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ANALYSIS OF THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR GENE AS A PROTO-ONCOGENE

by

John Howard Hunts

A Dissertation Submitted to the Faculty of the DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY
In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1986
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by John Howard Hunts entitled ANALYSIS OF THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR GENE AS A PROTO-ONCOGENE and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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June 16, 1986

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Yoshiohshi Teramae
Dissertation Director
June 16, 1986
STATEMENT BY THE AUTHOR

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SIGNED: John A. Smith
To my parents who supported me through hard times and especially to Stacy whose love made it all the more worthwhile.
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ABSTRACT

The epidermal growth factor receptor (EGFR) gene was examined as a proto-oncogene. Initially, the cellular homolog of the retroviral oncogene erb-B was shown to be localized to the same region of human chromosome 7 as the EGFR gene, giving support to the idea that these genes are closely related.

To determine how some cells can over-express the EGFR gene, somatic cell hybrids constructed between a human EGFR-overproducing cell line and a mouse EGFR-deficient cell line were examined. EGFR gene amplification was observed in one of these hybrids along with EGFR gene rearrangement, which is thought to generate an abnormal mRNA.

When examining tumor tissue, the EGF receptor (EGFR) was found to be elevated relative to normal adjacent tissue in 9 out of 15 primary human squamous cell carcinomas. Only 2 of these 9 tumors had EGFR gene amplification, suggesting alternative mechanisms potentially involved in increasing EGFR levels. Because placental tissue expresses high levels of EGFR, it was thought that some tissues may normally possess high EGFR levels and
that some cancerous tissues inappropriately mimic the mechanisms active in the placenta. From the examination of several tissue samples, EGFR mRNA levels and EGFR protein half-life were also postulated as contributing factors regulating the EGFR levels. The EGFR gene was implicated as a proto-oncogene by evidence which suggests that either a qualitative or a quantitative change in the receptor may be involved in tumorigenesis.

Finally, to begin to better understand what role EGFR hyperproduction plays in tumorigenesis, a DNA vector was constructed which produces antisense-RNA for inhibiting EGFR expression.
CHAPTER 1

INTRODUCTION

Cancer has been present and has caused havoc throughout the history of mankind. This devastating affliction has not occurred in epidemic proportions, but rather at a slower, somewhat constant rate. Archaeological evidence from Egyptian skulls suggests that osteosarcomas were present as early as 3,400 B.C. (Ackerknecht 1958). Similarly, there have been references to cancer in some of the earliest known written records of man (Shimkin 1977, p. 21). These observations suggest that there are certain inherent factors in the billions of cells that make up the human body and/or in the environment where man lives, which have a predisposing influence towards cancer. In this work, only an inherent factor in humans is dealt with. Specifically, this study was undertaken to better understand how a normal gene, that for the epidermal growth factor receptor (EGFR), can, when activated, potentially play a role in the process of tumorigenesis.

The word cancer is derived from the Greek word for crab, karkinos. A malignant tumor, in fact, resembles a
crab in that its daughter cells can metastasize to different regions of the body, similar to the arms of a crab. Two terms which describe the basic characteristics of cancerous tissue are anaplasia and autonomy (Shimkin 1977, p.5). Tumors usually contain disorganized masses of embryonic or undifferentiated tissues that are termed anaplastic. Furthermore, tumors often grow at a rate different than or autonomous from their tissue of origin. These two characteristics have an inverse relationship. As cells differentiate, their growth potential decreases and as tumor tissue becomes anaplastic, its growth potential usually increases (Ruddon 1981, p.53). Early attempts to characterize tumors as developing from parasitic bacteria failed. However, the notion that tumors have a parasitic nature is quite accurate. Cancerous tissues can grow and invade neighboring normal tissue. Therefore, cancer may be considered a type of internal parasite or the mutiny of a single cell in a sea of billions.

**Molecular Biology of Cancer**

The finding that DNA is the genetic blueprint of the cell (Avery 1945) and the solving of the structure of DNA (Watson and Crick 1953) have laid the foundation for the field of molecular biology in general. These important discoveries and others have greatly contributed to a
better understanding of the molecular biology of cancer. In 1911, Payton Rous first showed that a transmissible particle, now known to be a retrovirus, could cause cancer in chickens. Although these findings were not accepted at that time, they have now been well received.

Contributions of Retroviruses

The study of retroviruses has probably contributed more to the molecular biology of cancer than any other field (Bishop 1985). Retrovirology, however, has had its potential involvement with human cancer questioned. Although tumor induction was easily shown to be associated with the retrovirus, results were thought to represent laboratory artifacts (Marx 1984). Much more interest has since been generated in the study of retroviruses by the finding that the transformation induced by the virus is not an inherent property of only the virus. That is, transformation can occur by insertional activation of a cellular gene by the viruses long terminal repeats (LTR) promoter/enhancer or by virally transducing cellular genes (Bishop 1983). These activities of the retrovirus are due to its unique life cycle.

The retrovirus is an RNA virus, which replicates through a cellularly integrated DNA intermediate generated by the enzyme reverse transcriptase. This unique feature
of the retrovirus allows for the rare insertional activation of cellular genes or the erroneous acquisition of cellular DNA sequences. If the sequence acquired by the virus is somehow involved in regulating the cells proliferative capacity, this recombined viral/cellular DNA sequence may be selected for by its ability to induce a cancer in its host. However, because the recombination event usually deletes important sequences of the virus, only competent, unrecombined viruses will allow the recombined transforming virus to infect new cells. The cellular DNA sequence, once acquired by the virus, is termed a viral oncogene and the cellular-parental sequence is termed a cellular oncogene or proto-oncogene (Bishop 1985).

Still, however, the fact that no transforming retroviruses had been isolated from human tumors distracted from the idea that oncogenes may be involved in human cancer. Then, an important oncogene screening system, using mouse NIH-3T3 cells and DNA transfection, showed that in fact a human tumor did contain an activated oncogene independent of a retrovirus (Murray et al. 1981). The NIH-3T3 cells grow into tight clumps of cells, called foci, if an appropriate oncogene is present. The important finding here is that the oncogene identified, H-ras,
is thought to be identical to a retroviral oncogene. This has given greater credence to the idea that the oncogenes identified by the retrovirus may also play important roles in human tumors.

Oncogenic Conversion

By studying retroviral oncogenes and their cellular counterparts, important information has been gained with regard to how a normal gene is activated in such a way as to constitute an oncogene. Essentially two models for the generation of oncogenes have been postulated. One model, first suggested by Temin (1971), proposed that all cells contain potential oncogenes that could become activated by qualitative changes due to mutation or viral interaction. An example of this is the single point mutation that occurs in the proto-H-ras gene converting it to an oncogene (Tabin et al. 1982). The second model, suggested by Huebner and Todaro (1969), proposed that all cells have repressed oncogenes which can be induced by retroviruses or carcinogens. This model, on the other hand, emphasizes quantitative changes. Evidence for this hypothesis is the enhanced expression of the proto-oncogene c-myc. Activation of c-myc has been shown to occur by retrovirus insertional activation (Hayward, Neeland, and Astrin, 1981) or by activation due to chromosomal
translocation (Leder et al. 1983; Adams et al. 1983). Currently, any gene is classified as a proto-oncogene if a qualitative and/or quantitative change in the gene product is implicated in tumorigenesis.

Multi-step Process: Oncogenes Identify Key Elements

It has become apparent, however, that a single event is not sufficient to induce a cancerous state (Duesberg 1985). Land, Parada and Weinberg (1983) have shown that tumorigenic conversion of primary rat embryo fibroblasts requires at least two cooperating oncogenes. This idea is supported by the fact that tumor tissue has a large number of characteristics different from normal tissue (Ruddon 1981, p.4). In transforming primary rat fibroblasts, activated ras and myc oncogenes were used. The ras oncogene is thought to potentially control the cancer cells transformed morphology; whereas, the myc oncogene is thought to affect the cells life-span, converting the cell to an immortalized state (Land et al. 1983). These genes, when used individually, showed incomplete or intermediate transformed states. Used together, however, ras and myc appear to have complimentary activities in tumorigenesis. Therefore, it has been suggested that some oncogenes can induce tumors, while others act to maintain the tumor (Bishop 1983). It appears then that cellular DNA sequences indentified by the retrovirus re-
present genes whose protein products regulate crucial cellular functions, which, if disrupted, can direct the cell towards a tumorigenic state.

Autocrine Growth Control

Comparing several of the functions of oncogene products suggests that a large number of these proteins are potentially involved in controlling key steps which regulate cellular growth (Heldin and Westermark 1984). An oncogene product which mimics an otherwise normal mitogenic signal, could induce a type of autocrine growth control (Sporn and Roberts 1985). Two examples of autocrine growth control generated by oncogene activity are v-sis and v-erb-B. V-sis, originally isolated from monkeys and cats, encodes for an analogue of platelet-derived growth factor (PDGF) (Waterfield et al. 1983; Doolittle et al. 1983). In this case, cells containing v-sis are able to stimulate their growth by producing their own growth factor. V-erb-B, isolated from chickens, encodes a truncated form of the epidermal growth factor receptor (EGFR), which has a deleted epidermal growth factor (EGF) binding domain (Downward et al. 1984). In v-erb-B's case, the truncated growth factor receptor is thought to short circuit the normal pathway of the mitogen by its ability to function independent of EGF binding.
Anti-oncogenes

An opposite, but not exclusive, viewpoint to the origin of cancer is that important components of the cell, which regulate the normal state, may be lost leading to a tumorigenic state. These types of factors, whose functions oppose that of the oncogene products, can be termed anti-oncogenes. Evidence for this hypothesis has come from fusion studies between tumorigenic and non-tumorigenic cells. The resulting hybrids exhibit characteristics similar to the non-tumorigenic parent, suggesting that the transformed state is recessive (Harris et al. 1969). This recessive quality has also been found paradoxically in some cases with the ras oncogene product, which was originally isolated for its dominant character (Craig and Sager 1985). Although no gene has yet been isolated which shows tumor suppressing activity, recent observations suggest that specific chromosomes may contain anti-oncogenes. For example, both hereditary retinoblastoma and Wilm's tumor are thought to involve the loss of normal chromosomes 13 and 11 respectively (Knudson 1985; Koufos et al. 1985). A specific case of apparent oncogene suppression has been shown in hamster tumors induced by ras and myc oncogenes (Oshimura, Gilmer and Barrett 1985). In this case, full transformation correlates with loss of chromosome 15.
Therefore, although a great amount of evidence suggests that oncogenes play a significant role in tumor formation, anti-oncogenes or suppressing agents may also contribute to a neoplastic state.

