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GROWTH FACTOR- AND ONCOGENE-INDUCED TRANSFORMATION IN CHICKEN EMBRYO FIBROBLASTS AND NORMAL DIPLOID HUMAN FIBROBLASTS

by

MICHAEL RICHARD ANTCZAK

submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

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MAY 1993

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Michael R. Antczak

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degree.*

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GROWTH FACTOR- AND ONCOGENE-INDUCED TRANSFORMATION IN CHICKEN EMBRYO FIBROBLASTS AND NORMAL DIPLOID HUMAN FIBROBLASTS

Abstract

by

MICHAEL RICHARD ANTCZAK

Cotransfection of *neo* with various oncogenes resulted in CEF transformation in vitro and, in several instances, sarcoma formation in vivo. Transfection of a family of v-*src*, c-*src* and v/c-*src* chimeric constructs demonstrated the ability of the assay to discriminate between transforming and nontransforming genes. Transfection of a number of *erbB* variants showed that internal mutations, primarily in the kinase domain, contribute significantly to this oncogene's fibroblast transforming abilities. The tumorigenic potentials detected by direct oncogene transfection faithfully reproduced the findings of similar studies using infectious, oncogenic retroviruses. Our studies establish the utility of CEF transformation by direct DNA transfection.

Deregulated expression of the TGFα and EGF-R genes has been implicated in the development of a range of mammalian malignancies, most notably those of human origin. While introduction of the TGFα/EGF genes into immortalized rodent cell

lines has frequently resulted in cellular transformation, attempts to generate similar results following simple, exogeneous TGFα/EGF treatment have proven largely unsuccessful. The potential role(s) played by these growth factors in the transformation of normal cells, derived from primary cultures has, heretofore, not been closely examined. In chapter 3 it is reported that both normal chicken embryo fibroblasts (CEF) and normal, diploid, human foreskin fibroblasts (HFF) can be efficiently transformed, in an apparent single-step fashion, following exogeneous TGFα/EGF treatment. CEF cells infected with a retrovirus carrying the TGFα gene generated unusually large, aggressively growing soft agar colonies. The ligand-induced transformation of CEF and HFF is affected by culture age. Cultures consistently responded less efficiently as they underwent increasing numbers of population doublings, yet there was no detectable, concomitant, diminution in the number of receptors per cell. This is the first report of efficient transformation of normal cells derived from primary cultures by TGFα and EGF, without the need for other complementing growth factors or oncogenes.

DEDICATION

To my parents, for their unending support and sacrifice.

TABLE OF CONTENTS

CHAPTER 1

GROWTH FACTORS AND ONCOGENES

i) INTRODUCTION

As the oncogene and growth factor fields continue to mature, the boundaries between them grow progressively less well defined. There are now several examples of considerable overlap between the two groups, in form as well as function. In some instances, oncogenes elicit early events in growth factor signalling pathways by encoding molecules whose structures and functions closely parallel those of normal growth factors, as in the case of *v-sis*, a mutant form of PDGF (platelet-derived growth factor). In other instances, oncogenes encode altered forms of growth factor receptor molecules which function constitutively and independently of ligand stimulation, as in the case of *v-erbB*, a truncated form of the EGF-R (epidermal growth factor receptor). There are oncogenes whose normal counterparts are believed to play important roles in signal processing, receiving signals from activated receptors and converting them into suitable intracellular effector molecules, as in the case of *v-ras*. Finally, several oncogenes are believed to encode altered forms of nuclear transcription factors, such as *v-fos* and *v-jun*. In their unaltered, normal forms these genes likely represent the most distal members of signalling pathways. They are perhaps the ultimate effector molecules, physically

interacting with genetic control regions to adjust transcriptional programming in response to external stimuli.

Studies of the various classes of oncogenes have increased our understanding and appreciation of the balance and complexity of normal growth factor signaling mechanisms. In reviewing some of the major classes of oncogenes, I will first address the growth factors and growth factor-related oncogenes.

ii) GROWTH FACTORS AND GROWTH FACTOR-RELATED ONCOGENES

Growth factors were first identified approximately 40 years ago following the observation that mouse sarcoma cells released a factor, nerve growth factor (NGF), which promoted the growth of chick embryo nerve cells (85). Since that time the ranks of growth factors have increased considerably; a listing of the more commonly studied growth factors is given in Table #1. Of all the growth factor systems studied to date, the epidermal growth factor (EGF) and its receptor have been studied most extensively. EGF was first discovered in 1962 as a factor which promoted early eyelid opening and incisor eruption in newborn mice (21). In 1978 a functionally related growth factor, transforming growth factor alpha (TGFα), was found to be present in conditioned medium from murine sarcoma virus-transformed cells (28). Since that time there have been many

Table 1. Commonly studied growth factors. (from R. A. Weinberg (146))

Acronym(s)	Name	Sources	Typical targets
EGF	epidermal GF	embryos submaxillary gland	epithelia
TGFα	tumor GF-α	embryos oncogene-transformed cells	epithelia
PDGF-A, -B	platelet-derived GF	platelets, tumor cells	mesenchymal cells
aFGF	acidic fibroblast GF	brain, pituitary	mesenchymal cells
bFGF	basic fibroblast GF	brain, pituitary	mesenchymal cells
TGF-β 1,2,3	tumor GF-β	oncogene-transformed cells	wide spectrum
GM-CSF(CSF-2) (CSF-α)	granulocyte-macrophage colony-stimulating factor	T lymphocytes fibroblasts	mixed granulocyte - macrophage colonies
M-CSF (CSF-1)	macrophage colony-stimulating factor	fibroblasts	macrophage
G-CSF (CSF-β)	granulocyte colony-stimulating factor	monocytes	granulocytes
Multi-CSF (IL-3)	multipotential GF	T lymphocytes	multipotential progenitors
IGF-I,-II	insulin-like GF	extraembryonic membranes, fetal serum	mesenchymal, epithelial cells
NGF	nerve GF	submaxillary gland	neuronal cells
Il-1	interleukin-1	leukemia cells	B,T lymphocytes neutrophils
EPO	erythropoietin	kidney cells	erythroid precursors

reports concerning the secretion of TGFα from tumor cells and cells transformed by various oncogenes, including *ras*, *src*, *mos* and SV40 large T antigen (146). Interestingly, despite their fairly unique compositions (only about 33% amino acid homology in the mouse (30, 92)), EGF and TGFα are structurally quite similar and are believed to be ligands for the same receptor, the epidermal growth factor receptor. In an effort to explain the existence of two independent ligands for the same cellular receptors in vivo, it has been suggested that TGFα may play an autocrine or paracrine role, promoting cellular proliferation in the vicinity of secreting cells. EGF, on the other hand, may not be particularly well suited for the induction of large-scale cell growth and may instead function more like a hormone (92). This view is consistent with the known function of TGFα in wound healing and embryogenesis, but whether or not EGF truly functions in an endocrine fashion is still unclear.

A second growth factor, transforming growth factor beta (TGFβ), was also found in the medium of virally-transformed mouse cells. TGFβ is structurally unrelated to TGFα. It exists in at least three distinct forms in mammals and interacts with a unique set of cellular receptors (146). The role(s) of TGFβ has been, perhaps, the most perplexing of all the growth factors so far identified. TGFβ has been found to stimulate cell growth in some instances while inhibiting growth in others. For example, the growth of subconfluent NRK (normal rat kidney) cells is

inhibited by TGFβ (114) but confluent NRK are growth-stimulated (92). In another example, TGFβ acts synergistically with TGFα in inducing the transformation of immortalized NRK cells, which are not transformed by TGFα treatment alone (143). However, in normal chicken and human fibroblasts, that is, cells derived from primary cultures, TGFα treatment alone induces transformation and the addition of TGFβ has little or no effect on TGFα-induced transformation (this thesis). The varied effects of TGFβ treatment have led some to suggest that TGFβ might not function directly in cellular proliferation; rather, its role may be to modulate the activity of other growth factors (64). TGFβ is produced by a wide range of tissues and cell cultures, both normal and transformed (31, 38). *In vivo* it is secreted in an inactive form and complexes with serum proteins in the blood. The TGFβ binding factor is α_2-macroglobulin (64). TGFβ complexes are fairly abundant in platelets where they are believed to function in wound healing, perhaps by stimulating angiogenesis (87).

Growth factor mimicry by oncogenic proteins is illustrated by the oncogenes sis (PDGF), hst (FGF) and int-2 (FGF). PDGF exists in three distinct forms, either as an A- or B-chain homodimer or as an A/B heterodimer. The *sis* protein is a B-chain homodimer. PDGF is likely to function in an autocrine or paracrine fashion since it is rapidly cleared from the blood by complex formation with a $t_{1/2}$ of less than 2 mins (13). Some

PDGF complexes, for example those with α_2-macroglobulin, inhibit its activity (13, 66, 109), while others (for example, those with cellular matrix components (acidic glycoaminoglycans)) may serve to heighten local PDGF concentrations or preserve its mitogenic potential (115). PDGF induces a chemotactic response in fibroblasts, smooth muscle cells (54,122), monocytes and neutrophils (32), although in the case of the latter two it is not mitogenic but instead causes the secretion of their granular components (135, 153). In this way, platelet PDGF may serve to recruit cells to the site of an injury where it and other factors can direct the healing process. PDGF may also act as an indirect mitogen, causing the release of factors from responsive cells which are then mitogenic to nonresponsive cells. An example of this is can be seen in *in vitro* erythropoiesis. Bone marrow cells, which themselves do not respond to PDGF, are stimulated to grow in the presence of mesenchymal cells treated with the factor (24, 29). Interestingly, treatment of cells with other growth factors frequently stimulates secretion of PDGF-like molecules (126). PDGF induces some of the earliest events in the movement of cells from the G_0 to the *S* phase of the cell cycle. Minutes after exposure, cells turn on a series of genes, termed *immediate early genes*, whose ranks include the actin, *fos* and *jun* genes. That some of these *immediate early genes* are critical for cell division has been demonstrated in the case of *fos*, where antisense RNA or *fos* antobodies were shown to prevent

emergence of cells from G_0 (104, 112).

FGF actually represents a family of related factors currently designated 1 through 5. The *int-2* gene and the *hst* gene are FGF-3 and FGF-4, respectively. FGF and PDGF are termed *competence factors*. They are believed both to promote the emergence of cells from quiescence, G_0, and to prepare the cells to respond to a second series of factors termed *progression factors*. EGF and insulin-like growth factor-I, IGF-I, are both considered *progression factors*. Despite their name, FGFs affect the growth and function of a wide range of cell types, including mesenchymal cells, endocrine cells and neural cells. The first two members of the FGF family, FGF-1 and FGF-2, correspond to acidic and basic FGF, respectively. Normally, the basic form of FGF is more potent than the acidic (37) but when the two molecules interact with heparin the potency of the acidic form is increased and both forms display nearly equivalent activity (4). FGF-1 and -2 are potent mitogens for cells found in vascular walls, such as smooth muscle and endothelial cells (37). Additionally, in endothelial cells, FGFs also function as chemotactic agents. These properties suggest a seminal role for FGFs in angiogenesis. In short, FGFs play essential roles in the maintenance, function, performance and development of a wide range of cell types and tissues, a repertoire unmatched by most other growth factors.

Growth factors then are the shapers and formers, they

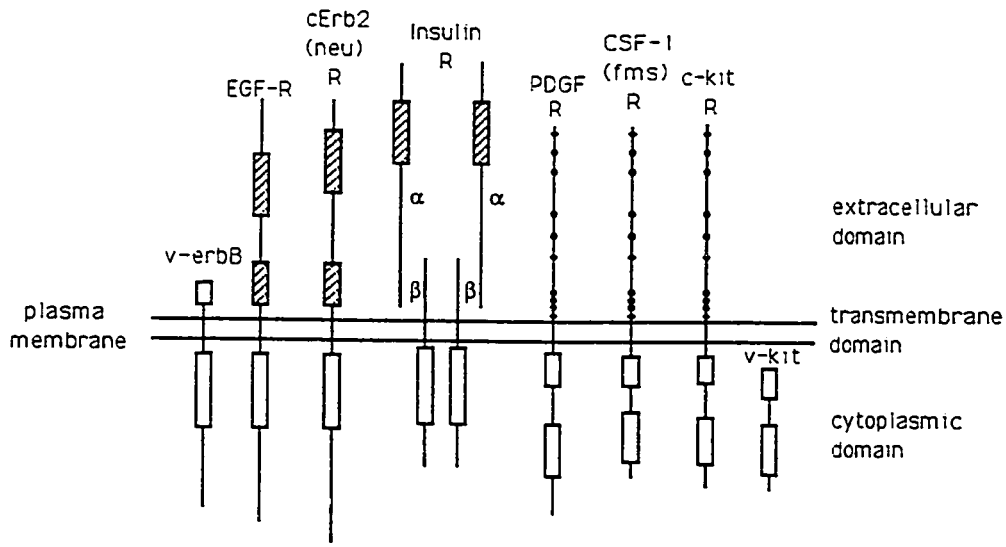
provide identity and purpose, they are the coordinators and caretakers of a sea of otherwise lost and helpless cells. That persistent signalling by growth factor-like oncogenes or oncogenic forms of their receptors can result in cellular transformation and tumorigenesis is a testament to the importance of the delicate balances and critical interrelationships which must be maintained among the various members of the group. A loss of that balance, a loss of moderation, can trigger some very serious trouble indeed.

iii) RECEPTOR PROTEIN-TYROSINE KINASE ONCOGENES

The protein-tyrosine kinase class of oncogenes consists of two major subclasses, those derived from receptor-like molecules and those from nonreceptors. Only the receptor tyrosine kinases and their oncogenic counterparts will be addressed here, with particular emphasis on the the EGF-R and the *erbB* oncogene. The receptor protein-tyrosine kinase oncogenes include *erbB*, *ros*, *erbB2/neu*, *fms*, *met*, *trk*, *kit*, *sea*, and *ret*. Corresponding ligands have been identified in only a few cases. EGF/TGFα, for the *erbB* gene, CSF-1 (colony stimulating factor-1) for the *fms* gene, and SCF (stem cell factor) for the *kit* gene. Schematic representations of the normal and oncogenic forms of several of these receptors are shown in Fig. 1.

Figure 1. Schematic representation of normal and oncogenic forms of several protein tyrosine kinase receptors.

