with antibody against the A431 EGF receptor. Immunocomplexes were then bound to insoluble protein A Sepharose and washed repeatedly. The final immunoprecipitates were analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis followed by fluorography. As shown in Figure 1, lane 3, mRNA selected by phEGFR-1 yielded an immunoprecipitable EGF receptor protein of M_r=160,000 after translation in oocytes. In contrast, hybrid-selected translation with an equivalent amount of filter bound pBR322 DNA did not yield this product nor any other (Figure 1, lane 4). This indicates that the result obtained for phEGFR-1 was not due simply to nonspecific binding of RNA to the nitrocellulose filter. Translation of total A431 poly(A)$^+$-RNA followed by

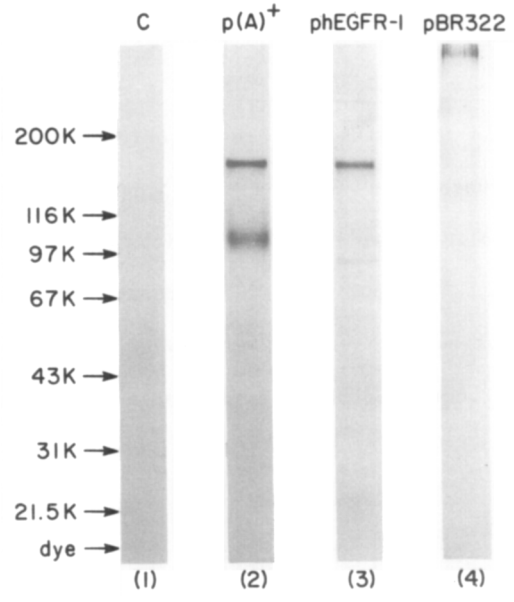

FIG. 1. Hybrid-selected translation of EGF receptor messenger RNA in Xenopus oocytes. Batches of 20 oocytes were injected with RNA, incubated in Barth's medium containing [^{35}S] methionine, and then processed for immunoprecipitation with antibody to the A431 EGF receptor (Materials and Methods). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. Lane 1 is the immunoprecipitate obtained from uninjected oocytes. Lane 2 is that obtained from oocytes injected with total A431 poly(A)$^+$-RNA. Lanes 3 and 4 are the immunoprecipitates obtained from oocytes injected with phEGFR-1 and pBR322 hybrid-selected mRNAs respectively. The migration of molecular weight standards is indicated.

immunoprecipitation with antibody to the EGF receptor resulted in the M_r = 160,000 protein and a second protein of M_r = 100,000. The smaller protein is structurally related to the EGF receptor and is present in A431 cells (19,20). It corresponds to the N-terminal EGF binding domain of the receptor and is encoded by a mRNA of 3.0 Kb in length (5). The receptor related protein was not observed after phEGFR-1 hybrid-selected mRNA translation in oocytes (Figure 1, lane 3), indicating a lack of homology of phEGFR-1 with this mRNA.

The cDNA insert of phEGFR-1 was characterized by restriction endonuclease mapping and partial DNA sequencing. The nucleotide sequence and the corresponding amino acid sequence are presented in Figure 2. This sequence agreed in its entirety with a portion of the EGF receptor nucleotide sequence recently reported by Ullrich and co-workers (5), who used synthetic oligonucleotide probes to isolate EGF receptor cDNAs. phEGFR-1 contains the sequence information for approximately 190 of the carboxyterminal amino acids of the EGF receptor as well as a portion of the 3' untranslated region of the

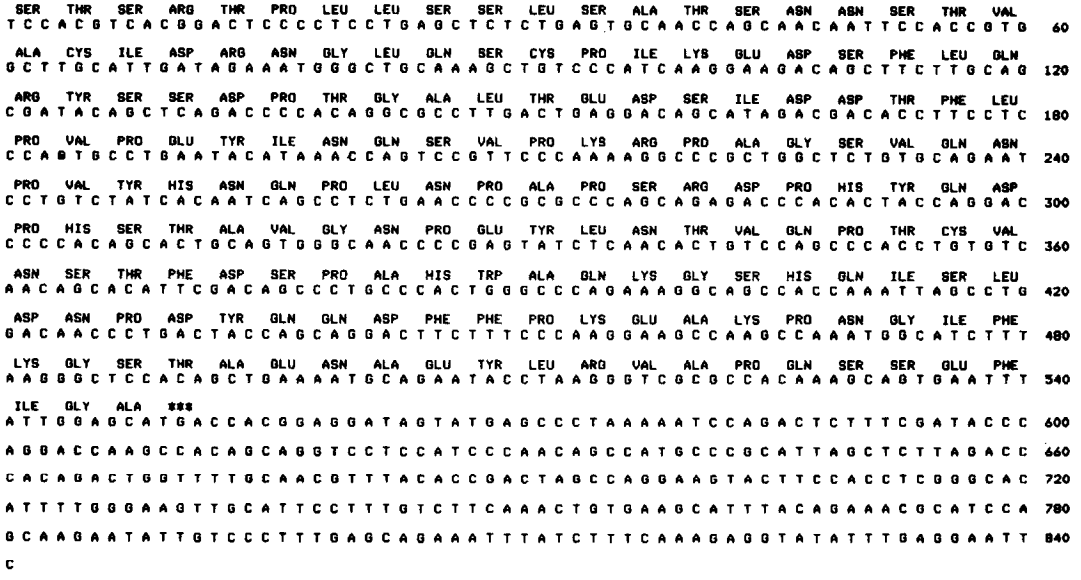

FIG. 2. Nucleotide sequence analysis of phEGFR-1. The insert of phEGFR-1 was excised with Eco RI and mapped with Hinc II, Pvu II and Pst I. After cutting with Hinc II (at position 358) and Eco RI, the fragments were subcloned into M13(mp18). Dideoxy sequencing was carried out in both directions from the Hinc II site. The nucleotide sequence obtained and the corresponding amino acid sequence are presented.