**Epidermal Growth Factor (EGF)**

EGF is a small polypeptide growth hormone of 6,045 molecular weight with three disulfide linkages (Carpenter and Cohen 1979). This polypeptide was first isolated because of its interesting action on inducing early eyelid opening and incisor eruption in newborn mice (Cohen 1962). When bound to its cell surface receptor, EGF induces a complex cascade of intracellular events, including tyrosine phosphorylation (Hunter and Cooper 1981), receptor clustering and internalization (Haigler et al. 1978; Schlessinger et al. 1978), increased glycolysis (Diamond et al. 1978), all eventually leading to DNA replication and cell division (Armelin 1973; Hollenberg and Cuatrecasas 1973).

**Epidermal Growth Factor Receptor**

The EGF receptor (EGFR) is a cell surface glycoprotein of 170,000 molecular weight (Das and Fox 1978). EGFR is found in all three germ layers, ectoderm, mesoderm and endoderm and distributed throughout mammalian and
avian species, suggesting that this receptor may play an important role in growth control (Adamson and Rees 1981). When the hormone is bound, EGF becomes internalized and takes a pathway which appears to be dependent on the cell's physiological state (Miskimins and Shimizu 1983). A specific protein tyrosine kinase activity intrinsic to the receptor is activated within 1 minute of EGF binding (Hunter and Cooper 1981).

**EGFR Gene Represents a Proto-oncogene**

Three major observations have suggested that the EGFR gene represents a proto-oncogene, and as such, when activated to its oncogenic form, may contribute to the genesis of some cancers. The first indication that the EGFR gene may be related to some oncogenes came when the receptor was shown to display protein tyrosine kinase activity (Hunter and Cooper 1981). The unique tyrosine kinase activity had, up to this time, only been associated with several of the transforming proteins from retroviruses. More recently, it has been shown that the v-erb-B oncogene is a truncated version of the EGFR (Downward et al. 1984). Furthermore, it appears that the enhanced expression of the EGFR gene may play a role in some squamous cell carcinomas (Cowley et al. 1984; Hunts et al. 1985b) and in some brain tumors (Libermann et al. 1984).
In this study, the EGFR gene is analyzed in the context of a proto-oncogene. Initially, the c-erb-B gene was regionally mapped on human chromosome 7 to give further evidence that the c-erb-B gene and the EGFR gene are identical. The hyperproduction of EGFR in an epidermoid carcinoma cell line, A431, was examined and found to be partially due to EGFR gene amplification occurring on a translocated chromosome. To study the possible involvement of elevated EGFR expression in squamous cell carcinomas, several tumor tissues were screened and 2 were found to contain EGFR gene amplification. Interestingly, some of the tumors expressed elevated levels of EGFR relative to normal adjacent tissue, but did not contain gene amplification. In these cases, two alternative mechanisms leading to increased EGFR levels, are postulated. These alternative mechanisms may represent normal cellular activities for some tissues, but are thought to be inappropriately mimicked by some squamous cell carcinomas. To better define the activities of EGFR and its possible involvement in some tumors, recombinant DNA vectors were constructed. These vectors generate antisense-EGFR mRNA for the purpose of inhibiting the cells endogenous EGFR gene.
CHAPTER 2

PROCEDURES

Cell Culture

Anchorage dependent cell cultures were maintained at 37 C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 ug/ml streptomycin in the presence of 10% CO₂. Hybrid cell lines were also maintained in the presence of HAT (1x10⁻⁴ M hypoxanthine, 4x10⁻⁷ M aminopterin, and 1.6x10⁻⁵ M thymidine).

Isolation of Genomic DNA

High molecular weight DNA was isolated by lysing cells in a buffer containing 1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA, and digested overnight at 37 C with 200 ug/ml proteinase K (Sigma). Large tissue samples were first disrupted with a polytron for 1 minute at medium speed in the above buffer. The amount of buffer used was 5 ml for either 1 g of tissue or a 150 mm dish. The lysate was then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). The
DNA in the aqueous phase was precipitated with 2 volumes of chilled ethanol and redissolved in 10 mM Tris-HCl, pH 7.5, incubated with 100 ug of RNase (Sigma) per ml for 4 h at 37 C, deproteinized as above and precipitated with ethanol. The concentration was then determined using a spectrophotometer at a wavelength of 260 nm (absorbance of 1.0 equals 50 ug/ml DNA).

**Isolation of RNA**

To a 1 g fragment of tissue or a cell pellet, 16 ml of 4 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, and 0.5% sarkosyl was added and lysed by homogenizing 1 minute full speed with a polytron for the tissue or by vortexing 10 seconds for the cell pellet (Chirgwin et al., 1979; Lizardi, 1983; Maniatis, Fritsch and Sambrook, 1982). A 3.5 ml volume of 5.7 M CsCl, and 0.1 M EDTA, pH 7.5, was placed in an SW41 rotor tube (Beckman) and overlayed with 8 ml of the cell lysate. Centrifugation was for 18 hours at 36,000 rpm and 20 C. The RNA formed a pellet at the bottom of the tube which was rinsed with chilled 70% ethanol and dried. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA and 1% SDS and then extracted with an equal volume of chloroform:1-butanol (4:1). A volume of ethanol (2.2) was added to precipitate the RNA at -20 C for 2 hours. The
RNA was then recovered by centrifugation in a microfuge for 10 minutes. Poly (A)+ selection was done as described by Maniatis et al. (1982). The concentration was determined by using a spectrophotometer at 260 nm (absorbance of 1.0 equals an RNA concentration of 40 ug/ml).

Isolation of Plasmid DNA

A 50 ml culture of overnight bacteria in LB-broth, containing the appropriate antibiotic, was added to 500 ml of the same sterile broth. After 2.5 hours shaking at 37 C, 2.5 ml of chloramphenicol (Sigma) (34 mg/ml in ethanol) was added and the culture was continued overnight. The bacteria was then pelleted, resuspended in 5 ml of 25% sucrose and 50 mM Tris-HCl, pH 8.0, and treated with 2 ml of lysozyme (Sigma) (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) for 2 minutes on ice. A detergent solution of 20 mM Tris-HCl, pH 8.0, 62 mM EDTA, 1% NP40, and 1 mM deoxycholic acid was used to lyse the bacteria. The lysate was then centrifuged at 37,000 rpm for 1 hour at 4 C in a Ti 80 rotor (Beckman). The supernatant was removed and added to a sufficient amount of CsCl to make a solution of 17 g CsCl per 16 ml supernatant. Ethidium bromide was then added to a concentration of 600 ug/ml. This solution was centrifuged at 45,000 rpm for 24 to 48 hours at 20 C. The lower DNA band in the tube was then removed with a sy-
Ringe. Ethidium bromide was removed by water saturated n-butanol extraction. The plasmid was then extensively dialyzed against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The concentration was determined by spectrophotometry.

**Southern Blotting and Hybridization**

**DNA Digestion**

High molecular weight DNA, 10 to 20 ug, was digested with a restriction endonuclease using the supplier's recommended conditions. A small aliquot was then electrophoresed as described below to determine if digestion was complete. The digested DNA was precipitated and resuspended in 20 ul of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA (TE) and mixed with a 5x concentration of 0.05% bromophenol blue, 1% SDS, 40% glycerol and 30 mM EDTA.

**Agarose Gel Electrophoresis**

Usually a 0.7% agarose (Sigma, type II) solution in 40mM Tris-acetate, pH 8.0, and 1mM EDTA was melted by microwave oven and poured into a horizontal running plate. When solidified, the buffer wells were filled with the same buffer and the samples were applied. The voltage used was 3 V/cm for 5 to 12 hours. The molecular weight marker used was wild type lamda bacteriophage DNA cut with the restriction enzyme Hind III. This generated DNA frag-
ments of 23, 9.4, 6.6, 4.4, 2.2, 2.0, and 0.56 kilobase pairs (kb). The gel was stained in 0.5 \text{ ug/ml} \text{ ethidium bromide} for 10 minutes and visualized using a UV illuminator at 300 nm and 360 nm.

DNA Denaturation

The DNA was denatured for hybridization by treating with several volumes of 0.5 M NaOH and 1.5 M NaCl for 15 minutes with 2 changes of buffer. The gel was then neutralized in 1 M Tris-HCl, pH 7.0, and 1.5 M NaCl with the same volume and 2 changes.

Transfer to Nitrocellulose

Nitrocellulose filter (Schleicher and Schuell BA85) was cut to the same size as the gel (Southern 1975). The filter was pretreated for 5 minutes in 2x SSC (20x SSC was 175.3 g NaCl and 88.2 g Sodium citrate in 1 liter of H$_2$O, pH 7.0). A blotting set-up was then used as described (Maniatis et al. 1982, p.385). A 1 kg weight and approximately 500 ml of 20x SSC was used to transfer the DNA to the filter in 16 hours. The filter was blotted dry on 3MM paper and placed under a heat lamp for 10 minutes. The nitrocellulose was baked at 80 C in a vacuum oven for 2 hours.
DNA Dot Blotting

Dot blotting was used to apply undigested DNA directly to nitrocellulose. DNA was first denatured in 0.3 N NaOH at 60 C for 10 minutes. An equal volume of 2 M ammonium acetate was added for neutralization. The DNA was then applied directly to the filter using a dot blot apparatus (Bio-Rad). The filter was baked as described above.

DNA Probe Preparation

The probe for hybridization was first isolated by digesting the vector with the appropriate restriction enzyme and separating by agarose gel electrophoresis. The probe fragment was recovered by electroelution into dialysis bags (Maniatis et al. 1982, pp. 109-12). Specific activity reached by nick-translation was 1-5x10^8 cpm/ug of probe, using alpha-^{32}P-ATP. Unincorporated label was removed by G-50 Sephadex chromatography (Maniatis et al. 1982, p. 466).

DNA Hybridization Conditions

Filters were first soaked in 2x SSC for 5 minutes and then prehybridized for 1-3 hours at 42 C in 50% formamide (BRL), 5x SSC, 10x Denhardt's (a 50x solution contains 5 g Ficoll 400, 5 g polyvinylpyrrolidone 40,000, and
BSA Pentax fraction V in 500 ml H₂O), 50 mM Tris-HCl, pH 7.5, 0.1% SDS, and 100 ug/ml denatured salmon sperm DNA (phenol extracted and boiled 5 minutes to denature). The volume of this solution was 0.05-0.1 ml/cm² of nitrocellulose. Hybridization was in the same solution for 24-48 hours at 42°C containing denatured probe (denatured by boiling 5 minutes and cooling 1 minute on ice). The concentration of the probe used was 25-50 ng/ml of hybridization solution. Prehybridization and hybridization was carried out in a seal and save food bag with all air removed. A lower stringency of hybridization solution was 35% formamide, 4x SSC, 10x Denhardt's, with other components and concentrations the same as above.