Hatched areas or closed circles in extracellular domains indicate locations of cysteine residues. Open boxes in cytoplasmic domain represent conserved protein tyrosine kinase core sequences. Note insertion in this area in PDGF, CSF-1, and *c-kit* receeptors. The C' terminus of *v-erbB* from AEV-H is truncated by 32 amino acids compared to the EGF receptor, the C' terminus of the *v-fms* oncogene is truncated by 40 amino acids compared to the CSF-1 receptor, and the C' terminus of *v-kit* is truncated by 49 amino acids compared to *c-kit*. (Taken from Gill (48).)


cErb2
(neu)
R
Insulin
R
CSF-1
(fms)
R
EGF-R
PDGF
R
c-kit
R
α
α
extracellular
domain
v-erbB
β
β
plasma
membrane
transmembrane
domain
v-kit
cytoplasmic
domain

The EGF receptor is a molecule of approximately 170 kd which contains a glycosylated, external binding domain of 621 amino acid , a single membrane spanning region of 23 amino acids and a 542 amino acid cytoplasmic domain which is responsible for its intrinsic tyrosine kinase activity (16, 47, 93, 136). The EGF-R binds its ligand, EGF, with high affinity (Kd = 10^{-10} M) in a 1:1 stoichiometric relationship, suggesting the existence of only a single external binding site (144, 151). From the plasma membrane to its carboxy terminus, the cytoplasmic portion of the molecule contains a kinase domain, a region highly conserved among members of the protein-tyrosine kinase family, and a regulatory domain, a region generally quite divergent (136, 69). Within the kinase domain are sites for binding ATP and protein substrates. The ATP-binding site is well conserved among the receptor protein-tyrosine kinases and is located 50 amino acids from the inner aspect of the plasma membrane (48). Substitution of a critical lysine residue within the ATP-binding site, believed to form a salt bridge with ATP, by an uncharged amino acid abolishes ligand-induced tyrosine kinase activity and other biological consequences of EGF-R/ligand interactions (19, 60). The receptor/ligand interactions themselves, however, appear unaffected. The cytoplasmic regulatory domain contains several major tyrosine autophosphorylation sites. Phosphorylation at these sites is believed to maximize receptor performance by improving access

to the substrate binding regions within the kinase domain (65).

The *erbB* oncogene was identified as the transforming component of two naturally occurring retroviral isolates, avian erythroblastosis virus (AEV) strains H and R (42, 155). Strain AEV-R contains both the *erbB* and *erbA* oncogenes. The *erbA* oncogene, a mutated form of the thyroid hormone receptor (118, 145), is believed to increase the potency of the *erbB* gene and block the differentiation of erythroid cells (45, 52, 74, 106, 155). The oncogenic form of the *erbB* gene has been shown to result from viral insertional mutagenesis into the chicken EGF-R gene (*c-erbB*). The consequence of this viral integration event is the expression of an amino-truncated protein lacking the major portion of its extracellular domain; most importantly its ligand binding site (44, 103). Apparently, loss of the ligand binding domain eliminates normal constraints on receptor activity and results in an *erbB* protein which is constitutively active. This is felt to be the basis for the transforming potential of the *erbB* oncogene and similarly constructed experimental forms of the EGF-R (50, 81, 147).

But why or how does a mutant, constitutively active receptor affect cellular transformation or tumorigenesis, or for that matter by what mechanism(s) does a normal receptor affect cell growth? The answers to these questions depend on the consequences of receptor activation, one of which is tyrosine phosphorylation. Protein phosphorylation at serine or threonine

residues is a fairly common event and is frequently used to modulate protein function. Approximately 10% of cellular proteins are modified in this fashion (70). Protein phosphorylation at tyrosine residues, though, occurs much less frequently and represents only about 0.03% of total cell protein phophoamino acids (69). Interestingly, many of the molecules found to possess protein-tyrosine kinase activity are cell surface growth factor receptors or their oncogenic derivatives. These findings suggested a potentially important role for tyrosine kinases in cellular growth control. Recently, considerable efforts have been launched to identify important substrates of the tyrosine kinases. Among the potential candidates are molecules shown to associate with and become phosphorylated by activated members of the receptor tyrosine kinases, namely, the GTPase activating protein (GAP), phospholipase C-γ (PLC-γ), phosphatydlinositol 3-kinase (PI-3-kinase), and the normal, 74 kd, *raf* protein (PDGF receptor references (97, 77, 141, 78, 82, 98, 99, 100); EGF-R references (9, 20, 35, 91)). It is important to note that normal and oncogenic forms of the nonreceptor tyrosine kinases are also known to associate with these enzymes, *c-fyn* , *v-src* , *v-fps* , *v-abl* , sometimes as part of a complex, *c-src* / and *c-yes* /middle t (15, 35, 43, 55, 58, 98, 107, 129, 138, 149).

A discussion of the GAP and *raf* proteins will take place in upcoming sections on oncogenes which appear to function as

membrane-associated G proteins and cytoplasmic protein-serine/threonine kinases. For now, I will address phospholipase C-γ and PI-3-kinase and their interactions with the EGF-R.

Phospholipase C (PLC) is a cytoplasmic enzyme of approximately 145 kd which plays a strategically important role in phosphatidylinositol (PI) metabolism. PLC catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdInsP$_2$) into inositol-1,4,5-trisphosphate (InsP$_3$) and diacylglycerol (DG). InsP$_3$ and DG are known as *second messenger* molecules. They are believed responsible for the liberation of intracellular Ca^{2+} and the activation of protein kinase C (PKC), respectively (8, 88, 105). There are three isozymes of phospholipase C - β, γ and δ. Of the EGF-R mediated tyrosine phosphorylation of PLC, approximately 95% occurs on PLC-γ, 5% on PLC-β and none is detected on PLC-δ (18). These results suggest that PLC-γ is the isozyme which associates with the activated EGF-R. There is a growing body of evidence which strongly supports the hypothesis that PLC-γ is activated following EGF-R-mediated tyrosine phosphorylation and is an important element in the EGF-R signalling pathway which leads to cell proliferation. Some of the supporting evidence is as follows: a) EGF treatment induces tyrosine phosphorylation of PLC-γ in cells; b) the EGF-R is coprecipitated with PLC-γ antibodies and PLC-γ with EGF-R antibodies; c) a tyrosine kinase inhibitor, tyrphostin, blocks EGF-induced PLC-γ phosphorylation,

the association of PLC-γ with the EGF-R and prevents EGF-induced Ca^{2+} release and DNA synthesis and d) removal of phosphate from serine residues of PLC-γ has no effect on activity but removal of phosphate from tyrosine residues results in the loss of activity (18, 91). By associating with the activated EGF-R, and other receptor and nonreceptor tyrosine kinases, PLC-γ, through its phospholipid hydrolysis, is believed to amplify and convert the original activating signal into a series of components, $InsP_3$, DG, $InsP_4$, which then elicit a series of more distant events further down the signalling pathway. One of these components, DG, triggers a regulatory loop of the EGF-R, through its ability to activate PKC. Activated PKC, a serine/threonine kinase, phosphorylates the EGF-R, primarily at thr-654 just inside the plasma membrane, suppressing its activity (148). Finally, it is important to note that EGF-induced phosphorylation of PLC-γ is physiologically relevant. It can be detected at levels as low as 1nM EGF and reaches maximal levels within 30 secs (91).

While the actual function of PI-3-kinase is not yet known, there is some suggestion that polyphosphoinositides phosphorylated at position 3 may play a role in the regulation of actin filament rearrangement. Perhaps the primary function of PI-3-kinase is to generate an additional class of *second messenger*-like molecules to influence events further down various signalling pathways (9). PI-3-kinase is a type I PI

kinase which phosphorylates the inositol ring at position 3 as opposed to the type II PI kinase, PI-4-kinase, which phosphorylates position 4 (3, 17, 150). Many phospholipids containing a phosphate at position 3 are resistant to the actions of the known PLC isozymes (51, 86, 123). PI-3-kinase came into the limelight when it was shown that it closely correlated with middle t transformation and that middle t mutants which could no longer associate with PI-3-kinase were also no longer transforming (23, 75, 76, 149). PI-3-kinase is a heterodimer of an 85 and 110 kd protein (17). The 85 kd subunit is believed to be responsible for association with the tyrosine kinases and middle t (17, 22, 51, 76), while the 110 kd subunit is believed to contain the catalytic domain. While phosphorylation of the 85 kd subunit itself is not thought to be essential for association, phosphorylation of the tyrosine kinase or middle t protein is required for close interaction with PI-3-kinase (51). In this regard, EGF-R autophosphorylation is required for tight association with PI-3-kinase (51). It is important to note that PI-3-kinases appear to have no activity on their own and seem to require interaction with tyrosine kinases or tyrosine kinase complexes to function (51). Finally, EGF-induced PI-3-kinase activity appears to peak 1-2 hrs after treatment and can be detected at an EGF dose as low as 10 pM, a level well within the range of phsiological relevance (9).

iv) G PROTEIN ONCOGENES - THE *ras* GENE.

G proteins or gaunine nucleotide regulatory proteins, are believed to transfer, and potentially amplify, signals from a wide variety of membrane receptors to internal mechanisms important in the generation and regulation of *second messenger* molecules or internal hormones (128). G proteins are made up of three unique subunits designated α, β and γ. The α subunit binds and hydolyzes GTP and the various families of α subunits determine the specificity of interaction with both receptor and effector molecules. The β and γ subunits function in membrane association and proper positioning of the α subunit for receptor association (12). The G protein is activated by binding GTP and, when that occurs, the α subunit separates from the $\beta\gamma$ complex. The α subunit, with its GTP molecule bound, is the form which influences the activity of the effector molecule. When the intrinsic GTPase of the α chain hydrolyzes GTP it loses its activitating potential and the α subunit with the bound GDP molecule returns to the $\beta\gamma$ complex. The cycle is complete when the receptor is activated once again and mediates the dissociation of GDP from the α subunit allowing another GTP molecule to bind (12).

Three closely related forms of the 21 kd *ras* protein are known to exist in mammalian cells, *H-ras*, *K-ras* and *N-ras* (36, 57, 124). Like the G proteins, normal and oncogenic forms of the

ras proteins bind GDP and GTP and also contain intrinsic GTPase activity. G proteins and *ras* proteins are found in similar locations within the cell, along the inner aspects of the plasma membrane. These observations led to the suggestion that the *ras* proteins might function like G proteins (121). Unlike the G proteins, however, where GTP hydrolysis is believed to occur independent of α subunit interaction with other proteins, GTP hydrolysis by *ras* is directly stimulated by a cytoplasmic protein (131). In comparison to G proteins, the GTPase activity of normal *ras* proteins is low and oncogenic forms are even lower (46, 90, 95, 130). The low GTPase activity of some mutant *ras* proteins, and therefore the increased half-life of the active *ras* -GTP complex, was found to be correlated with their ability to transform cells. Other mutant *ras* proteins were found to have an increased half-life of the active *ras* -GTP complex by virtue of their decreased affinity for guanosine nucleotides. Since cellular GTP is much more abundant than GDP, GDP is quickly replaced by GTP in these mutants, thereby prolonging the active state of the *ras* protein (39, 125, 142). However, when some transforming GTPase mutants were found to have GTPase activity only two fold lower than normal in an in vitro assay, a search was begun for a more encompassing explanation of *ras* / mutant *ras* function. These studies, done with living cells, led to the identification of the GTPase-activating protein or GAP. The GAP protein was found to greatly stimulate the

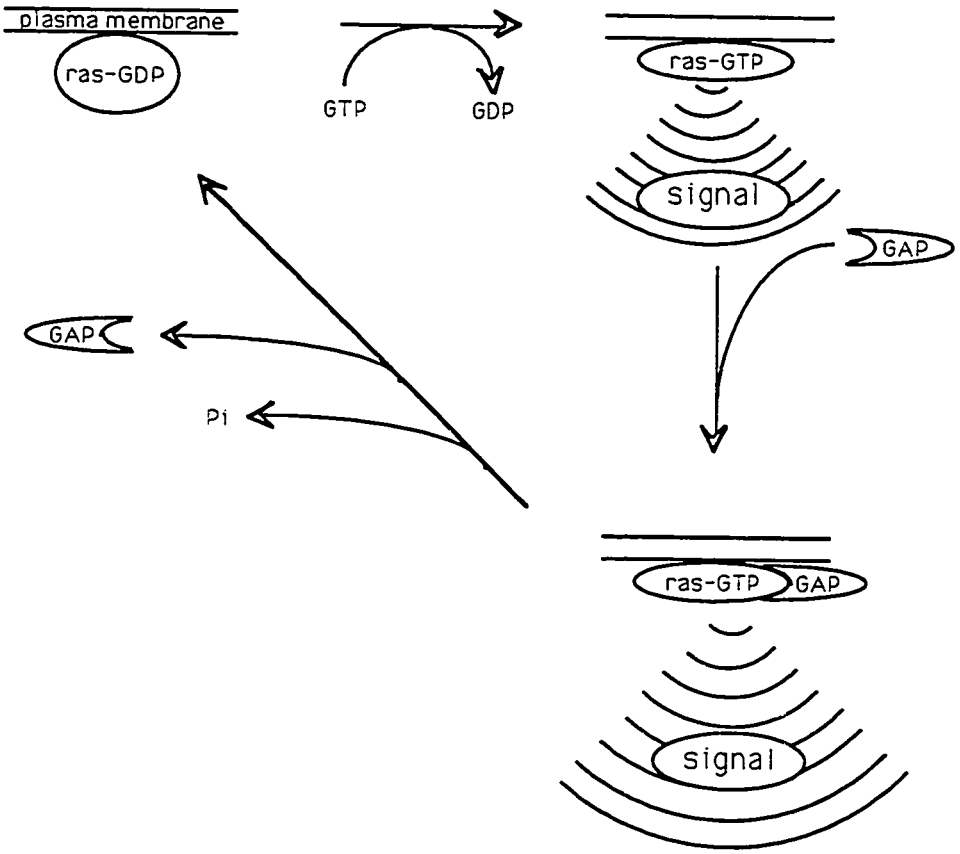
activity of normal *ras* GTPase but had little or no effect on the activity of mutant *ras* proteins (130). This led to adjustments in models of *ras* function. In one model, shown in Fig. 2, GAP which is normally free in the cytoplasm can associate with membrane associated *ras* -GTP complexes forming *ras* -GTP-GAP complexes. Since GAP only associates with active, GTP bound, *ras* molecules, the lifespan of the *ras* -GTP-GAP complex is dependent upon how rapidly GAP effects the hydrolysis of GTP by stimulating the GTPase activity of *ras* . For as long as it is maintained, the *ras* -GTP-GAP complex sends a mitogenic signal to the cell, through the activity of *ras* -GTP. Once GTP hydrolysis has occurred, GAP dissociates and mitogenic signalling by the *ras* protein ceases. In the case of mutant, transforming *ras* proteins, GAP does not complex with the mutant *ras* -GTP complex, the GTPase of the mutant *ras* is not stimulated and mitogenic signals are sustained for extended periods of time (94).