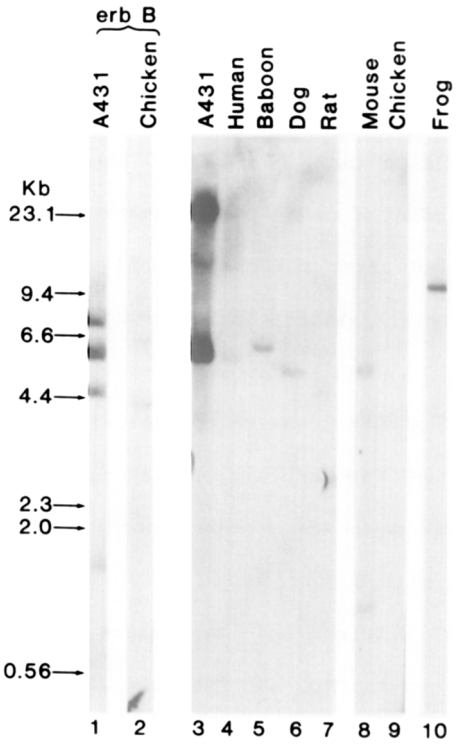

FIG. 3. Evolutionary conservation of EGF receptor cDNA sequence. 10 µg of genomic DNA from the indicated sources was digested with Bam HI, electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose and hybridized with nick-translated, [^{32}P]labelled probes corresponding to erb B (lanes 1, 2) or phEGFR-1 (lanes 3-10). After hybridization and washes, the filters were autoradiographed. Molecular weight standards were Hind III fragments of λ . Their sizes in Kilobase pairs (Kb) are indicated.

receptor mRNA. It does not extend 5' into the sequences encoding the kinase domain of the EGF receptor and which are highly conserved between the human EGF receptor and the v-erb B oncogene of avian erythroblastosis virus (4).

The phEGFR-1 sequence is evolutionarily conserved among vertebrate species, as shown by hybridization of [^{32}P]labelled, nick-translated phEGFR-1 DNA to a Southern blot containing Bam HI digested total genomic DNA isolated from human, baboon, dog, rat, mouse, frog and chicken (Figure 3). The human cDNA hybridized to one or more Bam HI restriction fragments in the DNAs from all but chicken (Figure 3). The failure to react significantly with chicken DNA was not due to DNA degradation as a [^{32}P]labelled probe derived from the avian erythroblastosis viral erb B oncogene (mixture of the Bam 0.5 and Bam -

RI 0.5 fragments, ref. 16) was found to hybridize to unique fragments in this DNA (Figure 3, lane 2). There is marked divergence at the nucleotide level between the carboxyterminal portion of the human and chicken EGF receptors, whereas the corresponding region is more conserved between humans and amphibians. It would be interesting to examine the gene in reptiles and other birds to determine the point at which this divergence occurred as well as how it occurred. Hybridization of A431 cellular DNA with the erb B probe revealed one fragment (~ 6 Kb) in common with those that hybridized to phEGFR-1 (compare lanes 1 and 3 of Figure 3). Several additional fragments were also observed with the erb B probe. These must lie upstream of the 6 Kb fragment in the EGF receptor chromosomal gene. Similarly, the large (~ 23 Kb) fragment, specific to the human probe, must lie downstream to the other fragments. It is evident that the chromosomal EGF receptor gene is very large.

Interestingly, only two out of the three human fragments that hybridized with phEGFR-1 are amplified in A431 cells. The origin of the unamplified fragment is unknown. It could conceivably be derived from an unamplified EGF receptor allele or closely related gene. We found no evidence of any rearrangement within the region of the chromosomal gene corresponding to phEGFR-1 (21). Our results indicate that the carboxyterminal portion as well as the kinase domain of the EGF receptor are evolutionarily conserved.

In conclusion, a cDNA corresponding to part of the human EGF receptor has been obtained by expression cloning in λgt11. The cDNA has been used to verify, by hybrid-selection, that the product of translation of EGF receptor messenger RNA in Xenopus oocytes is a M_r = 160,000 protein, previously demonstrated from studies involving total messenger RNA from A431 cells[1]. Its sequence is evolutionarily conserved, indicating that it may provide a useful probe for studies concerning the expression of EGF receptor mRNA in different cells and species.

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1. Simmen, F.A., Schulz, T.Z., Headon, D.R., Wright, D.A., Carpenter, G. and O'Malley, B.W. submitted.

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