Washing Conditions

The filter was removed from the bag and placed in 100-200 ml of 2x SSC and 0.1% SDS for 10 minutes at 22°C with gentle shaking. The filter was then placed in the same volume of 0.2x SSC and 0.2% SDS with 2 changes for 30 minutes each at 60°C. The filter was then exposed to Kodak XAR-5 x-ray film at -70°C with an enhancer screen.

Northern Blotting and Hybridization

Agarose Gel Electrophoresis and Transfer

Before separation, RNA was denatured by a 1 hour
treatment in 1 M glyoxal (deionized by Bio-Rad AG 501-X8 resin) and 10 mM sodium phosphate, pH 6.5, at 50°C (Thomas 1983). RNA was mixed with a 5x solution of 50% glycerol, 10 mM sodium phosphate, pH 6.5, and 0.05% bromophenol blue. Samples were electrophoresed using an apparatus as described (Maniatis et al. 1982, pp. 200-1) and a peristaltic pump. A 1.1% agarose gel was used with 10 mM sodium phosphate buffer at 90 V for 3-6 hours. The transfer of RNA from the gel to the nitrocellulose was the same technique as for DNA. Similarly, the filter was baked. However, after baking, the glyoxal adduct was removed from the RNA by treating with 20 mM Tris-HCl, pH 8.0 at 100°C for 5 minutes. Molecular weight markers used were denatured 28S and 18S ribosomal RNAs and 4S tRNA.

RNA Dot Blotting

The RNA was denatured as above and then applied directly to nitrocellulose using a dot apparatus. The filter was then processed as above.

RNA Hybridization Conditions

The filter was prehybridized at 42°C for 4 hours in 5x SSC, 50% formamide, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 5x Denhardt's, and 100 µg/ml denatured salmon sperm DNA. Hybridization was in the same buffer with the
denatured probe for at least 36 hours at 42 C. The volume of the hybridization fluid and the probe concentration was determined as described for DNA hybridization.

Washing Conditions

The filter was removed from the hybridization bag and placed in 2x SSC and 2% SDS for 10 minutes at 22 C. The solution was then changed twice using 0.1x SSC and 0.1% SDS at 45 C for 30 minutes each. The filter was then ready to expose to x-ray film.

**EGF Binding Assay**

**EGF Iodination**

EGF was labelled with $^{125}$I by Iodobeads (Pierce) as the oxidizing agent. A 100 ul reaction contained 5.5 ug EGF (receptor grade from Collaborative Research), 1 mCi sodium iodide ($^{125}$I, 13-17 mCi/ug, Amersham) and 0.25 M potassium phosphate, pH 7.5, and was reacted for 10 minutes on ice. The reaction was stopped by removing the iodobead and adding an equal volume of 1 mg/ml tyrosine. The low molecular material was removed by chromatography on Biogel P-4 equilibrated with Earle's balanced salt solution containing 1 mg/ml BSA and 5 mM HEPES, pH 7.4 (EBSS-BSA).
Binding Assay

Two binding assays were employed here, one for whole cells and one for tissue homogenates. For whole cells, the cells were grown to confluence in 30 mm dishes, washed twice with EBSS-BSA, and incubated with 2 ml of EBSS-BSA on ice for 2 hours in the presence of $^{125}$I-EGF (2x10$^{-10}$ M). In the case of non-specific EGF binding, unlabeled EGF (2x10$^{-7}$ M) was added 15 minutes before the addition of labeled EGF. After incubation, plates were washed twice with EBSS-BSA and solubilized with 1 N NaOH for counting in a gamma counter (Beckman). For the tissue homogenate binding assay, approximately 100 mg of tissue was homogenized for 15 sec using a polytron (medium speed) in 1 ml of EBSS containing 1 mM PMSF and the procedure was repeated 4 times. The homogenate was then filtered through nylon mesh and then metal mesh. The protein concentration was adjusted to 1 mg/ml using the above buffer. A 400 ul volume was used for the binding assay on ice for 2 hours. The concentration of labeled and unlabeled EGF used was the as above. The homogenate was then filtered using 0.2 um Millipore filters and counted to determine binding.

DNA Transfection

The night before transfection, 5x10$^6$ cells were
seeded into a 90 mm dish and grown overnight. The next day, calcium phosphate–DNA precipitates were prepared (Corsaro and Pearson 1981). A 2x concentration of 50 mM HEPES, pH 7.1, 250 mM NaCl, and 1.5 mM sodium phosphate, pH 7.5 was prepared. Then a 2x solution of 40 μg/ml DNA and 250 mM CaCl₂ was mixed dropwise with an equal volume of the 2x HEPES solution and the DNA allowed to precipitate for 30 minutes at room temperature. This mixture was then applied to the cells, 20 μg DNA per 90 mm dish containing 10 ml of DMEM supplemented with 10% FCS and absorbed for 24 hours at 37 C. The next day the medium was changed and the cells were incubated for 24 hours. After 48 hours from DNA addition, the cells were diluted 1:5 and placed under the appropriate selection conditions.

Recombinant DNA Techniques

All enzymes were used according to the supplier's recommended conditions. All techniques and procedures were performed as described by Maniatis et al. (1982). For screening bacterial colonies, an alkaline extraction procedure was used (Birnboim and Doly 1979).
CHAPTER 3

C-ERB-B/EGFR REGIONAL MAPPING

Because of the gene transducing ability of the retrovirus, a vast amount of information has been obtained implicating the association of cellularly derived sequences with the retroviral transforming activity (Bishop 1983). One of these viruses, the replication defective avian erythroblastosis virus (AEV), is an acutely tumorigenic virus, which, when injected into chickens, causes erythroid leukemias and sarcomas (Graf and Beug 1983). Initially, two viral gene products of cellular origin, erb-A and erb-B, were suspected of causing the cancer (Anderson et al. 1980). Since then, however, deletion studies have eliminated erb-A's involvement in transformation (Frykberg et al. 1983). Furthermore, a new AEV isolate AEV-H (Yamamoto et al. 1983a), containing only erb-B, has been shown to have transforming activity similar to AEV.

V-erb-B Protein Product

The erb-B protein product is a transmembrane glycoprotein of approximately 68,000 molecular weight with a
protein moiety of 62,000 molecular weight (Hayman et al. 1983). The protein sequence of erb-B has significant homology to the src gene family (Yamamoto et al. 1983b), a family of viral oncogene products which posses protein tyrosine kinase activity (Hunter and Cooper 1985), suggesting that this may also be an enzymatic activity of erb-B. Additional evidence supporting the idea that the erb-B gene may play a significant role in some chicken tumors is the finding that the c-erb-B gene can be activated by LTR insertion (Fung et al. 1983). In this case the replication competent avian leukemia virus (ALV) inserts its genome into the c-erb-B gene and, because of the viral LTR structural features and strong promoting activity, disrupts the normal regulation of this gene.

**Preliminary C-erb-B Regional Mapping**

The possibility that the oncogenes identified through the study of retroviruses could also be involved in some human cancers has made the study of these genes of great interest. One approach to studying oncogenes is to map their chromosomal location. A powerful technique used in gene mapping is the construction of interspecies somatic cell hybrids (Ruddle 1981). The characteristic of these hybrids, taking the example of human:mouse somatic cell hybrids, is that they preferentially loose human
chromosomes while maintaining the mouse chromosomes. This allows hybrids to be selected which maintain only a few human chromosomes. By studying the traits of interest in different hybrid isolates, while knowing the human chromosomes present through karyotypic analysis, a correlation can be made between the presence of a specific trait with that of a single chromosome or chromosomal fragment. Using this technique, Spurr et al. (1984) first reported that the cellular homolog of the v-erb-B gene, c-erb-B, is located on chromosome 7 in the pter to q22 region. This finding suggested that the c-erb-B gene may be close to the EGFR gene, previously located on the p13 to q22 region of chromosome 7 (Kondo and Shimizu 1983).

To determine if the c-erb-B gene was in the p13 to q22 region of chromosome 7, the panel of human:mouse cell hybrids used by Kondo and Shimizu (1983) was examined. Figure 1 shows the panel of hybrids used with A9 as the parental mouse EGFR deficient cell. The parental human cells GM1356 and GM2068 both contained translocation chromosomes involving fragments of chromosome 7. For example, hybrid B-21-3 contained the chromosome 7 fragment p13-qter with the human chromosome 1 fragment pter to p34 translocated to it. This hybrid could then be used to screen the p13-qter region of chromosome 7. The hybrids A-1 and D-
REGIONAL MAPPING OF c-erb-B

<table>
<thead>
<tr>
<th>HUMAN CHROMOSOME 7</th>
<th>GMA 1356 B-21-3 A9</th>
<th>GMA 2068 D-10-1 A-1</th>
</tr>
</thead>
</table>

EGF BINDING
- p3-qter
- pter-p13
- pter-q22
- c-erb-B q13-q22

RELATIVE % OF CONTROL
- 65
- 1
- 38
- 100

Fig. 1. Regional Mapping of C-erb-B

Human chromosome 7 fragments for each of the hybrids B-21-3, A-1 and D-10-1 are shown. The relative % of control value is obtained from densitometric tracing of Fig. 2.
10-1 were similarly used to screen different regions of chromosome 7. Because no biological activity had been associated with the human c-erb-B gene, however, a slightly different approach had to be taken, similar to that of Spurr et al. (1984). Often the restriction endonuclease pattern of a gene from one species will be different from that of another species. These differences can then be correlated with the presence or absence of a specific chromosome or chromosomal fragment.

Using the strategy described, DNA was isolated from both human and mouse cells and from the panel of hybrids shown in Figure 1. These DNAs were then digested with the restriction enzyme Eco RI, applied to a 0.7% agarose gel for separation by electrophoresis, and transferred to nitrocellulose filter paper for Southern hybridization (Figure 2). The probe used for hybridization was a 0.5 kb Bam HI fragment of v-erb-B (Yamamoto et al. 1983a). The results, however, did not show any difference in the Eco RI restriction pattern for the c-erb-B gene in the hybrids. The strategy then taken was to make densitometric tracings of the hybridization bands for differences in the amount of c-erb-B present (Figure 1, 2). That is, if the human c-erb-B gene was contained in any of the hybrids, the intensity of the restriction band should be
Southern Hybridization: probe v-erb-B

Southern blot with v-erb-B probe. DNAs (10 ug) were digested with Eco RI and electrophoresed in 0.7% agarose.
greater than if it was absent. The hybrid D-10-1 con­tained the fragment of chromosome 7 on which c-erb-B had been mapped (Spurr et al. 1984). D-10-1 was then used as a control and given an arbitrary desitometric value of 100% (Figure 1). The desitometric values given the other cells were relative to D-10-1. The preliminary results obtained suggested that the human c-erb-B gene was located in the p13-q22 region of chromosome 7 (Hunts and Shimizu 1984), similar to the EGFR gene. Finally, the band intensity for the human cell line A431 was very strong, suggesting that these cells may have more than the normal diploid content of this gene.