The *ras* GAP molecule has been shown to be tyrosine-phosphorylated in oncogene-transformed cells (35), as well as in cells stimulated by PDGF, EGF and CSF-1 (35, 77, 78, 97, 111) but there is yet no evidence that this modification affects its activity.

In some systems a correlation appears to exists between the activities of PKC and *ras* . In phorbol ester- or T antigen-

Figure 2. Control of p21*ras* activity in normal cells.

The *ras* protein bound to GDP represents an inactive form of the molecule. As a consequence of an incoming signal, i.e. a ligand/receptor interaction, GDP is displaced by GTP and the p21*ras* protein is activated. The activated, GTP-bound *ras* protein then becomes a target for GAP binding. The p21-GTP-GAP complex may represent the most important form of p21*ras* in terms of transmission of the mitogenic signal further down the signalling pathway. During its association with p21-GTP, GAP is believed to stimulate the conversion of p21-GTP to p21-GDP by enhancing the intrinsic GTPase of p21*ras*. Following hydrolysis of GTP to GDP, the GAP protein dissociates from the complex, returns to its free, cytoplasmic form, and mitogenic signalling by the ras protein is discontinued (Adapted from (94)).


plasma membrane
ras-GDP
GTP
GDP
ras-GTP
signal
GAP
GAP
Pi
ras-GTP
GAP
signal

stimulated cells, *ras* GAP is less able to stimulate the GTPase activity of *ras*, suggesting PKC may influence *ras* activity by affecting the ability of GAP to associate with the active *ras* - GTP complex (33).

A number of physiological responses to *ras* activation have been reported, including membrane ruffling (6), increased DG levels (40), induction of *c-fos* mRNA (127), and increased activity of phopholipase A_2 (6, 7), S6 kinase and $p34^{cdc2}$ serine kinase (5). Studies of low molecular weight GTP-binding proteins structurally related to *ras* have led to a very interesting, though rather speculative, model of *ras* function. This model suggests that *ras* proteins might control the assembly of a class of cell surface receptors, at specific sites on the plasma membrane, by interacting with their effectors (15). In normal cells, for example, receptor activation would in turn activate *ras* proteins, which would then trigger actin filament rearrangement resulting in receptor clustering and, ultimately, internalization. In transformed cells, constitutively active, mutant *ras* proteins would trigger receptor clustering and, since many receptor tyrosine kinases are activated by oligimerization and cross-phosphorylation (137, 156), they would indirectly trigger receptor activation in the abscence of ligand (15).

v) SERINE/THREONINE KINASES - REGULATORY PROTEINS AND ONCOGENES

Most protein phosphorylation occurs at serine (~90%) and threonine residues (~9%); as previously stated, phosphotyrosine is a relatively minor species (~0.1%) (69). The serine kinase family of regulatory proteins includes a diverse assortment of molecules, many of which are listed in table 2. While it appears as though serine and threonine phosphorylation is utilized primarily to regulate metabolic activity, at least two members of this kinase family, protein kinase C (PKC) and *raf*, are believed to play important roles in signal transduction, although the exact mechanisms involved remain unclear. PKC is activated following the generation of DAG which is formed as a result of mitogen-stimulated phospholipase C activity. Since virtually all mitogen stimulation results in membrane turnover, increased phospholipase C activity, and DAG production, a central role for PKC in early signal transduction is all but a certainty. Overexpression of PKC has been shown to result in increased cell growth potential (62, 63) and mutated forms of the enzyme have been shown to be transforming (96).

The *raf* oncogene represents an altered form of a protein-serine/threonine kinase which normally associates with activated, phosphorylated PDGF and EGF receptors. PDGF-associated *raf* proteins were found to be phosphorylated at

Table 2. Some cellular serine kinases (71).

Protein serine kinases.

cyclic nucleotide regulated
- cAMP-dependent protein kinase (a and b forms)
- cGMP-dependent protein kinase

Ca^{++}/calmodulin regulated
- myosin light chain kinase (skeletal and smooth muscle forms)
- calmodulin-dependent protein kinase I
- calmodulin-dependent protein kinase II (a and b forms)
- calmodulin-dependent protein kinase III
- phosphorylase kinase c subunit
- PSK-C3

diacylglycerol regulated
- protein kinase C (a, bI, bII, c, d, e, and z forms)

raf family
- c-*raf* protein
- A-*raf* protein
- B-*raf* protein

others
- casein kinase I and II
- proteinase-activated kinase I
- glycogen synthetase kinase 3 and 4
- double-stranded RNA- and double-stranded DNA-regulated kinases
- heme-regulated protein kinase
- S6 protein kinases
- b-adrenergic receptor and rhodopsin protein kinases
- MAP-2 protein kinases
- HMGCoA reductase protein kinase
- pyruvate dehydrogenase protein kinase
- c-*mos* protein
- *pim* -1 protein
- CDC2Hs protein (catalytic subunit of maturation promoting factor (MPF) and histone H1 growth-associated protein kinase)
- PSK-J3

serine, threonine and tyrosine residues but increased *raf* serine-kinase activity correlated best with tyrosine phosphorylation (99). The *raf* protein appears to move from the cytoplasm to the nucleus upon activation (72). Once in the nucleus, the raf protein would be stategically positioned for a critical role in transcriptional regulation; in that regard it has recently been shown to activate the c-*fos* (the normal, unaltered *fos* gene) gene (73).

vi) NUCLEAR ONCOGENES

A fairly complete listing of the oncogenic transcription factors is shown in Table 3. Three members of this group will be addressed here, namely, *myc* , *fos* and *jun* .

Of all the known oncogenes, *myc* , has been implicated in the widest variety of neoplasms across the broadest range of species. Normally, *myc* gene expression is induced shortly after mitogen stimulation, generally within 1-3 hrs. *Myc* expression can be detected in nearly all proliferating cells from the embryo to the adult. In many cases *myc* gene activity is a function of the relative rate of cell proliferation and there is generally an inverse relationship between the degree of cell differentiation and the level of *myc* expression. Though insufficient on its own, *myc* expression has been shown to be required for cell division and entry of cells into S phase. In this regard, treatment of cells

Table 3. Oncogenic transcription factors.

The oncogenes that encode bona fide or putative transcription factors have been listed according to the sequence motif that is found in their DNA binding domains. *fos*, *jun*, *myc*, *myb*, *rel*, *ets*, *erbA*, and *ski* are all retroviral oncogenes. *evi-1*, *myc*, *spi-1*, *Hox-2.4*, and *myb* have all been activated by retroviral insertion. N-*myc* and L-*myc* were found as *myc*-related genes amplified in human tumors. *gli* was found as an amplified gene in glioblastomas. *vav* was isolated as a "dominant" oncogene by transfection on NIH 3T3 cells. *myc*, *lyl-1*, *tal*, *scl*, *prl*, and *myl/RARα* were found at breakpoints in chromosomal translocations (not all of these have been formally been shown to be oncogenes) (taken from 72).

Oncogenic Transcription Factors

A) Leucine zipper/basic DNA binding domain

fos
jun

B) Helix-loop helix

myc
N-myc
L-myc
lyl -1
tal
scl

C) Zinc fingers

myl/RARa
erbA (dominant negative)
evi-1
vav
gli-1

D) Homeobox

pbx
Hox-2.4

E) Others

myb
rel (dominant negative)
ets-1
ets-2
spi-1 (ets-related = PU1)
ski

with antisense *myc* RNA was shown to block proliferation in both human HL60 cells (151) and, when introduced prior to growth factor stimulation, in human T lymphocytes (59). That the *myc* protein is a powerful, strategically important, and potentially dangerous commodity within the cell is strongly suggested by the unusually extensive network of regulatory sites located within the 5' untranslated region of the gene. These sites affect transcriptional initiation, message elongation, mRNA processing and stability, translational initiation and protein stability. In most cases the inadvertent loss of one or more of these regulatory mechanisms, the loss of carefully regulated expression, is the basis for *myc* - induced neoplasia.

Like those of most other nuclear oncogenes, *myc* proteins are believed to bind DNA, though the relevance and specificity of these interactions remains elusive. *myc* has been identified as having a helix loop helix structure (26, 79), and a leucine zipper (25), features known to be important for DNA association and protein-protein interaction, respectively. Finally, while the previous discussion has centered on c-*myc* and its oncogenic derivatives there are at least two other members of the myc gene family, L-*myc* and N-*myc*. L-myc and N-myc appear to be important in early cell lineage and embryological development and in most cases their functions are replaced by c-myc at later times in development (27, 158).

The *fos* and *jun* genes were first identified as the causative

elements in virally induced neoplasias in mice and in chickens. Both *fos* and *jun* are members of the *immediate early* class of genes. Expression of these genes is rapidly induced within minutes of growth factor or serum stimulation, even in the presence of agents which block protein synthesis (41, 53).

Following serum stimulation, the *fos* gene reaches peak levels of expression after 15 mins and returns to normal basal levels within 60 mins. Normally, the *fos* protein is autorepressive, following c-terminal serine phosphoesterification (113), but in the presence of protein synthesis inhibitors such as anisomycin and cycloheximide, elevated levels of *fos* expression can be maintained for prolonged periods of time (41). The *fos* gene contains a series of unique regions which are believed to play important roles in modulating its expression in response to various factors. One of these regions, which lies approximately 300 bases upstream from the start site of the *fos* gene, has been found to be important in responding to serum growth factors, termed the serum response element (SRE) (49, 132, 133). More recently, it has been shown that only a small portion of the SRE is required to mediate the serum response; this region is referred to as the dyad symmetry element, DSE. The DSE is the site for specific interaction with the serum response factor (SRF), the protein responsible for serum-induced *fos* expression (134). The *fos* gene is a member of a series of related proteins including *FosB*,

Fra1, and *Fra2* (80,140).

If not identical to AP-1, the *jun* gene encodes a protein very closely related to it (1, 10). AP-1, or activator protein-1, was initially identified as a transcription factor which bound specific sequences within the control regions of the SV40 enhancer and human metallothionein IIA genes (10). The carboxy terminus of the *jun* protein is fairly closely related to the yeast transcription factor GCN4 (89, 139), which plays an important regulatory role in amino acid synthesis (2, 61). *jun* /AP-1 are induced following phorbol ester treatment of cells (1, 84). This finding has provided some potentially important mechanistic information on the function of *jun* /AP-1. For example, phorbol ester activation can occur in the absence of protein synthesis, which means that existing molecules of *jun* /AP-1 must be modified or in some way affected to mediate the appropriate response. It is now known that treatment with phorbol esters, mitogens, etc., triggers the loss of inhibitory phosphate groups from *jun* 's carboxy terminus (14) and phosphorylation of activating sites near its amino terminus, at amino acids 62 and 72, a process recently attributed to the activity of MAP kinases (mitogen-activated protein-serine) (108). A potential role in this modification/activation process for the phorbol ester receptor, protein kinase C, is an intriquing possibility, though it is not at all clear whether this role would likely be excitatory or inhibitory. In addition to *c-jun* two additional members of the

gene family have been identified, *junB* and *junD* (102, 116). All members of the family are rapidly induced upon mitogen stimulation/phorbol ester treatment, form homodimers with themselves or heterodimers with each other, and bind AP-1 sites (83, 102, 116, 117).

Fairly recently, anti-*fos* antibody analysis led to the finding that *jun* and *fos* complex with one another, presumably by way of their "leucine zippers", and function as a transcriptional unit (110). The *fos* - *jun* complex binds AP-1 sites with higher affinity than *jun* homodimers; *fos* itself does not bind these sites as a monomer, nor is it believed to form homodimers (56, 79, 102, 119). Most intriguing are reports that the *fos* - *jun* complex may exert both positive and negative transcriptional effects (120, 154). Considering the possible numbers of interactions between the various members of the *fos* and *jun* gene families, the potential effects of various protein modifications and the abilities of *fos* and *jun* to associate with other proteins, it would seem that this system is well suited to mediate a wide range of transcriptional activities. While some of the mystery has already been revealed there is very much more to know before we can truthfully say we understand the mechanisms of *fos* , *jun* , and *fos* - *jun* transcriptional activation.

During this introduction I have attempted to describe a progression of activities which are likely to follow a ligand-receptor interaction; a series of events which ultimately affect

cell growth and behavior. An attempt was made to provide examples of anomalies which can occur at various points along the signalling pathways as cells internalize, process and act upon information they receive, following interactions with their extracellular environment.

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CHAPTER 2

Transformation of chicken embryo fibroblasts by direct DNA transfection of single oncogenes: comparative analysis of *src*, *erbB*, *myc* and *ras*.

i) INTRODUCTION

A number of in vitro and in vivo systems have been developed to characterize the transforming potential of oncogenes (for example, see references 3, 4, 6, 17 and 43). Assays for tumorigenesis are frequently conducted through the inoculation of whole animals with either viruses carrying putative oncogenes or cells transformed by oncogenes. Direct injection of animals with oncogene DNA has also proven useful in some instances (14).

The most widely used in vitro assays for transformation utilize oncogenes introduced into cells by infection with viral vectors or by transfection with DNA. These in vitro assays frequently employ established rodent cell lines (NIH 3T3 or RAT-1) and score for the transformed state on the basis of the loss of contact inhibition (44) or anchorage-dependent growth (23). These assays have been extended to primary or secondary rat embryo or kidney cells (25, 38), which usually require the cotransfection of two oncogenes to detect transformation. A single oncogene, however, can sometimes transform primary embryo cells when cotransfected with the neomycin resistance gene (neo) (24, 26). Land et al. (24) suggested that G418 selection might facilitate the growth of transformants by removing an inhibitory environment presented by the normal cells in the culture.

A number of important sarcoma-leukemia viruses are of

avian origin, and chicken embryo fibroblasts (CEF) have been used extensively to study the transformation parameters of these viruses (48). Thus far, transformation studies in CEF have been conducted primarily through the use of infectious viruses. In CEF, unlike rodent cells, direct transfection of oncogene DNA has previously met with limited success. Cooper and Okenquist (7) showed that calcium phosphate-mediated transfection of genomic DNA carrying Rous sarcoma virus produced transformants in CEF inefficiently, unless infectious virus was generated and allowed to spread throughout the culture. Similarly, Kawai and Nishizawa (22) showed that Polybrene-mediated direct transfection yielded higher but still marginal frequencies of transformation, despite the fairly wide range of doses tested. As a result of these studies, it was thought that CEF were refractory to DNA transfection and integration.