V-erb-B Possibly Related to EGFR

At this time a very interesting and important finding was made, indicating that the c-erb-B gene may be the EGFR gene or a related gene. Using protein sequence determination of peptide fragments, Downward et al. (1984) have shown that the amino acid sequence of v-erb-B closely matches that of EGFR. The v-erb-B protein, however, is significantly smaller than the 170,000 molecular weight of the EGFR glycoprotein (Carpenter and Cohen 1977). This suggests that the v-erb-B protein may be a truncated form of the EGFR (Downward et al. 1984). If this is the case, v-erb-B may owe its transforming activity to the ability
to send mitogenic signals independent of mitogen binding.

Determining if the c-erb-B gene mapped to the same chromosomal location as the EGFR gene would further support the hypothesis that the v-erb-B gene is derived from a truncated EGFR gene. To examine this possibility, restriction enzymes were screened to obtain different restriction patterns for the c-erb-B gene in human and mouse cells. Figure 3 shows the Southern blot for c-erb-B, using a 1.1 kb Sal I fragment of v-erb-B as a probe (Yamamoto et al. 1983a) and the restriction enzyme Hind III. The human, GM2068, c-erb-B hybridization bands obtained were 17 kb, 15 kb and 8 kb. Whereas, the mouse A9 bands did not contain the larger two bands 17 kb and 15 kb. This showed that the restriction enzyme Hind III could be used to distinguish between the human c-erb-B gene and the mouse c-erb-B gene. In the hybrids B-21-3 and D-10-1, the two large human specific bands are present. Whereas, in the hybrid A-1, these bands were not seen (Figure 3). This gives further support to the c-erb-B regional mapping to p13-q22 of chromosome 7 (Figure 1).

Because of the great interest in the EGFR gene, 3 groups have cloned EGFR cDNAs. Merlino et al. (1984) used the protein sequence homology between v-erb-B and EGFR to reason that their DNA sequences should also be similar. A
Fig. 3. Human C-erb-B Gene has Specific Bands

DNAs (10 ug) were cut with Hind III and show human specific bands of 15 and 17 kb. Lane 1 = placental; lane 2 = human fibroblast; lane 3 = mouse A9; lane 4 = hybrid B-21-3; lane 5 = hybrid D-10-1; lane 6 and 7 = hybrid A-1.
v-erb-B DNA probe was then used to screen an A431 cDNA library. A431 is a human epidermal carcinoma cell line which produces high levels of EGFR (Fabricant, Delarco and Todaro 1977). The next group, Lin et al. (1984), used immunoscreening of an A431 cDNA library in an expression vector. Ullrich et al. (1984), working backwards from the protein sequences of several EGFR peptide fragments, constructed oligonucleotide probes to screen A431 and human placental cDNA libraries. The general result of these studies is that A431 has a 15-30 fold gene amplification of the EGFR gene and also produces an abnormal truncated mRNA, which will be discussed in chapter 4.

**C-erb-B/EGFR Mapping**

With the cloning of the EGFR gene, the relationship between EGFR and v-erb-B could be further analyzed. Because of the great homology between the EGFR gene and v-erb-B gene, these probes can be used interchangeably (Merlino et al. 1984, Ullrich et al. 1984). Figure 4 shows the Southern blotting results for the same panel of hybrids as in Figure 5. However, in this case, the EGFR cDNA probe pE7 was used (Merlino et al. 1984). In each lane 1 Eco RI was used to digest the DNA and in each lane 2 Hind III was used. HeLa and A9 served as the human and mouse controls, respectively. The hybrids contain
Fig. 4. Human c-erb-B/EGFR Gene Localization

Lane 1 DNAs (10 ug) were digested with Eco RI and lane 2 DNAs (10 ug) were digested with Hind III. The probe used was the EGFR cDNA clone pE7 (see Fig. 10).
**Fig. 5. Regional Mapping of EGFR/C-erb-B Genes**

<table>
<thead>
<tr>
<th>HUMAN CHROMOSOME 7</th>
<th>HELA</th>
<th>A-9</th>
<th>D-10-1</th>
<th>A-1</th>
<th>B-21-3</th>
<th>C-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pter-q22</td>
</tr>
<tr>
<td>q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>q22-qter</td>
</tr>
<tr>
<td>EGF BINDING</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c-erb-B /EGFR GENE</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
different fragments of human chromosome 7 (Figure 1) and the hybrid C-9 contains the complete human chromosome 7. For the Eco RI digest of HeLa, a human specific restriction pattern can be seen which is repeated in hybrids D-10-1, B-21-3 and C-9, but not in A-1. This result confirms the previous regional mapping of the human EGFR gene to the p13-q22 region of chromosome 7 (Kondo and Shimizu 1983). In analyzing the Hind III restriction patterns, the c-erb-B specific bands of 17 kb and 15 kb were again observed in the hybrids which contain the p13-q22 region of human chromosome 7. The results of Figure 4 indicate that the EGFR gene and the c-erb-B gene are both located in the p13-q22 region of human chromosome 7 (Figure 5) and also supports the hypothesis that the two genes are in fact identical (Hunts et al. 1984).

**C-erb-B is EGFR**

One essential characteristic missing from the v-erb-B gene product which did not support a close association with the EGFR protein was its lack of measurable tyrosine protein kinase activity. The EGFR is thought to transfer the mitogenic signal, induced by EGF, through the membrane by way of its tyrosine protein kinase activity (Hunter and Cooper 1981). If v-erb-B transforms the cell by a type of constitutive mitogenic signal, it should have
an activity similar to EGFR when EGF binds. Only recently has protein tyrosine kinase activity been detected in v-erb-B (Decker 1985; Gilmore, DeClue and Martin 1985; Kris et al. 1985), although this activity is very low. This suggests that the v-erb-B protein is not constitutively active, but can become active independent of EGF.

The great homology between the EGFR and v-erb-B peptides indicate a close association between these proteins (Downward et al. 1984). Furthermore, the regional mapping of the human c-erb-B/EGFR gene (Kondo and Shimizu 1983, Hunts et al. 1984) and in situ data (Merlino et al. 1985) also suggests a close identity. Gene dosage amounts have been estimated suggesting only a diploid amount of c-erb-B/EGFR (Merlino et al. 1985). Finally, the v-erb-B protein has been shown to possess protein tyrosine kinase activity. Therefore, the information at this time strongly suggests, although does not prove, that the c-erb-B gene is the EGFR gene.
CHAPTER 4

TRANSLOCATION CHROMOSOME INVOLVED IN EGFR OVEREXPRESSION

The human cell line A431 has been used to study EGF action as mediated through its cell surface receptor. The main reason for the selection of A431 as a model for EGFR activity is that this cell line possesses an exceptionally high amount of receptors ($2-3 \times 10^6$) (Fabricant et al. 1977). This has allowed for the visualization of events occurring after the initial binding of EGF, such as clustering of receptors, internalization of the receptor-bound EGF, and its degradative processing through a lysosomal pathway (Haigler et al. 1978; Haigler, McKanna and Cohen 1979).

Another way that A431 cells have proved useful has been as a model for studying EGFR hyperproduction. The first genetic analysis of A431 by Shimizu et al. (1984) has shown that a translocation chromosome involving a segment of chromosome 7 correlated with high levels of receptor. In this work, hybrids were analyzed which had been constructed between A431 cells and mouse A9 cells, which are EGFR deficient. Each hybrid retaining the
translocation chromosome termed M4 consistently had higher levels of EGFR than hybrids retaining the normal chromosome 7 from A431 (Shimizu et al. 1984). A431 has also been used to construct cDNA libraries from which cDNA clones have been selected for the EGFR gene (Merlino et al. 1984; Lin et al. 1984; Ullrich et al. 1984). These groups have shown that A431 has a 15-30 fold EGFR gene amplification, and produces an abnormal transcript of 2.9 kb only found in A431. However, neither the chromosomes on which the EGFR gene amplification occurred in A431 nor the mechanism of abnormal transcript generation had been determined.

**Translocation Chromosome Contains EGFR Gene Amplification**

The hybrids constructed by Shimizu et al. (1984) represent an excellent tool to examine individual chromosomes from A431 for gene amplification. The hybrids examined were C2B5, AA-1, and AA-7 and the parental cells were A9 and A431. C2B5 contains a single human X:7 translocation chromosome, which is the only human chromosome present and allows for its continuous selection in HAT medium and use as a haploid standard for chromosome 7 (Shimizu, Behzadian and Shimizu 1980). AA-1 contains the translocation chromosome M4 involving chromosome 7 from A431. AA-7
contains an apparently normal chromosome 7 from A431. The only human chromosome that the AA hybrids have in common is X. DNA was isolated from these hybrids and applied to nitrocellulose filter paper by a dot blot apparatus. The EGFR cDNA probe pE7 (Merlino et al. 1985) was then used to screen the level of EGFR gene present in these hybrids (Figure 6a). As a control for the amount of DNA present, the probe pAM-91 for actin was also used (Minty et al. 1981). The hybrid AA-1, containing the translocation chromosome M4, had a significantly greater hybridization intensity than C2B5 or AA-7, suggesting that M4 had an EGFR gene amplification (Hunts et al. 1985a).

Elevated EGFR RNA Levels and EGF Binding

RNA was isolated from each of the hybrids and parental cells and applied to nitrocellulose filter for Northern hybridization analysis, using pE7 as probe. Again, the AA-1 hybrid had significantly more receptor mRNA than the other two hybrid cells (Figure 6a), supporting the idea of gene amplification on M4. Furthermore, the EGF binding for AA-1 was found to be approximately 4 times greater than C2B5 or AA-7 (Figure 6b).

Quantitation of EGFR Characteristics

Figure 6b shows the quantitation of the hybrids
Fig. 6. EGFR DNA/RNA and EGF Binding

A. DNA/RNA dot hybridization. DNA applied was 0.67 ug except for A431 (0.17 ug). RNA applied was 1 ug except for A431 (0.17 ug). B. Graphic representation.
and parental cells for EGFR gene/mRNA and receptor levels. To accurately estimate the human EGFR gene content/mRNA level, the dot blots for probes pE7 and pAM-91 were scanned by a densitometer. Equal amounts of material were applied to each dot except for A431. Human actin genes are not contained on any of the human chromosomes present in the hybrid cells. Hybridization intensities were found to be similar for pAM-91, but not for pE7 when comparing equal amounts of mouse A9 and human A431 ratios of DNA to RNA. The slight background hybridization observed in A9 DNA, using pE7, was subtracted from values given the hybrids. The mRNA levels represent the sum of the 10 kb plus 5.6 kb mRNAs (Figure 7). Approximately 70% of the A431 RNA hybridization represents the 2.9 kb mRNA. No hybridization was observed for A9 RNA, using pE7. C2B5 was used as a standard and given an arbitrary value of one for each of the three categories tested. Values obtained for the other cells were then adjusted relative to C2B5. All values obtained for the hybrids AA-1 and AA-7 were normalized to account for human chromosomal loss due to passaging in tissue culture according to a standard loss curve for hybrids (Shimizu et al. 1984).

AA-7 exhibited a twofold increase in the amount of the EGFR gene compared to C2B5. AA-1, however, had ap-
Fig. 7. EGFR Abnormal Transcript

A. Northern blot with pE7 probe. Lane a= A9; lane b=C2B5; lane c= AA-1; lane d= AA-7; lane e= A431. RNA (0.4 ug for A431 and 20 ug for others) was run on in 1.1 % agarose. Exposure to X-ray film; 24 hr e,c and 7 days a,b,d. B. Densitometric tracing values from each lane.

<table>
<thead>
<tr>
<th>mRNA Species</th>
<th>C2B5</th>
<th>AA-1</th>
<th>AA-7</th>
<th>A431</th>
</tr>
</thead>
<tbody>
<tr>
<td>10Kb</td>
<td>43a  (1.0)</td>
<td>20 (1.0)</td>
<td>27 (1.0)</td>
<td>26 (1.0)</td>
</tr>
<tr>
<td>5.6Kb</td>
<td>57 (2.3)</td>
<td>44 (3.9)</td>
<td>73 (4.8)</td>
<td>5 (0.4)</td>
</tr>
<tr>
<td>2.9Kb</td>
<td>0</td>
<td>36 (6.4)</td>
<td>0</td>
<td>69 (9.5)</td>
</tr>
</tbody>
</table>

a) % of total mRNA. b) Relative copy number per 10Kb mRNA.
proximately a six- to sevenfold amplification relative to C2B5. The additive amplification observed in AA-7 and AA-1 cells, representing chromosomes 7 and M4 respectively in A431, accounted for approximately 28% (Figure 6b) of the 31-fold amplification of the EGFR gene observed in the parental A431, which is tetraploid for chromosome 7. Similarly, AA-1 also had higher levels of EGFR relative to C2B5 and AA-7. The six- to sevenfold gene amplification of AA-1 leads to only a four- to fivefold increase in EGF binding relative to C2B5. There could be several reasons for this effect one of which could be that some of the amplification involves genes encoding the variant mRNA, whose potential protein product may not have cell surface EGF binding capacity. These results support the idea that M4 is, in part, responsible for the increased receptor level observed in A431 due to an amplification event involving the EGFR gene.

**Gene Rearrangement Responsible for Abnormal RNA**

A431 cells produce three EGFR related mRNAs, a 10 kb, a 5.6 kb, and a variant 2.9 kb message (Merlino et al. 1984; Lin et al. 1984; Ullrich et al. 1984). The two larger messages are present in normal cells and may only differ in their 3' noncoding regions. The 2.9 kb variant mRNA is observed only in A431 and has been shown to encode
the EGF binding domain of the receptor, containing a 3' fragment of unknown origin (Ullrich et al. 1984).

Upon examining the mRNA species produced in the hybrids (Figure 7b), it was found that the M4 chromosome correlated with the presence of the variant mRNA, which was not observed in either AA-7 or C2B5. The aberrant message was overexpressed in A431 approximately sevenfold relative to the sum of the larger messages (Figure 7a). However, in AA-1 the relative amount of the variant mRNA was just slightly higher than the sum of the two larger mRNAs. This indicates that either the 2.9 kb mRNA is also produced from A431's other translocation chromosome M14 (Shimizu et al. 1984), or, alternatively, the transcription of the truncated mRNA could be suppressed in the hybrid cells.

It has been reported that A431 cells secrete a 110 kd EGF-binding protein (Weber, Gill and Spiess 1984). The aberrant mRNA could encode this secreted protein. The higher gene amplification relative to EGF binding observed in both A431 and AA-1 (Figure 6b) suggests that these genes may encode proteins unable to bind EGF, which supports an association of the variant message with the secreted protein. However, the presence of the secreted protein does not strictly correlate with the translocation
chromosome M4, indicating that either the variant mRNA is not responsible for the secreted protein or that the message for the 110 kd protein is also produced from other chromosomes besides M4.

Detection of EGFR Gene Rearrangement

It has been postulated that a DNA rearrangement occurred in A431 cells involving the EGF receptor gene, generating the variant mRNA (Ullrich et al. 1984). Using the cDNA probe pE7, the restriction pattern from several restriction enzymes were examined for AA-1 and AA-7 in order to determine if a rearrangement had occurred on M4 (Figure 8). However, no detectable difference was observed in the restriction patterns for the enzymes used with the probe pE7.

The strategy then used was to reprobe with a 400bp Pvu II-Cla I fragment from the extreme 3' end of the cDNA encoding the variant mRNA (Merlino et al. 1985). The results obtained suggested that a DNA rearrangement had occurred on M4 which could be responsible for producing the varianat mRNA. Figure 9 shows that hybridization of this probe to A431 DNA generates a small number of prominent amplified fragments whether digested with Eco RI (lane a) or Hind III (lane f). A single band in each enzyme digestion pattern (marked by the arrowhead) has
Fig. 8. No EGFR Gene Rearrangement Using pE7

DNA (5 ug for A431 and 15 ug for others) was digested with the restriction enzymes shown. Lanes 1,5,10 are A431; lanes 2,6,11 are AA-1; lanes 3,7,12 are AA-7; lanes 4,8,13 are A9 and lane 9 is lamda DNA cut with Hind III. The DNA probe is pE7.
Fig. 9. EGFR Gene Rearrangement Using 3' Probe

Lanes a,f are A431; lanes b,g are AA-1, lanes c,h are AA-7; lanes d,i are C2B5; lanes e,j are A9 (10 ug DNA each). The probe used was the 3' Pvu II-Cla I fragment from pE15 (see Fig. 10). Arrowheads show rearrangement and crosses show unrearranged EGFR gene.
been shown to be unique to A431 cells (Merlino et al. 1985). These same two bands were found in AA-1 DNA containing the translocation chromosome M4 (lane b and g), but not in either AA-7 or C2B5, which contain apparently normal chromosome 7s (lanes c, d or h, i, respectively). These fragments are thought to contain the breakpoint region of the rearranged EGF receptor gene in A431.

The results suggest that in A431 cells the translocation chromosome M4 harbors an amplified, rearranged copy of the EGFR gene. There was several other bands, which were exclusively associated with the 400 bp probe representing the unique sequences of the 2.9 kb aberrant mRNA, that appeared in all the interspecies hybrid cells containing human chromosome 7 (Figure 9 crosses). Therefore, the unidentified DNA which is fused to the EGF receptor gene appears to have originated from chromosome 7 as well, and thus the rearrangement responsible for the production of the variant 2.9 kb mRNA is probably the result of an intrachromosomal event.

**Structure of Rearranged EGFR Gene**

The normal and rearranged EGFR gene from A431, both thought to be contained on M4, have been sequenced to determine their structure (Merlino et al. 1985). Figure 10 shows the structure of the normal and abnormal cDNAs.
Fig. 10. Structure of Normal and Rearranged EGFR cDNA

A, C and D show cDNA fragments of the EGF receptor gene called pE15, pE3, pE62 and pE7. B shows the EGFR rearranged gene on top and the normal gene on the bottom. pE15 is a cDNA from the variant mRNA in A431 (see fig. 7 and text).
To further analyze the gene amplification on chromosome M4, DNA dot blots for the hybrids were again constructed (Figure 11). In this experiment probes used were a Cla I-Pvu II 5' fragment from pE15, a Cla I-Pvu II 3' fragment also from pE15 and pAM-91 (or pACT). The lower amount of the 2.9 kb mRNA in AA-1 relative to A431 as observed (Figure 7a) could be due to either M4 containing only one copy of the rearranged gene or that the transcription is less. However, the amount of the rearranged gene, as determined by the 3' probe of pE15 was similar to that determined by the 5' probe of pE15 (Figure 11). This suggests that both the normal and rearranged gene are amplified as a unit and that the reason for the reduced level of 2.9 kb transcript in AA-1 may be the result of some factor affecting its transcription.

**Significance of EGFR Gene Amplification/Rearrangement**

Chromosomal translocation and DNA rearrangements have been shown to be associated with chromosomes containing gene amplification (Cowan et al. 1982; Mushinski et al. 1983). Morphological evidence suggests that M4 is a translocation involving chromosome 7, which has the distal end of the p arm (p22-qter) replaced by a chromosomal fragment of unknown origin (Shimizu et al. 1984). Because of the large distance between this translocation site and
Fig. 11. Normal/Rearrangement Genes Co-amplified

DNA (2 ug) was applied. The 5' Pvu II- Cla I probe (p5') and the 3' Pvu II-Cla I probe both from pE15 were used. The pACT probe was used as a control.
Fig. 11. Normal/Rearrangement Genes Co-amplified
the region containing the EGF receptor gene (p13-q22) (Kondo and Shimizu 1983), it was felt that the rearrangement event generating the variant message and the translocation event were separate. The results from Figure 9 support this idea as well as an association between DNA rearrangement and DNA amplification.

It is conceivable that the amplification event involving the EGFR gene may have a significant role in tumor induction. Furthermore, this amplification may be associated with the intrachromosomal rearrangement event. However, because of the late identification of tumor tissue, it is difficult to prove a cause and effect relationship between chromosome rearrangement, gene amplification, and tumor induction. Chromosome rearrangement is probably both a cause and result of tumor induction and expansion. Therefore, with only EGFR gene amplification observed at this time in A431, no judgement can be made as to its association with transformation. The rearrangement event's generation of the variant message and the secreted protein may not have any role in tumorigenesis or any biological function.
CHAPTER 5

ELEVATED EGFR LEVELS IN SQUAMOUS CELL CARCINOMAS

Several of the oncogene protein products have protein tyrosine kinase activity (Hunter and Cooper 1985). Because of this, it has been thought that this activity may be central in these transforming enzymes abilities. When the EGFR was shown to have protein tyrosine kinase activity, this implicated the EGFR as also having some potential activity in the tumorigenic process (Hunter and Cooper 1981). Furthermore, the EGFR has also been implicated in tumorigenesis by an event which deletes the receptor's binding domain (Downward et al. 1984).

Recent results indicating that the intact EGFR may also have a role in some cancers has been suggested by the finding of Cowley et al. (1984) that squamous cell carcinomas have enhanced levels of EGFR. However, the data presented by Cowley et al. was not quantitative with regards to the primary tumors examined and did not address any of the mechanisms which may be involved in increasing EGFR levels. More recent findings by Libermann et al. (1985) have shown that there is increased receptor kinase
activity in some brain tumors. These tumors often had associated EGFR gene amplification and, in some cases, EGFR gene rearrangement. Therefore, abnormalities in receptor quality and/or quantity appear associated with tumorigenesis.