In our analysis of CEF transfection, we found that the neo gene (pSV2neo) (45) could be efficiently introduced into CEF cells to yield G418-resistant cells in a dose-dependent fashion. Transfection frequencies on the order of one drug-resistant cell per thousand cells have been achieved routinely throughout the course of our studies (see below). Furthermore, other avian cells, QT6 (a chemically transformed quail cell line) are noted for successful transfection (31). These findings prompted us to reexamine oncogene transformation of CEF using standard calcium phosphate transfection protocols. We report here that

CEF can be transformed efficiently by a number of oncogenes (*src, myc, erbB, ras*) when cotransfected with and selected for neo. The specificities of transformation based on studies of oncogenic and nononcogenic variants faithfully reproduced those generated by infectious virus.

This transformation assay for oncogene activity in avian embryo fibroblasts provides an important complementary and/or alternative approach to the use of rat embryo cells. It provides a more suitable host for avian oncogenes, especially those which do not tranform established rodent cells efficiently (e.g., *myc* and *erbB*) (8) and in conjunction with these systems it provides an opportunity to study the underlying basis of the species-specific transformation of certain oncogenes.

ii) MATERIALS AND METHODS

Plasmids. Plasmids were kindly provided by J.M. Bishop (pMC38), L. Sealy (pAEV 11-3), T. Yamamoto (pAE7.7), R. Ellis (pH1), A. Zelenetz and G. Cooper (psrc11), H. Hanafusa (pN4, pB5, pBB4, pHB5 and pTT501) and R. Pelley (pAEV-R/C and pAEV-C). psrc-11 (36) represents a nonpermuted SR-D Rous sarcoma virus genome with portions of the gag-env sequences deleted. pN4 (see Fig. 2 and reference 20) carries the cloned *v-src* gene of NY-SRA strain of Rous sarcoma virus. The *src* genes of pTT501 and pHB5 (20) are derived from *c-src* sequences.

pB5 and pBB4 are chimeric constructs of *v-src* and *c-src*, exchanged at the C-terminal PstI and BglII sites, respectively (20). pH1 (12) and pMC39 (51) are plasmids carrying, respectively, the permuted genomes of Harvey sarcoma virus and avian leukemia virus strain MC29. In both plasmids, the viral inserts are bounded by EcoRI sites; digestion with EcoRI followed by ligation restores the functional viral transcriptional units. pAEV-R (avian erythroblastosis virus vector) is the same as pAEV11-3 described by Sealy et al. (40), which carries an entire AEV-R genome in a nonpermuted form. To replace the *v-erbB* sequence of pAEV11-3 by insertionally activated *c-erbB* (*IA-c-erbB*), an XbaI site was introduced to replace the XmnI site located at the 3' untranslated region of pAEV11-3 (R. Pelley et al., manuscript in preparation). pAEV-C was generated by excising the ApaI (located at the very 5' end of v-erbB) to XbaI fragment of pAEV 11-3 and replacing it with a corresponding fragment from *IA-c-erbB* (33). The 3' XbaI site of *IA-c-erbB* was generated by placing an XbaI linker into the NsiI site (located at the 3' untranslated region of Ia-c-erbB cDNA). pAEV-R/C was similarly constructed, except an EcoRI (located at the 3' end of *v-erbB*) to XbaI fragment was exchanged with a corresponding fragment of *IA-c-erbB*. pAEV- B is identical to pAEV 11-3, except a BamHI fragment within the *erbB* gene is removed to generate a nonfunctional *erbB* gene (40).

CEF culture. CEF cells were prepared from the skin of 10- to

11-day-old embryos following removal and collagenase digestion (100U/ml; type IV; Worthington Diagnostics). Good tissue dispersal was generally achieved after 3 h of digestion at 37° C with intermittent pipetting action to mechanically dissociate large tissue fragments. Collagenase solutions were made up in complete medium.

CEF cells were grown in medium consisting of 50% medium M199 (GIBCO Laboratories) and 50% Dulbecco's modified Eagle medium (low glucose; GIBCO), supplemented with 3 to 5% fetal calf serum (GIBCO), 2% heat-inactivated chicken serum (GIBCO) (incubated at 55°C for 30 mins), and antibiotics (penicillin-streptomycin; GIBCO). The sodium bicarbonate level of this medium was 2.95 g/liter. Cultures were maintained at 37°C in 5% CO_2 environments and fed every other day with fresh growth medium.

CEF transfection. For transfection experiments, early (4th to 8th-) -passage CEF cells were used because of the limited number of population doublings prior to senescence (25 to 30 in our experiments). Primary cultures were not employed because other contaminating cell types represent an appreciable fraction of their total cell population. At the outset, four to five 100-mm-diameter plates containing approximately 0.5 X 10^6 cells were plated for each construct or set of constructs to be analyzed. At 16 to 20 hrs after seeding or 4 to 8 hrs prior to the addition of the transfection cocktails, all cultures were refed

with fresh growth medium. The transfection cocktails were prepared as follows. For solution A, to 2.5 ml of 2X HEPES-buffered saline (42mM HEPES (n-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid) (ph 7.1), 270 mM NaCl) was added 50 ul of 100X phosphate solution (1:1 mixture of 70mM Na_2HPO_4 and NaH_2PO_4). The resulting solution was mixed thoroughly and then set aside. For solution B, a final volume of 2.5 ml was achieved through the addition of water, DNA solutions (in 10mM Tris - 1mM EDTA, pH 8.0) and 300 ul of 2M $CaCl_2$ (in that order), followed by vigorous agitation. The initial concentrations of DNA solutions were maintained sufficiently high so that their volumes did not account for more than 15% of solution B. The transfection cocktails were completed by mixing solutions A and B together, vigorously agitating and allowing the precipitate to form for 20 to 30 mins at room temperature. The cocktails were then forcefully expelled from a pipette four to five times, in an attempt to create as fine a particulate as possible. A 1.0 ml volume of the transfection cocktail was then delivered to each 100-mm-diameter plate and allowed to remain for 20 hrs. After the treatment period, the medium containing the calcium phosphate precipitate was removed and replaced with fresh growth medium. The cultures were allowed to recover for 24 hrs before the onset of selection, the addition of 400 ug/ml G418 (100% activity; GIBCO). Selective conditions were maintained sufficiently long (about 10 days) to ensure the elimination of all

nonresistant cells. During this time, cultures were fed on every third day or as needed when maximal cell killing occurred, generally at 4 to 7 days. Following selection, cells were grown 1 to 2 days in standard growth medium or further expanded prior to soft-agar or tumorigenic analysis.

Soft-agar assay. For analysis in soft agar, 0.5 X 10^6 cells in 4.0 ml of 0.35% agar (Bacto-Agar or Noble agar; Difco Laboratories) were plated into 60-mm-diameter dishes containing 4.0 mls of prehardened 0.5% agar. After 1 to 3 days, an additional 3.0 mls of 0.35% agar was added to immobilize cells floating freely in the film of fluid on the surface of the previous 0.35% layer. All agar suspensions were prepared in complete growth medium (described above). Approximately 1 week later, 5 drops of complete growth medium (about 300 ul) were added to each dish and again as needed to prevent dessication. Results were determined after approximately 21 days and only colonies containing 50 or more cells were counted.

Tumorigenesis assay. To assay the tumorigenic potential of transfected CEF cells, the wing-webs of young chicks (6 days old or less) were inoculated subdermally with 10^7 $G418^r$ cells in a volume of 0.1 ml. The transfected cells were delivered in growth medium supplemented only with gentamicin at 100 ug/ml. The birds were examined at 2- to 3-day intervals and the final result was determined 2 weeks postinjection.

There are three important criteria which in large part

determine the success and reliability of this assay: (i) the use of very young chicks, 6 days old or less; (ii) introducing sufficient numbers of transformed cells as concentrated inoculum, on the order of 10^7 cells in 0.1 ml; and (iii) utilizing populations of cells selected for G418^r alone and not further selected for anchorage-independent growth as the source of cells for this assay.

iii) RESULTS

A)*neo* dose-resonse and transfection efficiency. We initially observed that calcium phosphate-mediated transfection of CEF cells with pSV2neo (45) rsulted in the generation of a substantial number of G418-resistant cells. This suggested that, contrary to previous belief, CEF could rather efficiently acquire and stably integrate foreign DNA into their genomes. To fully document this phenomenon and optimize conditions for CEF transfection, we conducted the following detailed analysis with the *neo* gene.

The *neo* gene was introduced at levels ranging from 1 ng to 10 ug/100-mm-diameter plate of cells. The total input DNA level was maintained constant at 20 ug per plate through the introduction of salmon sperm DNA. Two sets of dishes were utilized for each dose of the *neo* gene examined; one set was selected with G418 to determine the number of G418^r cells in the transfected population and the other set was grown in the

abscence of G418. This second set provided correction factors for cellular toxicity incurred as a result of the transfection protocol and made it possible to express each final result as the number of G418^r cells per 100% surviving cells. Untreated CEF grown in the abscence of G418 were used to determine the intrinsic cloning ability of these cells.

The results of a representative *neo*^{r} dose-response experiment are shown in Table 1. In general, a 10-fold increase in the input level of the *neo* gene resulted in about a 3-fold increase in the number of G418^{r}cells. At a level of 10 ug of pSV2neo per 100-mm-diameter plate, the transfection efficiency reached approximately 1 in 1,000 cells. Increasing the level of carrier DNA to 50 ug per plate had little influence on the number of G418-resistant cells which developed.

Toxicity induced by the calcium phosphate transfection protocol varied significantly between experiments, with 20 to 30% killing being typical. Within a given experiment, however, cell killing was relatively constant and appeared little affected by the identity or amount of plasmid utilized. The toxicity appeared to be primarily a consequence of exposure to the solution containing the calcium phosphate precipitate. Untreated CEF had cloning efficiencies of 20 to 30%. These experiments indicated that CEF could be transfected by the calcium phosphate method and, under the appropriate

TABLE 1. CEF transfection by pSV2neo DNA

Amt of pSV2neo/plate[a]	# neo^r colonies per 10^6 cells[b]
0 ng	0
1 ng	17 ± 34
10 ng	57 ± 73
100 ng	350 ± 184
1 ug	930 ± 188
10 ug	3,300 ± 587
10 ug + 20 ug of SS DNA	4,400 ± 601
10 ug + 50 ug of SS DNA	3,400 ± 340

a Except where indicated, all included additions of salmon sperm (SS) DNA to maintain the total input level at 20 ug per 100-mm-diameter plate.

b Corrected for cloning efficiency and cytotoxicity; results shown are the average of four dishes plus or minus standard deviation of experimental results.

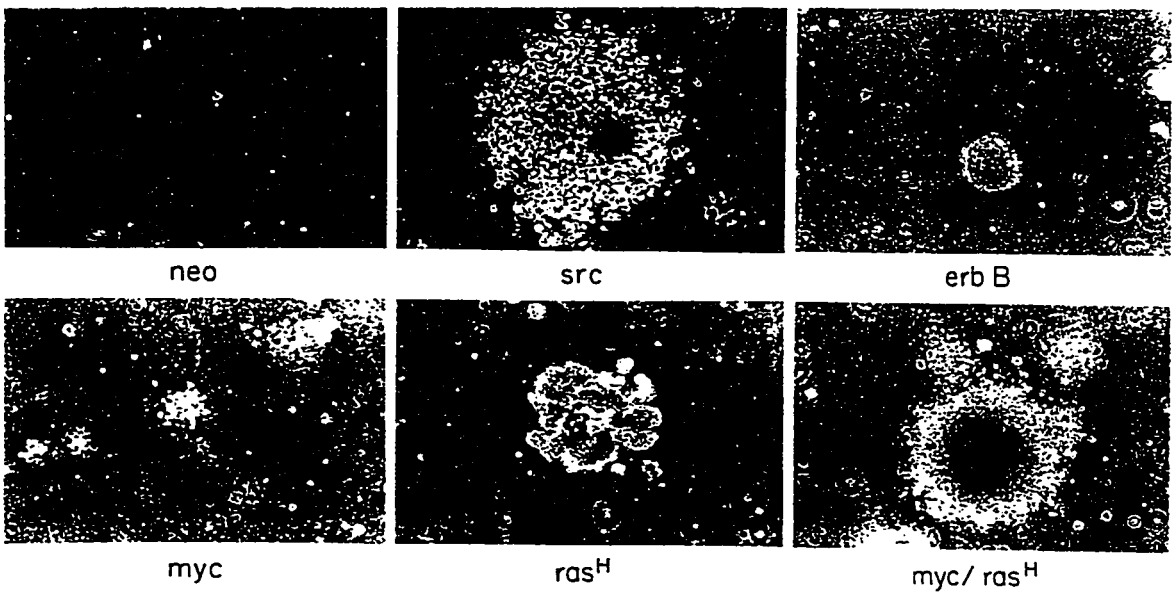
conditions, with an efficiency comparable to that of NIH 3T3 cells.

B)*v-src* dose-response curve. We next proceeded to develop an oncogene transformation assay in CEF by direct DNA transfection. We chose to study the prototypic oncogene *v-src* in psrc11, a construct in which the oncogene is flanked by Rous sarcoma virus long terminal repeats (LTRs) (36). psrc11 (20 ug/100-mm-diameter plate) and pSV2neo (6 ug/100-mm-diameter plate) were cointroduced into CEF by calcium phosphate precipitation. The transformation of CEF was monitored by anchorage-independent growth in soft agar. Without G418 selection, 73 colonies per 10^6 cells were detected. The colonies displayed typical *src* morphology (Fig. 1, panel src). This level of transformation is comparable to that previously reported by Kawai and Nishizawa (22), who used a Polybrene-mediated transfection protocol. When transfected cells were first grown in the presence of G418, however, a substantial yield of soft-agar colonies (approximately 20,000 per 10^6 cells) was generated. G418 selection increases the sensitivity of detection by as much as 3 orders of magnitude. We believe this is accomplished by removing the background of unsuccessfully transfected cells from the population.

We then measured the response of CEF to various doses of oncogene, to document that the transformation was src mediated. The amount of the psrc11 plasmid was varied from 2

FIGURE 1. Morphologies of oncogene-transformed CEF soft-agar colonies.