High Levels of EGFR Correlate with Squamous Carcinomas

To examine the association of EGFR with squamous cell carcinomas, 27 primary human tumors, including 15 squamous cell carcinomas and 12 other tumor types were examined. For screening EGFR levels in tumor tissue, a direct approach of measuring EGF binding to a tissue homogenate was taken. To determine the reliability of a binding assay using tissue homogenate, Scatchard analysis was first performed on A431 cells grown in nude mice and on placental tissue. The results obtained were similar to the reported data (Fabricant et al. 1977; Rao et al. 1985). This method was also found to be consistent when measuring large numbers of normal tissue samples (Table 1).

Enhanced EGF binding was found to be associated only with squamous cell carcinomas at a frequency of 9 out of 15. Two of these tumors, SCL1 and SCE1, had an EGF binding capacity approximately 15- to 20-fold higher than
normal adjacent tissue (Table 1). The 7 other EGF receptor amplified squamous cell carcinomas (SCL2, 3, 4, 5 and SCE3, 4, 5) had increased binding in the range of 2- to 6-fold higher than that of normal adjacent tissue. The finding that the normal adjacent tissue in all 9 cases had less EGF binding supports the idea that amplified EGF receptor levels may have some role in the genesis and/or maintenance of squamous cell carcinomas. However, amplified receptor levels were not observed in all the squamous cell carcinomas, suggesting that this abnormality may not be necessary for transformation. To further determine how specific EGFR amplification is to squamous cell carcinomas, 12 other tumor types were examined (3 pancreatic tumors, 1 colon tumor, 4 breast tumors, 1 stomach tumor and 3 astrocytomas). In each case, an EGF binding level in the same range as normal tissue was observed (Table 1). This indicates that increased receptor levels are indeed specific for squamous cell carcinomas.

**EGFR Gene Amplification**

To determine the potential mechanisms by which SCL1, SCE1, SCE2 and SCL3 increased their receptor levels over that of the normal adjacent tissue, DNA was isolated and digested with the restriction enzyme Eco RI for Southern blot analysis (Southern 1975). The probe used
<table>
<thead>
<tr>
<th>Tissue abbreviation</th>
<th>Source</th>
<th>EGF binding (fmol EGF/mg protein homogenate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>Human placenta</td>
<td>125</td>
</tr>
<tr>
<td>A431</td>
<td>Nude mouse</td>
<td>150</td>
</tr>
<tr>
<td>Lung carcinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCL1</td>
<td>Squamous cell carcinoma</td>
<td>74</td>
</tr>
<tr>
<td>SCL2</td>
<td>Squamous cell carcinoma</td>
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<td>SCL3</td>
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</tr>
<tr>
<td>SCL4</td>
<td>Squamous cell carcinoma</td>
<td>10</td>
</tr>
<tr>
<td>SCL5</td>
<td>Squamous cell carcinoma</td>
<td>1.4</td>
</tr>
<tr>
<td>Esophagus carcinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCE1</td>
<td>Squamous cell carcinoma</td>
<td>115</td>
</tr>
<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>3.6</td>
</tr>
<tr>
<td>SCE2</td>
<td>Squamous cell carcinoma</td>
<td>30</td>
</tr>
<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>5.5</td>
</tr>
<tr>
<td>SCE3</td>
<td>Squamous cell carcinoma</td>
<td>13</td>
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<td>10</td>
</tr>
<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>3.0</td>
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<tr>
<td>SCE6</td>
<td>Squamous cell carcinoma</td>
<td>4.2</td>
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<td>--</td>
<td>Normal adjacent tissue</td>
<td>2.3</td>
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<tr>
<td>SCE7</td>
<td>Squamous cell carcinoma</td>
<td>3.6</td>
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<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>0</td>
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<tr>
<td>SCE8</td>
<td>Squamous cell carcinoma</td>
<td>2.9</td>
</tr>
<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>3.5</td>
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<tr>
<td>SCE9</td>
<td>Squamous cell carcinoma</td>
<td>1.9</td>
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<td>--</td>
<td>Normal adjacent tissue</td>
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<tr>
<td>SCE10</td>
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<td>1.0</td>
</tr>
<tr>
<td>--</td>
<td>Normal adjacent tissue</td>
<td>1.3</td>
</tr>
</tbody>
</table>
was a cDNA specific for the EGFR gene, pE7 (Merlino et al. 1985). In Figure 12 it was observed that SCL1 and SCE1 showed approximately 15- to 30-fold amplification of the EGFR gene. Surprisingly, however, SCE2 and SCL3 had only a normal level of EGFR gene relative to placental tissue. No rearrangements were observed in the DNA restriction patterns for any of these tumors. Table 2 shows the EGFR gene level in several of these tumors as determined by densitometric tracing of Figure 12 and DNA dot blots not shown. These results suggest that in the case of SCE1 and SCL1 EGFR gene amplification may be the mechanism which induces elevated EGFR levels.

**Increased EGFR RNA**

RNA was then isolated for Northern analysis. Because of limited tissue availability RNA could only be isolated from SCE1 and SCL1. Figure 13 showed that the 15- to 30-fold amplification in the receptor genes of SCL1 and SCE1 was accompanied by a similar increase in receptor mRNA. The variant 2.8 kb mRNA observed in A431 has been shown to be due to an apparent intrachromosomal rearrangement in chromosome 7 (Merlino et al. 1985; Hunts et al. 1985a). However, no abnormality in transcript sizes were detected for these squamous cell carcinomas.
Fig. 12. EGFR Gene Amplification in Human Tumors

DNA (15 ug) was digested with Eco RI and probed with pE7. Lane 1 = Placenta; lane 2 = SCE2; lane 3 = SCL3; lane 4 = SCE4; lane 5 = SCE1; lane 6 = SCL1; lane 7 = A431. Exposure to X-ray film was for 24 hr.
Table 2. Characteristics of Human Tumors: EGFR gene levels.

<table>
<thead>
<tr>
<th>Tissue abbreviation</th>
<th>Source</th>
<th>EGF receptor gene copy number (relative to HP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP</td>
<td>Human Placenta</td>
<td>1</td>
</tr>
<tr>
<td>A431</td>
<td>Nude mouse</td>
<td>15-25</td>
</tr>
<tr>
<td>Lung carcinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCL1</td>
<td>Squamous cell carcinoma</td>
<td>20-30</td>
</tr>
<tr>
<td>SCL3</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>SCL5</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Esophagus carcinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCE1</td>
<td>Squamous cell carcinoma</td>
<td>15-25</td>
</tr>
<tr>
<td>SCE2</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>SCE4</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>SCE5</td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig. 13. High EGFR RNA Levels in Tumors

RNA (2 ug) was applied to a 1.1 % agarose gel. Lane 1 = A431; lane 2 = SCL1; lane 3 = SCE1; lane 4 = placenta. The probe used was pE7.
SCE2 and SCE3 have increased receptor levels which are apparently independent of gene dosage. The mechanisms by which these tumors increase their receptor levels may involve enhanced mRNA stability and/or receptor stability. Characteristics similar to those of SCE2 and SCL3 have been observed in the cell line UCVA-1 (Gamou, Kim and Shimizu 1984), which will be discussed further in the next chapter.

**Significance of EGFR Levels**

The increase of EGFR levels seems to be specific for squamous cell carcinomas on the basis of these results, but has also been observed in glioblastomas at a high frequency (Libermann et al. 1985). Because elevated receptor levels are associated only with the tumors, this suggests some role of enhanced EGFR levels in human neoplasia. Enhanced receptor levels are the result of gene amplification in many cases (Yamamoto et al. 1986). However, increased receptor levels could also result from mechanisms independent of gene amplification, as observed here in SCE2 and SCL3 and elsewhere for UCVA-1 (Gamou et al. 1984). Another example is placental tissue, which seems to be a target tissue for EGF (Rao et al. 1985). This tissue has increased receptor activity but normal EGFR gene content (Libermann et al. 1985). SCE2 and SCL3
may mimic the mechanism(s) used by placental tissue to increase their receptor levels. If so, this suggests that receptor hyperproduction may be appropriate for the proper functioning of some tissues but inappropriate for others. Therefore, the proposal is made that along with gene amplification, any inappropriate alteration in the mechanism(s) controlling the EGFR, leading to elevated levels, may have some role in human tumorigenesis (Hunts et al. 1985b).
CHAPTER 6

MOLECULAR MECHANISMS REGULATING EGFR LEVELS

One of the major strategies for the oncogenic disorganization of normal cellular growth control appears to be the unregulated expression of proto-oncogenes (Bishop 1983). In the case of the EGFR, hyperproduction has been associated with squamous cell carcinomas and glioblastomas (Cowley et al. 1984; Hunts et al. 1985b; Libermann et al. 1985). In this chapter, the mechanisms are addressed which may be responsible for increasing the EGFR levels on the cell surface of squamous cell carcinomas and other tissues.

Various tissues and cell lines were analyzed to determine factors which regulate the EGFR level on the cell surface. At least 3 mechanisms are postulated in the control of EGFR level: gene amplification, receptor mRNA levels, and receptor half-life (Hunts et al. 1986). The abnormal activation of any or all of these mechanisms may contribute to the high level of EGFR observed in squamous cell carcinomas.
In the previous chapter it was found that EGFR gene amplification was present in 2 primary squamous cell carcinoma tissues, which also had high EGFR levels. For the epidermoid carcinoma cell line A431, receptor gene amplification seems to be directly responsible for the enhanced EGFR levels (Lin et al. 1984). However, because it was also observed that high EGFR levels were present in placental tissue without EGFR gene amplification (Table 2), the assumption that receptor gene amplification was responsible for the enhanced receptor levels in these squamous cell carcinomas could not be assumed a priori. Therefore, an approach similar to Lin et al. (1984) was taken in selecting variants from a squamous cell carcinoma cell line NA, using EGF toxicity (high levels of EGF are toxic to cells which over-express EGFR). The reasoning used for this approach is that variants selected for reduced receptor levels should also have a reduced EGFR gene copy number if, in fact, the gene amplification is directly responsible for the receptor's high expression level. NA cells have been shown to possess high levels of EGFR and also contain the respective gene amplification (Yamamoto et al. 1986). Using NA, several variants were selected, all showing similar characteristics. The analy-
sis of one of these variants, ER-6, is presented here. Initially, it was found that ER-6 had approximately 10% of the EGF binding capacity as the parent, NA (Table 3). DNA was isolated from ER-6 and NA, digested with the restriction enzyme Hind III, fractionated on an agarose gel and Southern blotted (Southern 1975), using the EGFR cDNA probe pE7 (Merlino et al. 1985) (Figure 14, lanes 1 and 2). Similarly, mRNA was isolated, fractionated on agarose, Northern blotted, and hybridized again to pE7 (Figure 15, lanes 1 and 2). A summary of these results is given in Table 3 and 4. Densitometric analysis showed a reduction in both the EGFR gene and the respective mRNA levels in ER-6 relative to NA. These results suggest that receptor gene amplification is the mechanism used by NA to increase its receptor levels. This evidence strengthens the argument that the EGFR gene amplification is also a potential mechanism used by squamous cell carcinomas to increase their receptor levels.

**EGFR RNA Levels**

It has been observed here and elsewhere (Libermann et al. 1985) that placental tissue possesses high EGFR levels independent of gene amplification. This indicates that a normal mechanism is active in this tissue which maintains high EGFR levels. This mechanism is of interest
Table 3. EGFR Characteristics in Several Tissues.

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>EGF Binding (fmol/mg prot.)</th>
<th>EGF Receptor mRNA (relative to HP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP Human Placenta</td>
<td>125</td>
<td>9</td>
</tr>
<tr>
<td>HF Human Fibroblast</td>
<td>2.6</td>
<td>1</td>
</tr>
<tr>
<td>NA Squamous Cell Carcinoma</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>ER-6 EGF-selected NA</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>UCVA-1 Pancreatic Adeno-Carcinoma</td>
<td>75</td>
<td>13</td>
</tr>
</tbody>
</table>
Fig. 14. EGFR Gene Levels

Southern blot with pE7 probe. DNA (10 ug for lanes 1, 2 and 20 ug for lanes 3, 4, 5) were digested with Hind III. Lane 1 = NA; lane 2 = ER-6; lane 3 = UCVA-1; lane 4 = fibroblast; lane 5 = placenta.
Fig. 15. EGFR RNA Levels

Northern blot with pE7 probe. RNA (2 ug) was applied to 1.1 \% agarose. Lane 1 = NA; lane 2 = ER-6; lane 3 = placenta; lane 4 = fibroblast; lane 5 = UCVA-1.
Fig. 15. EGFR RNA Levels
### Table 4. EGFR Gene Levels.

<table>
<thead>
<tr>
<th>Tissue abbreviation</th>
<th>Source</th>
<th>EGF receptor gene copy number (relative to HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>Human placenta</td>
<td>1</td>
</tr>
<tr>
<td>HF</td>
<td>Human fibroblast</td>
<td>1</td>
</tr>
<tr>
<td>NA</td>
<td>Squamous cell carcinoma</td>
<td>20-30</td>
</tr>
<tr>
<td>ER-6</td>
<td>EGF-selected NA</td>
<td>2-3</td>
</tr>
<tr>
<td>UCVA-1</td>
<td>Pancreatic adenocarcinoma</td>
<td>1</td>
</tr>
</tbody>
</table>
because it was observed that some squamous cell carcinomas had high EGFR levels but did not contain receptor gene amplification (Table 2). A similar observation has been made by Libermann et al. (1985) for some brain tumors. To determine the possible mechanisms involved, we compared placental tissue to normal human fibroblasts, which only had approximately 2% of the EGF binding relative to the placenta (Figures 15 and 17, lanes 3 and 4). The results indicate that a second mechanism, which can potentially enhance EGFR levels, involves increasing the amount of receptor transcript. The higher EGFR mRNA concentrations in placental tissue could be generated by a greater mRNA stability and/or a greater rate of transcription.

**EGFR Half-life**

Further analysis of Table 3 and 4 reveals a discrepancy between EGF binding and the ratio of EGFR DNA to RNA levels when comparing NA, placenta (HP), fibroblasts and UCVA-1. That is, the EGF binding for HP and UCVA-1 were higher than would be expected solely from their DNA/RNA ratios relative to NA and fibroblasts. UCVA-1, a pancreatic adenocarcinoma cell line, has been shown to possess a high level of EGFR (Gamou et al. 1984), but a normal diploid content of receptor gene (Figure 14, lane 5), and only a slightly higher level of receptor mRNA
relative to HP (Figure 15, lane 5). The data shown here implicates a third mechanism, independent of both EGFR gene and mRNA levels, potentially capable of increasing receptor levels.

Evidence is presented here which suggests that EGFR half-life may also play a key role in regulating the EGFR levels on the cell surface. Near confluent cells, NA and UCVA-1, were pulse labeled with $^{35}$S-methionine for 2 hours. The label was then chased in DMEM containing fetal calf serum for 0, 4, 8, 21, 32, and 45 hours. Cells were then lysed with RIPA buffer (Richert et al. 1979), and the EGFR immunoprecipitated with the monoclonal antibody B4G7, specific to EGFR (Behzadian and Shimizu 1985). The precipitates were washed and applied to an SDS-polyacrylamide gel for electrophoresis. The gel was fixed and treated for exposure to X-ray film for 1 week. The gel was then cut to remove EGFR bands and solubilized for liquid scintillation counting. Counts were then used to determine values for the EGFR half-life for UCVA-1 and NA in the presence and absence of EGF (Table 5). It was observed that UCVA-1 had a significantly longer receptor half-life in the absence of EGF compared to NA. In the presence of EGF, however, similar half-lives were observed, suggesting that a mechanism regulating receptor stability on UCVA-1
Table 5. EGF Receptor half-life.

<table>
<thead>
<tr>
<th>Cells</th>
<th>+EGF(a)</th>
<th>-EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCVA-1</td>
<td>10</td>
<td>30-35</td>
</tr>
<tr>
<td>NA</td>
<td>10</td>
<td>10-15</td>
</tr>
</tbody>
</table>

(a) Values were determined by linear regression of the radioactive counts obtained from precipitation. EGF was added at the beginning of chase.
may be active. This mechanism is thought to be at least partially responsible for the discrepancy between the cell surface EGFR levels relative to the receptor gene and transcript levels.
MOLECULAR APPROACH TO STUDY EGFR ACTIVITY

One technique which may prove useful in studying EGFR function and activity is the use of complimentary or antisense-RNA. Antisense-RNA has been used with some success for the thymidine kinase (TK) gene and an actin gene (Izant and Weintraub 1985). By reversing the direction of the gene of interest relative to its promoter, the opposite strand of DNA will be transcribed, generating a complimentary antisense-RNA. This antisense-RNA can then hybridize with the sense-RNA to inhibit its translation into protein. In the case of antisense-RNA which is generated in the nucleus, the hybridization is thought to inhibit gene expression by preventing RNA transport out of the nucleus (Kim and Wold 1985). Depending on the efficiency of this technique, it may prove to be a useful tool for future analysis and dissection of EGFR function and activity.

Construction of pEN

Initially, a general purpose vector was constructed using the pSV2neo plasmid (Southern and Berg 1982). The
pSV2neo plasmid was chosen because it has a selectable marker, neo, which gives cells resistance to the drug G-418. However, the vector must integrate into the cell's genome to be maintained because the plasmid has no site for DNA replication and will be lost during cell division. Figure 16 shows the outline of the construction of the general purpose expression vector pEN. A Hind III site was first deleted from pSV2neo. This change did not disrupt the neo gene activity. An Eco RI-Bam HI fragment was then added to this plasmid from pBR 322, which had been cut first with Eco RI and Bam HI. An SV40 poly A site was put into the filled Eco RI site, generating pEN. The pEN vector has 3 unique restriction sites Cla I, Hind III, and Bam HI. The dark regions are derived from pBR322. The stippled regions are derived from SV40 and the lined box is the neo gene. Any gene can be inserted in the Cla I site and any promoter can be inserted in the Hind III or Bam HI sites.

Antisense-EGFR-RNA

The vector pEN was then used to construct antisense-EGFR vectors. Figure 20 shows the vectors which were constructed. All the vectors contain the AEV LTR as a promoter inserted in the Hind III site of pEN. Three fragments from the EGFR cDNA were chosen (Figure 17). A
Fig. 16. Construction of pEN
Fig. 17. Antisense-RNA EGFR Vectors
Fig. 18. Fragments of EGFR Used for Antisense RNA
5' fragment was used, Pvu II-Cla I, and filled in and inserted in the filled Cla I site of pEN, generating pERR5. Likewise, a middle Bam HI fragment was isolated from pE7 and filled in and inserted in pEN at the filled Cla I site, giving rise to pERRM. Similarly, a 3' Cla I fragment was isolated from pE62 and inserted in the Cla I site of pEN, giving pERR3. All EGFR inserts were selected for in the opposite orientation to the promoter relative to the normal gene (Figure 17).

**Reduction of EGF Binding**

The vectors were introduced into NA cells, EGFR hyperproducers, by calcium phosphate-DNA precipitation (Corsaro and Pearson 1982). Cells recovering the integrated vector were then selected for by using the drug G-418 (Southern and Berg 1982). Single colonies were isolated using cloning rings. These G-418 resistant colonies were then checked for EGF binding relative to the parental NA cells. If the EGFR antisense-RNA is effectively inhibiting the transport of EGFR mRNA outside of the nucleus, EGF binding should be decreased. The results of the binding assays from several different clones selected from pERR5 are shown in Table 6 and from pERRM and pERR3 are shown Table 7. The results indicate that pERR5, containing the 5' end of the EGFR cDNA was the most efficient at
Table 6. EGF Binding: pERR5.

<table>
<thead>
<tr>
<th>Clone</th>
<th>% input bound/10^6 cells</th>
<th>% binding relative to NA (+ increase, -- decrease, -- no change &gt;10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent NA</td>
<td>10.5</td>
<td>--</td>
</tr>
<tr>
<td>5-1</td>
<td>5.0</td>
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<tr>
<td>5-2</td>
<td>7.7</td>
<td>-27</td>
</tr>
<tr>
<td>5-3</td>
<td>8.9</td>
<td>-15</td>
</tr>
<tr>
<td>5-4</td>
<td>7.6</td>
<td>-28</td>
</tr>
<tr>
<td>5-5</td>
<td>8.4</td>
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<td>-17</td>
</tr>
<tr>
<td>5-17</td>
<td>9.6</td>
<td>--</td>
</tr>
<tr>
<td>Clone</td>
<td>% input bound/ $10^6$ cells</td>
<td>% binding relative to NA (+ increase, - decrease, -- no change &gt;10%)</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Parent</td>
<td>NA</td>
<td>--</td>
</tr>
<tr>
<td><strong>pERRM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-1</td>
<td>11.6</td>
<td>--</td>
</tr>
<tr>
<td>M-2</td>
<td>11.4</td>
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<td>12.1</td>
<td>+15</td>
</tr>
<tr>
<td><strong>pERR3</strong></td>
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<td></td>
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<tr>
<td>3-11</td>
<td>11.0</td>
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Table 3. Total Average % Change in EGF Binding.