The colonies were derived from cells transfected with the following DNAs in the presence of 6 ug of pSV2neo per 100-mm-diameter plate: 20 ug of salmon sperm DNA (panel *neo*), 20 ug of psrc11 (panel *src*), 20 ug of pAEV-R (panel *erbB*), 20 ug of pMC38 (panel *myc*), 20 ug of pH1 (panel *ras* ^{H}), and 10 ug each of pMC38 and pH1 (panel *myc* /*ras* ^{H}).


neo
src
erb B
myc
ras^H
myc/ ras^H

ng to 50 ug per plate, but the amount of pSV2neo was kept constant, 6 ug per plate, and the total amount of DNA was held constant at 65 ug per plate through the use of salmon sperm DNA. At 20 ng per plate, psrc11 gave a low but significant number of transformed colonies (Table 2). Increasing psrc11 to 200 ng per plate brought about a near-maximal response (4.0 X 10^4 colonies per 10^6 G418-resistant cells). At a psrc11 dose of 50 ug per plate, a decrease in colony number was seen; the colonies were markedly reduced in size and aberrant in appearance. These results suggest the existence of both a critical threshold level and a tolerable level of *src* gene expression in the transformation of CEF cells. The apparent deleterious effects of high level psrc11 are presumably due to the toxic effects of overexpression of *v-src* noted by others (47).

We were next concerned about the origin of the transformed population of cells. Did these cells arise from the expansion of a very small number of *src* -transformed cells or did they develop from a sizeable number of cells independently transformed by the *src* gene, representing an appreciable portion of the total $G418^r$ population? Cells were cotransfected with 20 ug of psrc11 and 6 ug of pSV2neo per 100-mm-diameter plate. The cells were then selected with G418 and allowed to form colonies in their original dishes without replating steps. In this way the fate of each G418-resistant cell could be monitored. Only frankly transformed colonies with distinct *src* morphologies

TABLE 2. CEF transformation by psrc11 DNA.

Expt. and amount of psrc11/plate[a]	No. of soft-agar colonies/10^6 cells[b]
A	
20 ug[c]	73 ± 13
20 ug	20,680 ± 2,992
B[d]	
0 ng	0
2 ng	0
20 ng	53 ± 5
200 ng	38,360 ± 3,207
20 ug	56,600 ± 2,394
50 ug	12,680 ± 2,081

[a] In the presence of 6 ug of pSV2neo per plate.

[b] Plus or minus standard deviation of experimental results.

[c] G418 was added to all other plates.

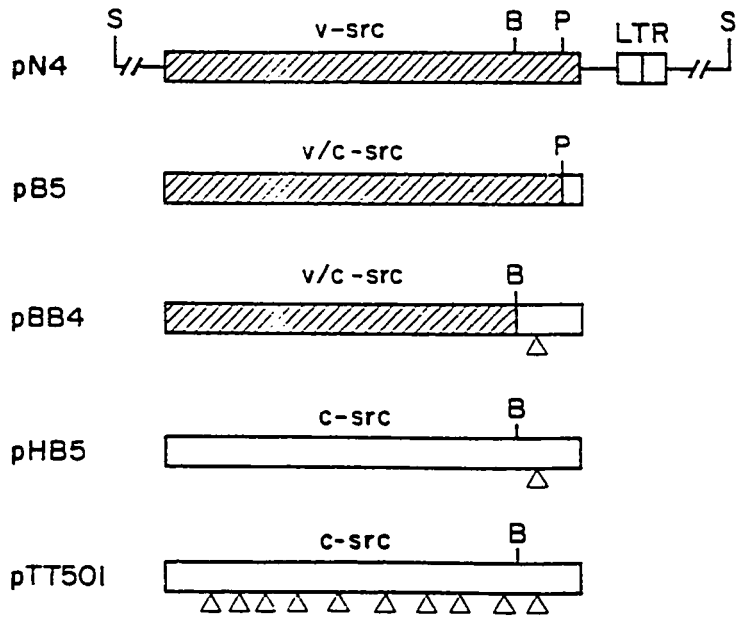
[d] Sufficient amounts of salmon sperm DNA were added to maintain total DNA input levels at approximately 65 ug per 100-mm-diameter plate.

were scored as positive. As a consequence, the result is likely to be considerably underestimated. Nevertheless, approximately 1 of every 10 G418^r clones displayed a frankly transformed phenotype (data not shown). This frequency is roughly comparable to that for soft-agar colony development at an equivalent *v-src* input level. These results demonstrated that, under the conditions of the analysis, the transformed population always represented an appreciable fraction of the total G418^r population.

C) Transforming potential of *v-src* and *c-src* and their chimeras. To test the ability of this transformation assay to discriminate between transforming and nontransforming genes, a family of *v-src*, *c-src* and *v/c-src* constructs were used (Fig. 2). These constructs were derived from permuted viral clones that required ligation prior to transfection to restore the 5' LTR promoter (20, 21). The *v/c-src* chimera consisted of the 5' end of *v-src* and the 3' end of *c-src* fused at the PstI or BglII site (pB5 and pBB4, respectively). The *v-src* and *v/c-src* constructs were transforming, whereas the *c-src* constructs scored negatively. The presence or absence of intron sequences (Fig. 2, triangles) had no effect on the transforming ability of *c-src*. These results are in good agreement with infection assays using viruses harboring these genes (20, 21). The transformation efficiencies in this experiment were lower than we observed for psrc 11, presumably, in part, because of the requirement for

FIGURE 2. CEF transformation by *v-src* and *c-src* and their chimeras.

All the *src* constructs are contained within a *Sal* I fragment, as shown for pN4 (20). The constructs were first digested with *Sal* I to liberate the inserts and ligated to restore a functional 5' LTR promoter. The *src* fragments were not purified prior to ligation. All except pN4 were introduced at a level of 5 ug per plate in the presence of 1.7 ug of pSV2neo per plate. pN4 was introduced at 8 ug per plate with 2.6 ug of pSV2neo. *v-src* (strain NY-SRA) sequences which carry the *v-src* -specific C-terminal 12 amino acids and eight internal mutations (▨), *c-src* sequences (□), and locations of *c-src* introns (△) are shown. S, *Sal* I; P, *Pst* I; B, *Bgl* II. Only those sites involved in the construction of chimeras are shown. The LTR of avian sarcoma virus is indicated.



	Soft Agar Colonies per 10^6 $G418^r$ cells
pN4	2.5×10^3
pB5	3.4×10^3
pBB4	3.5×10^3
pHB5	0
pTT501	15

ligation to generate expressible configurations of these plasmids. The *v/c-src* chimeras that retain tyrosine 527 were previously reported to be incapable of transforming NIH 3T3 cells (36) yet as virus were capable of transforming CEF (20). This led to the postulate that there is a chicken versus mouse species specificity in transformation by these constructs. Our data support this thesis, ruling out the possibility that the previous disparity (20, 36) was due to differences in the approaches used (infection versus transfection).

D) Tumorigenic potentials of *src*-transformed CEF.

Having demonstrated the utility of using direct transfection of CEF cells to study the transforming properties of oncogenes in vitro, we wished to test the tumorigenic potential of the transfected cells. While tumorigenic assays in nude mice have frequently been used for in vitro-transformed rodent cell lines, we knew at the outset that similar efforts in the chicken system would be compromised by several factors. First, the single-oncogene-transformed CEF are not immortalized and so will have limited population doublings in vivo. Second, no immunocompromised chicken line similar to nude mice exists. To avoid possible complications with viral rescue of the transfected constructs, we chose to use CEF obtained from line 0 embryos, a line free of endogeneous viral loci (1). Line 0 is not sufficiently inbred to be completely histocompatible with the injected cells. Nevertheless, taking several precautions

(discussed in Materials and Methods), including the use of young recipient birds (6 days or less) and an inoculum consisting of cells selected only for G418^r (but not for anchorage-independent growth), we were able to demonstrate the tumorigenic potential of *v-src*-transformed cells. At 2 weeks postinjection, psrc11-transfected cells (10^7 G418^r cells) induced wing-web sarcomas in six of nine birds, whereas pSV2neo-transfected cells were completely nontumorigenic (Table 3). Several of the *src*-induced tumors grew progressively and reached a size of 2.0 cm. As a comparison, in another experiment *v-src*-transformed cells were selected for both G418 resistance and anchorage-independent growth prior to injection; these cells induced sarcomas in five of six birds. These tumors, however, in general, were of smaller size and regressed.

E) Transforming potential of *v-myc* and *v-ras*^{H}.

v-myc and *v-ras* (oncogene of Harvey sarcoma virus) (18) represent two extensively studied oncogenes. When introduced into CEF via avian retroviral vectors, each gene can independently induce transformation (19, 26, 37). However, in transfection experiments, cooperation between *ras* and *myc* DNA is required for transformation of rat embryo cells in the absence of a selectable marker (24). We have tested the transforming potential of *v-ras*^{H} and *v-myc* in our assay system. Results from these experiments are shown in Table 4. When individual oncogenes were cointroduced with *neo* and

TABLE 3. Tumorigenesis assays of transfected CEF

Gene or oncogene and plasmid(s) used for transfection[a]	No. of birds with tumors/ no. inoculated	Tumor characteristics	
		Growth[b]	Dimensions[c] (cm)
neo			
pSV2neo	0/5	—[d]	
src			
psrc11	6/9	+, ++	0.5 - 2.3
	5/6[e]	+	< 0.5
myc, ras			
pMC38	0/8	—	
pH1	2/7	+	≤ 0.5
pMC38 + pH1	7/7	+, ++	0.5 -2.0
erbB			
pAEV-7.7	1/4	+	≤ 0.5
pAEV-R	4/4	+	≤ 0.5
pAEV-R/C	5/5[e]	+, ++	0.5 - 2.5

[a] pSV2neo plasmid was present as a cotransfectant in all experiments. Except where indicated, transfected, G418^r cells were injected into birds without prior selection through soft agar.

[b] +, Tumors decreased in size with time; ++, tumors continued to enlarge throughout the course of the experiment.

[c] Maximum dimensions determined 2 to 6 weeks postinoculation.

[d] —, No tumor formed.

[e] Injected cells were derived from pooled soft-agar colonies which were grown on plastic plates for several passages prior to inoculation.

TABLE 4. CEF transformation by *v-myc* and *v-ras*^{H} plasmids

Plasmid[a]	ug of DNA/plate	Soft-agar colonies: Morphology	Soft-agar colonies: No./ 10^6 G418^r cells
pMC38 (v-*myc*)	20	Small, loose association, irregularly shaped	4.7×10^4
pH1 (v-*ras* ^{H})	20	Medium to large, tight association, lobulated	6.0×10^3
pMC38 + pH1 (v-myc + v-*ras* ^{H})	10 each	Medium to large, loose association, irregularly shaped	2.7×10^4

[a]In the presence of 6 ug of pSV2neo per plate.

grown under selective conditions, *v-ras* ^{H} and *v-myc* transformed CEF at moderate (6,000/10^6 cells) and high (47,000/10^6 cells) frequencies, respectively. In CEF, without G418 selection, transfected populations failed to produce soft-agar colonies. The morphologies of soft-agar colonies induced by *v-ras* ^{H} and *v-myc* were distinct. The *v-ras* ^{H} colonies were of medium to large size, irregularly shaped, lobulated and consisted of closely associated cells (Fig. 1, panel *ras* ^{H}). *v-myc*-derived colonies were small and irregularly shaped and consisted of loosely associated cells (Fig. 1, panel *myc*). When both *v-ras* ^{H} and *v-myc* were introduced simultaneously (each at half the level of that used singularly) and selected for G418 resistance, the colonies displayed novel morphologies. They were generally large and spherical and consisted of small cells in loose association (Fig. 1, panel *myc/ras* ^{H}). The distinct phenotype of *v-myc/v-ras* ^{H}-transformed cells was further manifested by heightened tumorigenicity (seven of seven birds) and more aggressive growth properties in vivo (Table 3). In contrast, cells transfected by *v-ras* ^{H} alone induced only small, regressive tumor nodules in two of seven birds tested. *v-myc*-transfected cells were nontumorigenic. The lack of tumorigenicity of MC29-infected CEF has been previously noted (37) and is consistent with the present finding.

F) Transforming potential of *v-erbB* and *1A-c-erbB* and their chimera. A major impetus for this work was our

search for an efficient method with which to study the transformation properties of the *erbB* gene. Viruses carrying different forms of *erbB* often display different tissue specificities; some are leukemogenic, while others sarcomagenic (2, 16, 30, 33, 53). These tissue specificities are manifested only in chicken cells, thus precluding their analysis in established rodent cell lines. The *erbB* gene encodes the epidermal growth factor receptor (11). We have previously shown that truncation of the ligand-binding domain of the receptor by retroviral insertion leads to the activation of its leukemogenic potential (32, 34). This *Ia-c-erbB* represents the 3' half of the receptor and is strictly leukemogenic. Transducing viruses carrying variant forms of *IA-c-erbB* have been isolated and shown to have expanded disease tropisms (2, 15, 26, 35, 49, 53). These viruses are additionally able to transform fibroblasts and induce sarcomas. The *erbB* genes present in these viruses invariably carry structural alterations at their 3' termini (to include at least one of the three major autophosphorylation sites), but they also carry other scattered point mutations internally. We wished to confirm and extend this analysis by using direct DNA transfection to avoid the possible complications encountered in previous studies due to the potentially different replicative abilities and tropisms of the various virus isolates. We took advantage of pAEV-R vector DNA (41, 50), which carries a *v-erbB* ^{R} gene that is both fibroblast and erythroblast

transforming. This particular avian erythroblastosis virus isolate also carries the *v-erbA* gene, a gene which is related to the thyroid hormone receptor and does not have transforming potential on its own (13, 40). To test other *erbB* genes via this vector, we removed the *v-erbB* ^{R} gene and replaced it with *IA-c-erbB* or a chimeric construct bewtween *verbB* ^{R} and *IA-c-erbB*. The structures of these constructs and the results are summarized in Fig. 3.

pAEV-$\triangle$B, which carries a functional *v-erbA* but a defective *v-erbB*, was not transforming, consistent with previous work which showed that *v-erbB* is the oncogenic determinant (40, 41). pAEV-R is the DNA of the transforming virus (41, 50), which transformed CEF and induced sarcomas in vivo as expected (Table 3). The sarcomas induced by inoculation with these pAEV-R-transfected CEF, however, were small and regressed after approximately 2 weeks. pAEV-C was created by removing the *v-erbB* gene in pAEV-R and replacing it with *IA-c-erbB* (Fig. 3). This construct was not transforming. Inspection of the structural differences between the *erbB* gene of pAEV-R and pAEV-C revealed that the former carries multiple lesions: a 72-amino-acid truncation at the C terminus, a 21-amino-acid internal deletion and eight point mutations (5, 32). It is not yet clear which of these mutations activate the fibroblast-transforming potential, although the C-terminal truncation is a likely candidate, since this lesion which affects the autophos-

Fig. 3. CEF transformation by v-erbB and IA-c-erbB and their chimera.