<table>
<thead>
<tr>
<th>Vector</th>
<th>% change in EGF binding</th>
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</thead>
<tbody>
<tr>
<td>pERR5</td>
<td>-26</td>
</tr>
<tr>
<td>pERRM</td>
<td>-3.5</td>
</tr>
<tr>
<td>pERR3</td>
<td>+7.0</td>
</tr>
</tbody>
</table>
inhibiting EGFR expression. A decrease of up to 52% was seen in pERR5 clone 5-1 (Table 6). The average for the pERR5 clones was a reduction of EGF binding by 26% (Table 8). The other vectors pERRM and pERR3 showed little or no reduction in EGF binding. These results suggest that the 5' fragment of the EGFR cDNA is the most efficient for antisense-RNA inhibition of EGFR gene expression.

Integrated Vector DNA

DNA was then isolated from some of these selected colonies to see the form of the integrated vector. Figure 19 shows the Southern blot obtained from the parental NA and clones 3-8, 3-4, 3-5, and 3-6 from pERR3. The DNA was digested with the restriction enzyme Hind III and probed with the pEN vector containing the AEV LTR. If the vector integrated intact, a 1.8 Kb Hind III fragment for the AEV LTR should be seen as in clone 3-4 (Figure 19). The other clones do not have this band, suggesting that the vector was deleted during integration.

The cell line used for testing these vectors, NA, has a 20-30 fold EGFR gene amplification (Figure 14) accompanied by a similar high level of EGFR mRNA (Figure 15). This suggests that in order for the EGFR levels to be reduced more in NA, a larger amount of EGFR antisense-RNA must be produced. One way to do this would be to use an
Fig. 19. Integrated Antisense Vector DNA

Southern blot with pEN (containing AEV LTR) as probe. DNA (15 ug) was digested with Hind III.
amplification vector (Kim and Wold 1985). The antisense-RNA is linked to the mRNA for the dihydrofolate reductase (dhfr) gene by inserting the antisense-gene between dhfr and its poly A site, generating a bi-functional mRNA. As the drug methotrexate is increased, the cell must espress more of the dhfr enzyme, which co-amplifies the antisense-RNA. Reducing the EGFR levels in NA could change some properties of NA and give more information as to what effect high EGFR levels have in these transformed cells, such as changing the growth characteristics. Similarly, if the endogenous EGFR gene expression could be reduced to zero, rearranged or altered EGFR genes could then be introduced to better characterize receptor function.
CHAPTER 8

CONCLUSION

The results of this study support the following conclusions regarding the epidermal growth factor receptor (EGFR) proto-oncogene: 1) The c-erb-B proto-oncogene and the EGFR gene have both been regionally localized to the p13–q22 region of human chromosome 7, supporting the idea that the v-erb-B oncogene was derived from a truncated c-erb-B/EGFR gene; 2) The translocation chromosome M4 of A431 is at least partially responsible for the high EGFR levels observed in A431, which is due to EGFR gene amplification on M4; 3) The M4 chromosome also has a gene rearrangement which is thought to be responsible for the abnormal mRNA observed in A431; 4) A significant number of human primary squamous cell carcinomas had increased levels of EGFR relative to normal adjacent tissue, suggesting that elevated EGFR levels probably play some role in this type of cancer; 5) At least 3 mechanisms are postulated to be involved in increasing EGFR levels in tumor tissues which are gene amplification, increased EGFR mRNA levels and increased EGFR half-life; 6) Because placental tissue
normally possesses high levels of EGFR, it is postulated that some cancer tissues may inappropriately mimic the mechanisms active in the placenta; 7) Evidence is given which supports the notion that the EGFR gene does represent a proto-oncogene; 8) The 5' region of the EGFR gene was been found to be most efficient at inhibiting EGFR gene expression by antisense-RNA.

**Chromosomal Location of C-erb-B/EGFR**

Uncovering the location of the genes for c-erb-B and EGFR was important in providing evidence to support that these genes are identical. Both the v-erb-B gene probe and the EGFR gene probe identified 15 kb and 17 kb bands, by Southern blotting, unique to the p13-q22 region of human chromosome 7. All other evidence strongly supports the idea that the c-erb-B gene and the EGFR gene are one in the same. The comparison of the v-erb-B gene with the EGFR gene gives valuable information as to how a proto-oncogene can be converted into an oncogene. From deleting the EGF binding domain from the receptor, this allows the receptor to send mitogenic signals independent of the polypeptide growth hormone EGF. The disruption of a regulatory region on a proto-oncogene protein product appears to be a general strategy for oncogenic conversion.
EGFR Gene Amplification in A431

The reason for A431's high level of EGFR has been shown to be due to EGFR gene amplification (Merlino et al. 1984; Lin et al. 1984; Ullrich et al. 1984). Part of the 15-30 fold gene amplification in A431 was shown to be associated with the translocation chromosome M4 (Hunts et al. 1985a). The translocation chromosome M4 also correlated with the presence of an abnormal mRNA observed only in A431 cells. This abnormal transcript is thought to be generated by a gene rearrangement, which alters the restriction pattern observed for the hybrid containing M4 and for A431. This rearrangement was detected only by using the unique nucleotide sequence found in the abnormal mRNA as probe. This could be due to the EGFR gene structure or other limitations. Both the EGFR gene and the rearranged gene appear to be co-amplified on M4. This suggests that the rearrangement event occurred prior to the amplification event. The rearrangement may have induced the gene amplification, because gene rearrangement and amplification have been previously observed together. The EGFR gene amplification may have contributed to the tumorigenic state of these A431 cells by giving them a selective growth advantage over other cells. However, the production of a secreted EGFR like protein due to the rearrange-
ment event may have no significant role in the tumorigenic process and no biological affect.

**Increased EGFR in Squamous Cell Carcinomas**

EGFR hyperproduction was observed in 9 out of 15 primary squamous cell carcinomas examined. The large levels of EGFR were present only in the tumor and not in the normal adjacent tissue, suggesting some role of enhanced EGFR levels in human neoplasia. EGFR levels in the tumors were found to be 2-20 fold higher than normal adjacent tissue. The frequency with which EGFR hyperproduction was observed (60%), is less than the 100% observed by Cowley et al. (1984) but higher than that reported by Libermann et al. (1985) for glioblastomas. A possible explanation for this is that Cowley et al. (1984) used a very sensitive immunostaining technique that may score positive for small populations of cells, which represent only a fraction of the tumor. The assay used here may not detect high EGF binding by small subclones. There is a high incidence of increased EGFR levels and squamous cell carcinomas, which indicates that this tissue may have a greater propensity for increasing the EGFR relative to other tumors.
Factors Controlling EGFR Levels

By analyzing several tissues it became apparent that there are mechanisms independent of receptor gene amplification which can increase EGFR levels. Gene amplification is thought to be an abnormal mechanism for increasing EGFR levels because it was only observed in tumor tissue. Increased EGFR mRNA levels were observed in placental tissue relative to fibroblasts. This mechanism, increased transcription or mRNA stability, is thought to be a normal mechanism which allows placental tissue to increase its receptor levels. The mechanism of increased EGFR protein half-life was observed in the transformed cell line UCVA-1, so it is not known whether this may also be a normal way in which cells can increase receptor levels. The two alternative mechanisms of increased receptor transcript and receptor half-life were suggested to account for the discrepancies observed between the cell surface EGFR levels and the respective gene and mRNA levels. These mechanisms may be able to increase the receptor levels to as high a level as that observed when receptor gene amplification is present, and are thus important to consider. Recently, Clark et al. (1986) have obtained evidence which suggests that receptor mRNA stability is increased by exposure of KB cells to a phorbol
ester. A mechanism similar to this may be active in tissues which express high receptor levels without receptor gene amplification.

**Placental Tissue Expresses High EGFR Levels**

Increased EGFR levels were also observed in placental tissue, suggesting that some tissues may normally express high EGFR levels while others, such as tumor tissues, may inappropriately express high EGFR levels. Also, because placental tissue did not contain EGFR gene amplification, this suggests that there are alternative mechanisms that can regulate the level of EGFR on the cell surface, which may be erroneously mimicked by some squamous cell carcinomas. It is not known whether the high receptor levels on UCVA-1 were a characteristic acquired during transformation. However, it is thought that placental tissue may normally have these mechanisms active to maintain high receptor levels independent of gene amplification. Because placental tissue is thought to be a target tissue for EGF (Rao et al. 1985), the high receptor levels may be essential for the proper functioning of this tissue. It has been proposed that these same mechanisms may be inappropriately mimicked by some squamous cell carcinoma tissues during the process of neoplasia (Hunts et al. 1986). One question arising from this work is that
if high levels of EGFR are associated with some cancerous tissues, why is this present in the placenta? In fact, high levels of several proto-oncogene products have been observed in this tissue (Goustin et al. 1985), indicating that placental tissue is exceptional and must have regulatory factors which control these products.

**The EGFR Gene is a Proto-oncogene**

EGFR hyperproduction is not sufficient for transformation and may be important for the normal growth of some tissues, such as placenta. Furthermore, EGFR hyperproduction is not necessary for squamous cell carcinomas, because not all of the 15 primary carcinomas analyzed had high EGFR levels. However, no oncogene by itself has been shown to be sufficient or necessary for any type of cancer (Duesberg 1985). The simplest interpretation of the EGFR hyperproduction in squamous cell carcinoma is that to EGFR over-expressors have a selective growth advantage over EGFR normal-expressors. This would be a secondary step in tumor progression. EGFR hyperproduction may also play a primary role in tumor progression by firing mitogenic signals independent of EGF action. Clearly the EGFR gene has been implicated as a proto-oncogene. The work by Downward et al. 1984 suggests that qualitative changes in the receptor, such as removing the EGF binding domain, can
short circuit the receptor into sending uncontrolled growth signals. Furthermore, the study presented here suggests that quantitative changes in the receptor may also play a role in the tumorigenic transformation of some tissues. It remains to be determined, however, what the exact role EGFR hyperproduction plays in transformation.

**Antisense-RNA Reduction of EGFR**

Anti-EGFR RNA, depending on the efficiency of gene suppression, may prove useful for better understanding what role EGFR hyperproduction plays in the tumorigenic process. Using an integration vector for antisense-RNA production, it appears that the 5' end of the EGFR gene is most efficient at EGFR inhibition. The EGFR levels were reduced in NA, an EGFR hyperproducing cell line, up to 50%. To make any conclusions, however, a greater reduction in the EGFR levels must be achieved. Therefore, this technique awaits further modification before any conclusions can be made regarding the function of EGFR hyperproduction in tumor cells.
REFERENCES CITED


Avery, O.T., C.M. MacLeod, M. McCarty. 1944. Studies on the chemical nature of the substance inducing trans­ formation of pneumococcal types. J. Exp. Med. 79:137-57.