In each case, CEF were transfected with 20 ug of each of the following plasmids and 6 ug of pSV2neo per 100-mm-diameter plate. pAEV-△B, pAEV-R, pAEV-C, and pAEV-R/C are all based on pAEV 11-3 (40, 41), a plasmid carrying a nonpermuted AEV-R viral genome. pAE7.7 carries a permuted AEV-H genome (53) and requires Hind III digestion followed by ligation to recreate a functional form. P1, P2, and P3 indicate the approximate locations of three C-terminal tyrosines, corresponding to the major autophosphorylation sites (10). TM, Transmembrane domain; gag, the first six amino acids of the gag coding sequence of the avian sarcoma-leukosis virus. This gag-erbB fusion, found in virtually all erbB transducing viruses and in the cDNA of IA-c-erbB, is generated via splicing (32, 39). The AEV-R-derived (▥), AEV-H-derived (▨), and IA-c-erbB-derived (□) sequences are shown.

Construct	Structure of erbB	v-erbA	Soft Agar Colonies per 10^6 G418^r cells
pSV2neo	–	–	0
pAEV-ΔB	–	+	0
pAEV-R		+	4.4×10^4
pAEV-C		+	0
pAEV-R/C		+	1.2×10^4
pAE7.7		–	9.2×10^2

phorylation sites is commonly found in *erbB*- transducing viruses, including another avian erythroblastosis virus isolate, AEV-H (53). Indeed, AEV-H DNA, in the form of pAE7.7, when tested in our CEF assay was transforming, albeit with a reduced efficiency compared with that of AEV-R. The lower efficiency is again likely to be in part due to the requirement of prior ligation to make this permuted plasmid (pAE7.7) functional. To test whether the C terminus of *erbB* is inhibitory to fibroblast transformation, pAEV-R/C DNA was constructed. In this construct, the C terminus of *v-erbB* was restored, while the internal lesions remained. To our surprise, pAEV-R/C was very transforming in CEF and in several instances induced progressively growing sarcomas in vivo (Table 3). This provided evidence that truncation of the C terminus of *erbB* is not obligatory for fibroblast transformation and drew our attention to the importance of internal mutations. In a more detailed study to be published elsewhere (32a), we have confirmed these observations and uncovered single point mutations in the kinase domain that can activate the fibroblast-transforming and sarcomagenic potentials of *erbB*. These analyses fully established the sensitivity and reliability of studying tissue-specific transformation of CEF by direct oncogene transfection.

The *erbB*- induced soft-agar colonies were characteristically small, spherical and compact; they were typified by colonies derived from pAEV-R (Fig. 1, panel erbB). These colonies are

similar to those induced by pAEV-R viruses but very different from those induced by Rous sarcoma virus or MC29. All the data generated from our direct transfection assays are thus in complete agreement with viral infection data. This approach should prove very useful in delineating the region of erbB critical for fibroblast transformation, without resorting to more tedious and complicated viral assays.

G) DISCUSSION

Avian acute retroviruses have been a rich source for studying oncogenes. At least 15 such oncogenes have been isolated, with the most recent additions being *v-jun* (28) and *v-crk* (29). Most of these viruses can induce sarcomas in vivo and transform CEF in vitro. Due to the previously perceived difficulty of stable transfection of CEF, the characterization of oncogenes in CEF was carried out almost exclusively in the viral form. While there are obvious advantages to using viruses to study oncogenes (namely, in vivo analysis and assay of target cells refractory to transfection in vitro), the dependence on helper virus for replication, the rapidity of generation of mutations during viral propagation and the generally more time-consuming cloning steps are some of the drawbacks of this approach. In this study, we have defined conditions for efficient transfection of CEF and demonstrated its utility with the

characterization of several oncogenes, both of avian and rodent origin and under control of avian and murine LTRs.

Under our conditions, using a conventional calcium phosphate transfection method and the *neo* gene as a selection marker, the transfection efficiency of CEF reached one in a thousand. In retrospect, the inability of previous workers to demonstrate high-efficiency CEF transfection was likely a consequence of their source of DNA (i.e., cellular DNA from transformed cells) and the now generally accepted notion that for primary cells, in the absence of selection markers, cooperation of two oncogenes is required for efficient transformation (25, 38). In our studies, the use of cloned oncogene DNA and the *neo* gene helped overcome these difficulties. In our experiments, single oncogene fragments, in the abscence of the *neo* gene for selection, were essentially incapable of transforming CEF. The only exception was the *v-src* gene (psrc 1 1), which yielded a low number of colonies, consistent with the previous report (22). The pronounced enhancement of colony formation brought on by the inclusion of the *neo* gene and selection for G418 resistance can be explained in at least two ways. First, selection for *neo* increases the sensitivity of detection by eliminating unsuccessfully transfected cells. Second, as previously suggested in rat embryo fibroblast transformation (24), the imposition of G418 selection is likely to remove the inhibitory environment provided by the surrounding normal cells and

allow the effects of single oncogenes to become manifest. Efficient infections with acute retroviruses that transform virtually all normal cells can be viewed as producing a similar effect. This may explain the apparent paradox that viral infections can lead to CEF transformation with single oncogenes in the absence of selection markers. The recent report (27) that the phenotypes of *v-myc*- transformed cells can be suppressed by coculturing with normal cells lends further support to this hypothesis.

With the present assay, we have examined the effects of individual oncogenes on CEF more closely. Due to the various constructs and promoters used, it is difficult to compare the transforming potentials of all the tested oncogenes. Among similar constructs, however, the relative strengths of the individual oncogenes were reproducible. CEF transformed by individual oncogenes have distinct morphologies and tumorigenicities; for instance, *myc* colonies are characteristically loosely associated, whereas *ras* colonies are more compact in nature. CEF transformed by *myc* or *ras* individually have no to low tumorigenicity when injected into young birds. A combination of the *myc* and *ras* genes, on the other hand, yields a novel in vitro phenotype and significantly enhanced tumorigenicity. Since the transfected cells used for in vivo tumorigenic analysis were not preselected for anchorage-independent growth, the in vivo tumorigenesis and the in vitro

soft-agar assays offer two independent measures of the degree of transformation. We noted an apparent correlation between in vitro colony size and the tumorigenic potential, but there were exceptions.

The transformation specificities of our in vitro soft-agar data completely parallel those obtained by means of infectious viruses. We show by direct transfection that *v-src* and *v/c-src* chimeras were transforming, yet *c-src* was not. An additional rigorous test came from the tissue-specific transformation by the *erbB* gene. Previously, on the basis of viral infection analysis, we and others (2, 30, 32, 33) have demonstrated that an N-terminally truncated but an otherwise unmutated *erbB* was strictly leukemogenic. When additional mutations were introduced within the truncated molecule, viral *erbB* genes acquired fibroblast-transforming potential. This tissue-specific transformation pattern was reproduced in our transfection assay, which conclusively rules out the possibility that the obseved difference is due to viral tropism. Instead, our studies implicate the importance of the conformation of the catalytic domain of *erbB* in determining the accessibility or kinase activity toward tissue-specific substrates (this study and reference 32a).

In summary, we have optimized conditions for CEF transformation by individual oncogenes via direct DNA transfection. This assay provides an important alternative or

complementary system to the use of rodent cells. Recently, a number of *src* mutants which transform only rat cells but not chicken cells have been isolated (9, 52). These mutants are of potential value in the identification of cellular proteins involved in transformation. Our assay should greatly facilitate this effort.

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Chapter 3

One Step Transformation of Normal Human and Chicken Fibroblasts by TGFα and EGF.

i) INTRODUCTION

Deregulated growth factor and/or growth factor receptor genes have been implicated in the development of a number of human malignancies. Examples of these include the release of PDGF (platelet derived growth factor)-like molecules from human osteosarcoma (6, 22), glioma and sarcoma cells (5, 32); the production of FGF (fibroblast growth factor) in human chondrosarcoma (42); the over production of IL2 (Interleukin 2) in T-cell leukemia (24, 48) and of relevance to this paper, the production and release of TGFα (transforming growth factor alpha) from several types of human carcinoma (9, 10, 12, 14, 53, 54). These and other examples demonstrate the potential consequences of structural or regulatory modifications of individual growth factors or their receptors.

One of the most extensively studied growth factor receptor systems is the EGF-R and its activating ligands, TGFα and EGF (epidermal growth factor). In this system, oncogenic conversion is believed to occur as a result of at least two independent mechanisms. First, in chickens, retroviral insertion into the interior of the chicken EGF-R (also known as c-erbB) locus can result in the generation of 5' truncated receptor molecules with constitutive tyrosine kinase activity (19, 29, 31, 36). These truncated molecules, which have lost the major portions of their extracellular ligand binding domains, display both transforming

ability in vitro and tumorigenic potential in vivo (20, 21, 35, 37, 46). The second mechanism, frequently observed in human carcinomas, involves heightened expression of the activating ligand TGFα (9, 10, 12, 14, 53, 54); in some instances this is accompanied by overexpression of the EGF-R receptor (7, 12). The result of both mechanisms is greater cell self-sufficiency and independence, factors likely to promote early stage tumor progression.

Efforts to examine the role(s) played by TGFα and EGF in tumor development led to analyses of these growth factors in immortalized, rodent cell lines of either fibroblastic (e.g, NIH3T3 (15, 17, 38, 49), NRK (2, 3, 28, 53), Rat 1 (39), Fisher rat 3T3 (44)) or epithelial origin (NOG-8 (8, 28)). When these cells were transfected with genes encoding TGFα or EGF, a significant number of transformed, soft agar colonies were detected, in most cases. (In this paper, anchorage-independent growth in soft agar will be used as an operational definition of in vitro transformation). However, when the effects of exogeneous additions of TGFα and EGF to the culture medium were examined, more complicated scenarios developed. It was found that NIH3T3 cells could not be transformed by exogeneous ligand treatment unless additional copies of the EGF-R gene were introduced (15, 38, 49) and NRK (normal rat kidey) cells could be transformed only if additional growth factors such as TGFβ were also present (2, 28, 51). These and other results suggested

that an unusually high level of ligand-receptor interactions and, presumably, an equally elevated level of kinase activity were required to transform immortalized cell lines. Since, in general, primary cells are not as easily transformed as immortalized cells, this led to the speculation that cells derived from primary cultures would be refractory to transformation by TGFα or EGF (13).

We have been using normal fibroblasts derived from primary cultures to study EGF receptor-mediated transformation. We previously showed that normal chicken embryo fibroblasts (CEF) can be readily transformed by truncated and mutated erbB/EGF-R genes in a seemingly single-step fashion, following introduction of the genes either by direct transfection (1) or viral infection (35). The truncated erbB gene is thought to assume an active conformation which mimics the ligand-bound form of the normal receptor and is likely to utilize the same signal pathways. We therefore decided to test whether sustained application of TGFα (or EGF) to CEF would result in transformation. If successful, the analysis of cells derived from primary cultures should provide a system more relevant than those involving immortalized cell lines in attempting to understand events in cellular transformation and tumorgenicity. We have also extended our studies to include an examination of the effects of TGFα and EGF on normal, diploid, foreskin-derived, human fibroblasts (HFF).

Here we report that both normal chicken embryo fibroblasts and normal, diploid, human fibroblasts can be efficiently transformed by simple exogeneous ligand treatment using either TGFα or EGF. This ligand-induced transformation appears to involve a single-step mechanism and does not require the introduction of additional, complementing genes or factors. We also demonstrate the unusually vigorous transforming response of CEF cells following infection with a TGFα virus. While HFF responded nearly identically to TGFα and EGF, CEF cells were, in general, more efficiently transformed by TGFα, as expected from the greater binding affinity (approx 100 fold (27)) of the chicken erbB/EGF-receptor for TGFα versus EGF. The transforming responses of both HFF and CEF appeared to display an age-dependent component. Cultures became less responsive to ligand-induced transformation with increasing passage even though the average number of EGF receptors per cell remained constant, as did receptor binding affinities. In this regard, antiphosphotyrosine analysis revealed that the kinase activity and phosphorylation of abundant substrates were also indistinguishable. Thus, the basis for the "age-dependent" alteration in TGFα or EGF responsiveness is likely to be located at a point somewhere beyond initial ligand/receptor interactions.

ii) MATERIALS AND METHODS

CEF and HFF culture. CEF cells were isolated and cultured as previously described (1).

HFF cells were isolated from the circumcised foreskins of newborn males. The tissues were collected in small sterile bottles containing approximately 20 mls of Eagle's MEM medium (GIBCO Laboratories) supplemented with 100 ug/ml gentamicin. Tissue samples were processed within 24 hrs of collection according to the following protocol: The tissues were washed 3 times by serial transfer. At each transfer the tissues were agitated in an attempt to dislodge debri and reduce the level of potential contaminants. The tissues were then cut into small pieces and placed into a collagenase solution (300 U/ml; type IV; Worthington Diagnostics) for enzymatic dissociation. Collagenase solutions were prepared in complete growth medium. At hourly intervals, the tissues were pipeted up and down, using a moderate level of force, to dislodge cells and decrease the size of remaining tissue fragments. Following each round of pipeting, the collagenase solutions were removed for cell recovery and replaced with fresh solutions until the tissues were completely dissociated, approximately 3 to 4 hrs. In the recovered cell suspensions collagenase was diluted with complete growth medium and removed by centrifugation. HFF cells were grown in Eagle's MEM containing 10% fetal calf serum (GIBCO) and

antibiotics (penicillin-streptomicin; GIBCO). Cultures were maintained at 37°C in 5% CO_2 environments and fed every third day with fresh growth medium.

Soft-agar assay. For analysis in soft agar, cells in 4.0 mls of 0.35% agar (Bacto-Agar; Difco Laboratories) were plated into 60 mm dishes containing 4.0 mls of prehardened 0.5% agar. After 1 to 2 days, an additional 3.0 mls of 0.35% agar was added containing sufficient amounts of growth factor to bring the 11.0 ml total volumes to the desired levels. For comparative studies within a single experiment, all cell strains were subjected to identical preparations of agar, growth medium and growth factors. At approximately weekly intervals, unless otherwise specified, agar cultures were given 0.3 mls of medium containing sufficient levels of growth factor to treat the 11.0 ml totals at the appropriate doses. In order to help ensure equal distribution of growth factor during these refeeding steps, individual dishes were gently rocked from side to side until a thin layer of fluid was seen coating the entire surface of the agar. Results were determined after approximately 21 days, and only colonies containing 50 or more cells were counted.

EGF (GIBCO) and TGFα (Collaborative Research Incorporated) were obtained as lyophilized powders. Both growth factors were solubilized in complete growth medium to give stock solutions at 10 ug/ml. Following sterile filtration, stock solutions were stored as aliquots at -70° C for no longer than 1 month. Growth

factor stock solutions were subjected to only a single freeze-thaw cycle.

Binding Analyses. For competition analyses, determination of binding affinities, and the number of EGF-receptors per cell, cells were plated into 24-well cluster dishes (Fisher Scientific) and allowed to grow to near confluency. Growth medium was then changed and replaced with a medium containing 1% fetal calf serum. This "deficient" medium was allowed to remain on the cultures until the addition of the ^{125}I-TGFα–containing medium, approximately 24 to 36 hrs later. For binding competition studies, the "deficient" medium was replaced with 1.0 ml of binding medium (growth medium supplemented with 25mM Hepes and 2 mg/ml BSA) containing ^{125}I-TGFα (Biomedical Technologies Inc.) and either 0.0, 1-fold, 10-fold or 100-fold excess of cold TGFα or EGF . The plates were incubated for 2hrs at 4^{0} C after which time the binding liquor was removed and the wells were washed 4X with 2.0 mls of cold binding medium. For determination of ligand binding affinities and the number of EGF-receptors per cell, the "deficient" medium was replaced with 0.5 mls of graded doses of ^{125}I-TGFα in binding medium, including saturating ^{125}I-TGFα doses. The plates were incubated for 90 mins at 4^{0} C then washed as described for binding competition studies. For each type of analysis, the cells in individual wells were lysed in 0.5 mls of 1N NaOH incubated at 37^{0} C for 30 mins. Experimental results were

determined following analysis of cell lysates in a gamma counter (Beckman Instruments).

Western Analysis. For western analysis of induced tyrosine phosphorylation, cells were plated into 100-mm Petri dishes and allowed to grow to near confluency. Cultures were then refed with "deficient" medium, as described above for binding studies, and were maintained in the presence of this medium for 48 to 60 hrs. At the beginning of the exposure period, appropriate levels of stock solutions of TGFα or EGF were added directly to the existing culture fluid to generate the desired ligand concentrations. After the predetermined period of ligand exposure, cultures were washed 3X with PBS then lysed in 1.0 ml of lysis buffer @ 100^0 C. Lysis buffer consisted of: 50mM Tris-Cl, pH 6.8; 1% SDS; 1% 2-mercaptoethanol; 0.1 M dithiothreitol; 5% sucrose; 300 uM sodium orthovanadate; 0.03% bromophenol blue. Cell lysates were processed, electrophoresed, transferred and analyzed according to previously described protocols (43). The mouse anti-phosphotyrosine monoclonal antibody PY20 (ICN) was used as probe, followed by ^{125}I-labeled protein A (Amersham) to detect the mouse antibody.

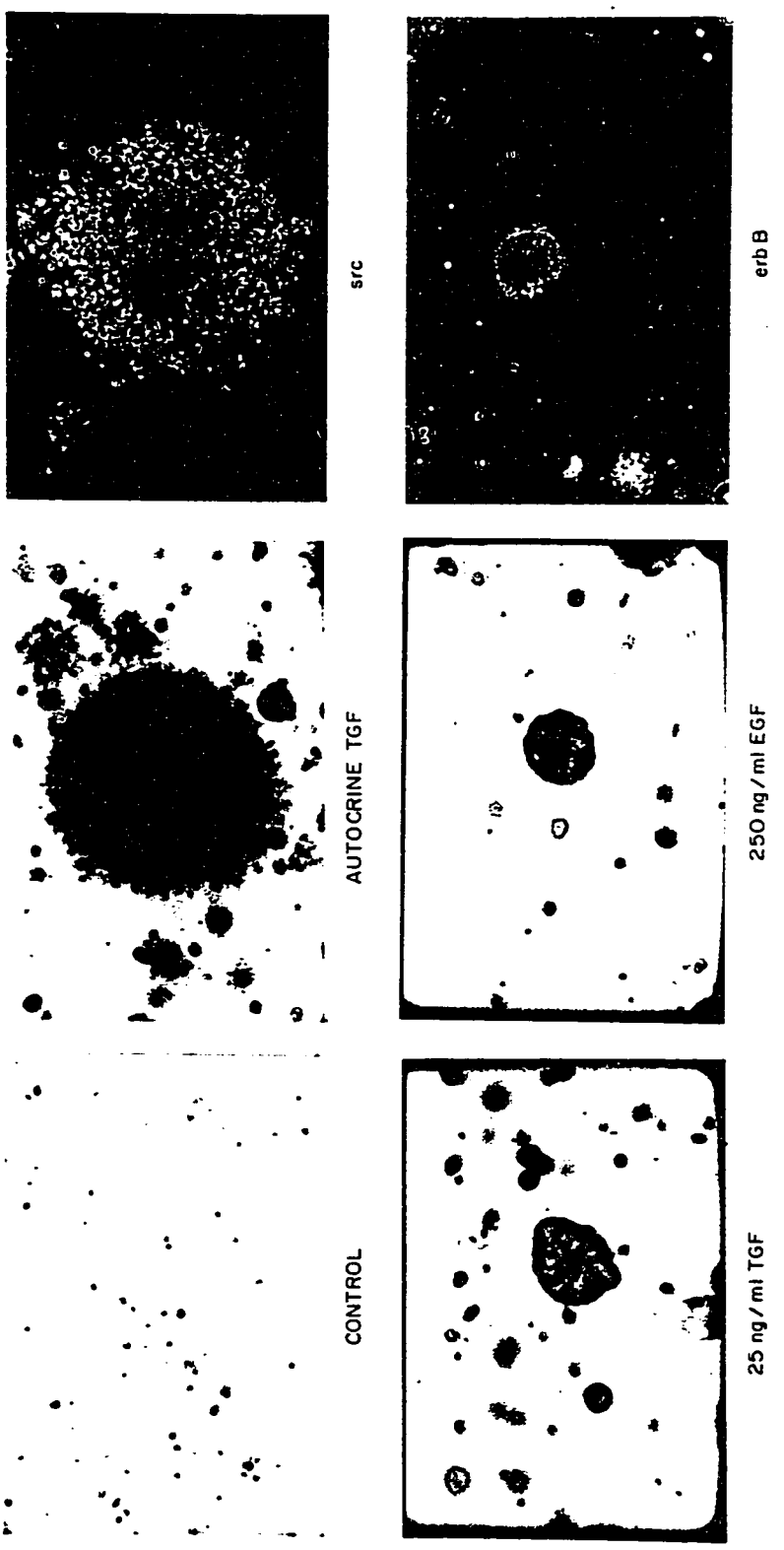
iii) RESULTS

A) Single step TGFα/EGF induced transformation of CEF. Normal chicken fibroblasts have been used extensively to analyze retroviral oncogenes (45). To date, the vast majority of this work has been done by way of infectious virus. Recently, we demonstrated the sensitivity of CEF cells in detecting the transforming potential of several viral oncogenes, including *v-src*, *v-ras*, *v-myc* and *v-erbB*, using a DNA transfection protocol without associated virus or cooperating oncogenes (1). Not only did CEF cells detect the transforming potentials of the various transfected oncogenes, additionally, they displayed unique phenotypes for each of the genes examined. We and others (20, 29, 31, 34, 35, 43, 52) have performed detailed analyses of the *v-erbB* genes, N-terminally truncated forms of the chicken EGF-R. These genes, which have lost their ligand binding domains and incurred several internal mutations, display constitutive tyrosine kinase activity and are transforming in fibroblasts and erythroid cells. It is thought that the deregulated tyrosine kinase activities of the various *v-erbB* genes are primary determinants of their transforming potentials. This led us to ask whether it might not be possible to achieve EGF-R-mediated transformation in CEF by creating a system which would establish constitutive, ectopic expression of one of the receptor's activating ligands, TGFα.

To accomplish this objective a one-round retroviral vector carrying a synthetic rat TGFα gene (SW355 (51)) was employed. These viruses were used to infect CEF cells following their release from the packaging cell line D17C3 (50) transfected with vector DNA. As a result of these infections, large, aggressively growing soft agar colonies developed at high frequency (Fig. 1, panel Autocrine TGF). The unusually vigorous growth properties displayed by these colonies were equalled only by v-src-transformed cells (Fig.1, panel *src*). This provided the first evidence that TGFα, when overexpressed in an autocrine fashion, could mimic the effects of potent oncogenes in normal fibroblasts. We then proceeded to test whether exogeneous additions of either TGFα or EGF would also be effective in inducing CEF cell transformation. CEF were plated into soft agar and treated with TGFα and EGF, 25ng/ml and 250 ng/ml, respectively, 24 hours later and at one week intervals thereafter. The ten fold higher dose of EGF was chosen because of the higher binding affinity of the chicken EGF-R for TGFα versus EGF (approx. 100 fold). After approximately 21 days of growth, large colonies were detected at high frequency following treatment with either TGFα (Fig. 1, panel 25 ng/ml TGF) or EGF (Fig. 1, panel 250 ng/ml EGF). The morphologies of the ligand-induced colonies were similar to those derived from *v-erbB*-transformed cells (Fig.1, panel *erbB*) but were considerably smaller in size than those generated with the TGFα virus (Fig. 1,

Fig. 1 Growth factor and oncogene-induced transformation in CEF: a comparative analysis.

The results from three independent analyses are shown: a) CEF cells were plated into soft agar and maintained without the addition of growth factors (control), or given periodic doses of either 25 ng/ml TGFα (TGF) or 250 ng/ml EGF (EGF); b) The consequence of CEF cell infection with a viral vector expressing the rat TGFα gene (Autocrine TGF); c) CEF cells transformed following transfection of the *v-src* (src) and *v-erbB* genes (erbB); these results are from previous studies (1).


src
erb B
AUTOCRINE TGF
250 ng / ml EGF
CONTROL
25 ng / ml TGF

panel Autocrine TGF). These results were initially surprising since previous attempts by others to transform rodent, fibroblast cell lines (NIH3T3 and NRK) by simple treatment with either TGFα or EGF were unsuccessful. To ensure that our results were characteristic of CEF in general and not merely the anomolous response of a particular CEF culture, we extended our analysis to include three independent CEF cell preparations. The three cultures were derived from different inbred strains of chicken (lines 0, $151XI4$ and $15I5X7_1$). All three of the independent CEF cell cultures were potently transformed following exogeneous TGFα/EGF treatment (see below). As an additional precaution, we also studied the transformation of NIH3T3 cells following exogeneous TGFα/EGF treatment and found them to be unresponsive, consistent with previous reports (15, 38, 49). These data suggested that CEF, unlike immortalized rodent cell lines, were highly sensitive to TGFα- and EGF-induced transformation and that they would provide a useful model to study the growth factor mediated transformation of normal cells.

B) TGFα and EGF binding analysis and the induction of differential and dose-dependent transformation of CEF. An interesting property of the chicken EGF receptor is its differential affinity towards TGFα and EGF. Lax et al. previously reported that the chicken erbB receptor binds human EGF with

an affinity 200 fold lower than that for human TGFα, using a chicken receptor cDNA clone overexpressed in a rodent cell line (27). To ensure that this differential binding was a general property of chicken cells and not a property specific to a particular cDNA clone, the following competition experiment was conducted. CEF cells were treated with medium containing ^{125}I-TGFα (5 ng/ml) in the presence of various levels of competing, nonradioactive TGFα and EGF (0.0, 5, 50 and 500 ng/ml). As shown in Fig. 2, excess cold TGFα was a much more efficient competitor than EGF, approximately 100 fold better, against bound ^{125}I-TGFα molecules. The relatively loose binding of EGF to the chicken receptor prevented us from carrying out the reciprocal competition experiment with an acceptable degree of accuracy. From binding experiments and Scatchard analysis we esimated the Kd of the chicken EGF-receptor for TGFα to be approximately 0.65-0.70 nM and the average number of EGF-receptors per CEF cell to be around 2.0-3.0 X 10^4, Table 1. To study whether the differential binding affinity of CEF cells for TGFα and EGF would translate into a differential transforming response we analyzed the dose relationship of this transformation phenomenon. These studies were done with line 0 CEF, a strain which lacks endogeneous viral loci (4). CEF were plated into agar then treated 24 hours later with various doses of TGFα and EGF (0.2, 1, 5, 25 and 1, 5, 25, 250 ng/ml, respectively). In these and all other

Fig. 2. TGFα/EGF competitive binding analysis in CEF derived from line 0, $15I_5x7_1$, and 15_1xI_4 chickens.

Binding of 5ng/ml ^{125}I TGFα to the chicken EGF-R was competed by the addition of cold TGFα or EGF at equivalent levels, 10 fold and 100 fold excess. Line 0 CEF, ○; line $15I_5x7_1$ CEF, ◇; line 15_1xI_4 CEF, □. Competing ligands: open symbols, EGF; filled symbols, TGFα.

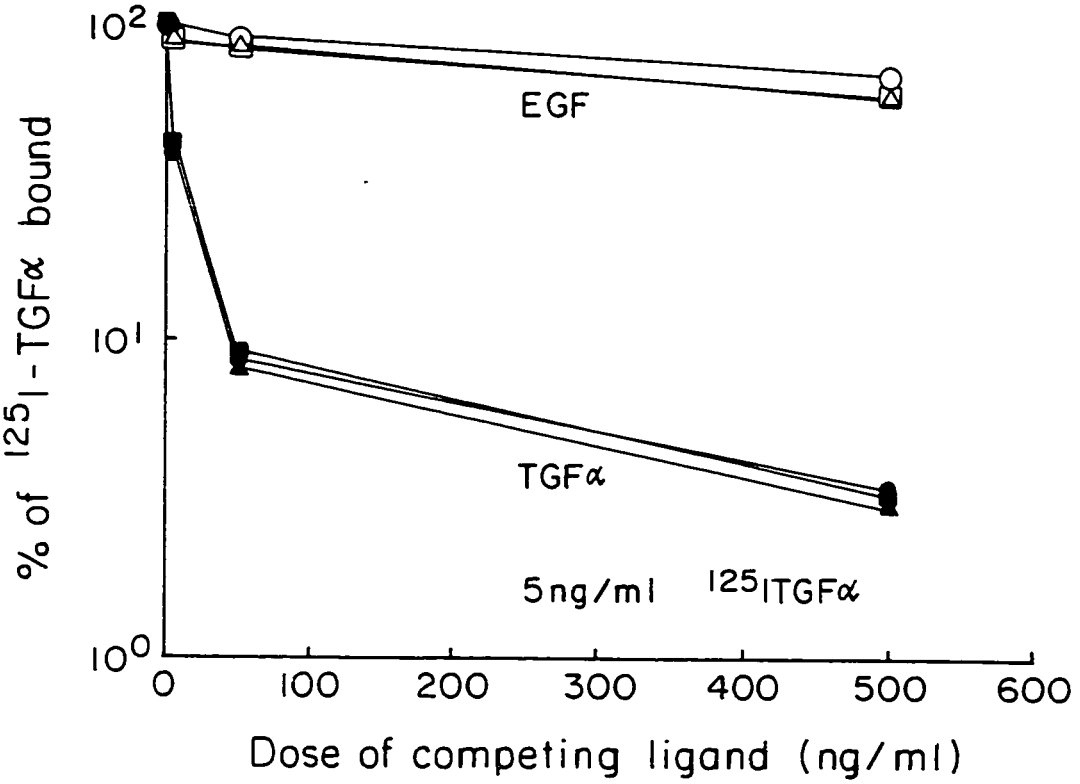

10^2
10^1
10^0
% of ^{125}I-TGFα bound
EGF
TGFα
5ng/ml ^{125}ITGFα
0
100
200
300
400
500
600
Dose of competing ligand (ng/ml)

Table 1. Determination of ligand binding affinity and EGF receptor number in CEF and HFF.

Cell/receptor	growth factor	Kd (10^{-9} M)[c]	No. receptors
CEF line 0, strain C; CEGF-R [a]	TGFα	.17	2.3×10^4
CEF line 15_1 X I_4; CEGF-R	"	.15	2.4×10^4
CEF line $15I_5$ X 7_1; CEGF-R	"	.23	3.3×10^4
CEF line 0, strain F;			
CEGF-R - p2	"	.12	1.1×10^4
p14		.12	1.4×10^4
p21		.12	1.0×10^4
A431; HEGF-R [b]	EGF	2.6	2.9×10^5
HFF1; HEGF-R	"	2.13	2.8×10^4
HFF15; HEGF-R	"	2.16	4.1×10^4

a chicken EGF receptor
b human EGF receptor
c calculations derived from duplicate samples at each dose examined; doses were chosen to include saturating ligand concentrations in each case.

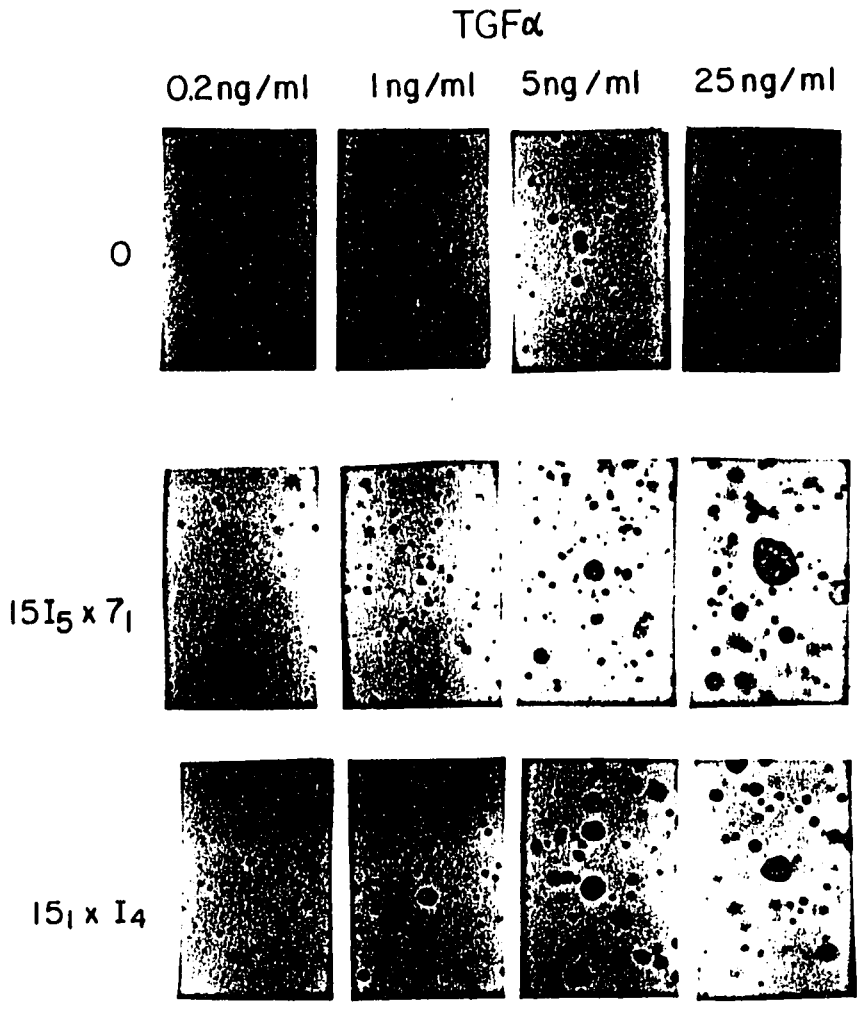
experiments, soft agar colonies comprised of at least 50 to 100 cells set the threshold for colonies scored as positive. Both qualitatively and quantitatively, TGFα was considerably more efficient than EGF in the induction of colony formation at a given dose (Fig.3). This was particularly true at the lower doses examined (0.2, 1, and 5 ng/ml TGFα versus 1.0 and 5 ng/ml EGF). To ensure these results were representative of line 0 CEF in general and not the individual peculiarity of this particular culture, prep C, we similarly examined two additional, independent, line 0 preps, preps E and F. The results of all three of these analyses were very similar (prep E and F results not shown). In approximate terms, an EGF dose an order of magnitude greater than that of TGFα was required to achieve 50% of the maximal, numerical response and to support the development of equivalent numbers of similarly large, transformed colonies. This value was smaller than expected based on the 100 to 200 fold difference in binding affinity of the chicken erbB/EGF-receptor for these two ligands and suggested that ligand-receptor interactions were not the sole rate-limiting determinants of transformation.

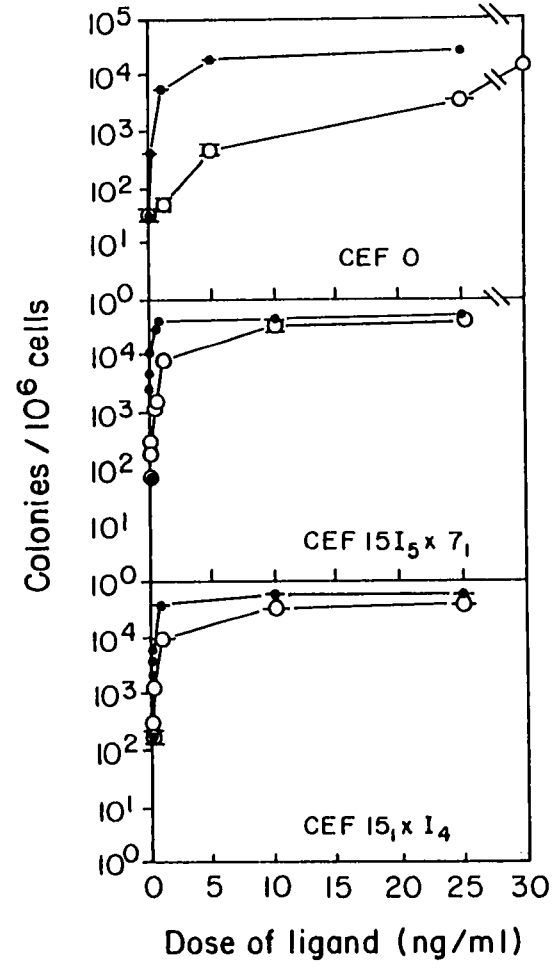
C) The differential responses of different CEF strains.

As a result of the unexpected results with line 0 CEF we once again examined CEF from other chicken lines, namely 151XI4 and 15I5X71, for their responses to graded levels of TGFα and

Fig. 3. TGFα/EGF-induced transformation in three independent CEF cell cultures.

CEF preparations from three unique strains of chickens, lines 0,151x71, and 151xI4, were plated into agar and examined for their responses to graded doses of TGFα and EGF. For each CEF strain, quantitative and qualitative dose responses are shown. Results achieved after approximately 21 days of growth, following weekly applications of growth factors. Each experimental point reflects the results from four to five individual dishes; error bars represent standard deviation from the mean.


TGFα
0.2ng/ml
1ng/ml
5ng/ml
25ng/ml
0
15I5 x 71
151 x I4


Colonies / 10^6 cells
CEF 0
CEF 15I5 x 71
CEF 151 x I4
Dose of ligand (ng/ml)

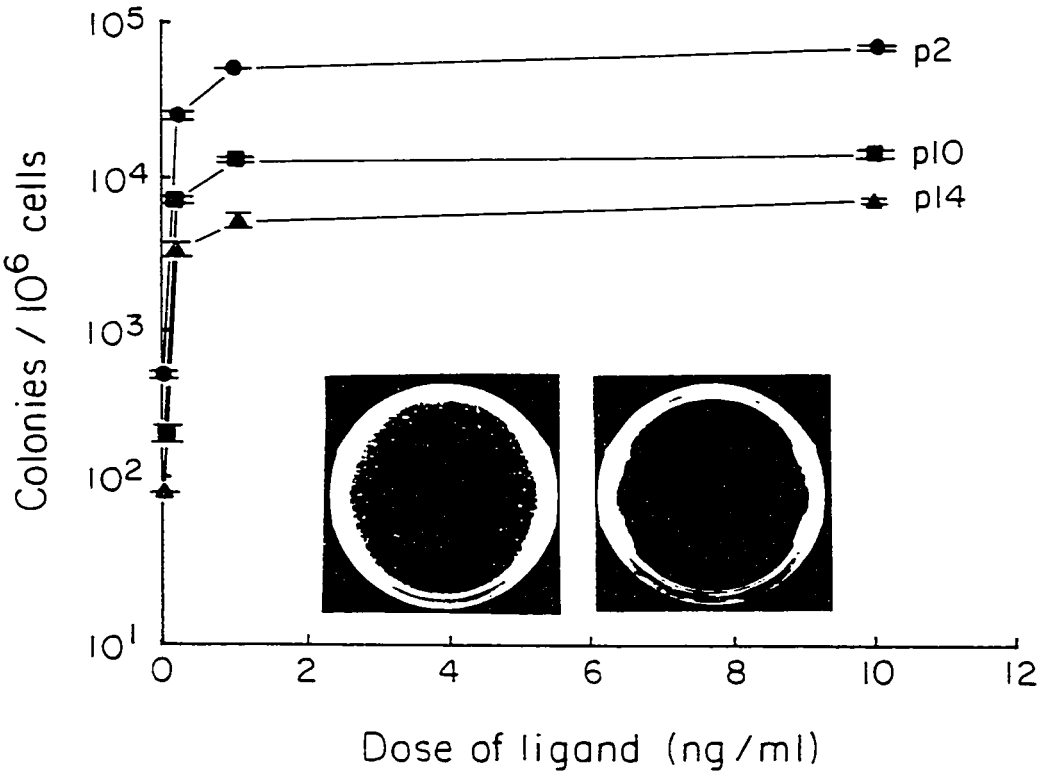
EGF. Both chicken lines were bred for their high susceptibility to retrovirally-induced leukemia or lymphoma. As in the case of line 0, CEF derived from these two lines were readily transformed by TGFα and EGF, but their responses were different from that of line 0, in particular their responses to EGF (Fig 3). In these CEF strains the sizes of the soft-agar colonies elicited by TGFα at 5 ng/ml were comparable to those of line 0 at ~25 ng/ml. In addition, when the number of colonies induced by TGFα and EGF were analyzed, the two results were very similar except at the lower concentrations of ligand, where the effect of TGFα was consistently greater than EGF. Qualitatively, TGFα always induced larger colonies than EGF, at a given dose. The higher sensitivity of strains 151XI4 and 1515X71 to TGFα and EGF cannot be explained on the basis of increased receptor number or enhanced kinase activity since these parameters were found to be virtually identical to those of line 0 (see Table 1 and below). The EGF receptors on strains 151XI4 and 1515X71 bind TGFα with 100-fold higher affinity than they do EGF, yet the two ligands engender very similar responses when present at higher doses (10-25 ng/ml). These data reinforce the notion that ligand-receptor binding is not the rate-limiting step in signal-transduction. It is conceivable that the high responders, 151XI4 and 1515X71, may more abundantly express certain limiting substrates which result in amplification of the receptor activation signal. In these cells the hundred-fold difference in

binding affinity between TGFα and EGF may be offset by a heightened internal response mechanism.

D) The effect of culture age on CEF transformation by TGFα and EGF. CEF cultures enjoy only a limited number of population doublings (ca. 20 to 30) before entering cellular senescence. To study the effects of cell passage on TGFα/EGF-induced transformation in CEF, groups of cells were assayed at their 2nd, 10th and 14th population doublings. The three groups of cells were plated into agar and treated identically throughout the course of the analysis. The results achieved following TGFα treatment are shown in Fig. 4; a similar result was obtained with EGF. As the cultures underwent increasing numbers of population doublings their sensitivities to ligand-induced transformation decreased. This decrease was always slightly greater in the case of EGF versus TGFα, resulting in an ever larger differential ligand response as the cultures aged (data not shown). The response of CEF to TGFα at their 2nd population doubling was approximately 10-fold greater than that at their 14th population doubling. Qualitatively, the differences in responsiveness at the two passages were quite striking. When assayed at its 2nd passage the CEF culture produced many more transformed colonies of larger size versus those generated from the same culture at its 14th passage (see photo inserts, Fig. 4). We were curious to determine whether

Fig. 4. The effect of increasing cell passage on TGFα/EGF-induced transformation in CEF.

Line 0 CEF cells at their 2nd, 10th and 14th population doublings were assayed for their response to various doses of TGFα and EGF (only TGFα results shown). Shown are both qualitative (photo inserts) and quantitative results generated during the analysis. At their 2nd population doubling, CEF cultures formed much greater numbers of larger agar colonies (left photograph insert) than were formed when asayed at their 14th population doubling (right photograph insert). For quantitative results, each experimental point reflects the results from four to five individual dishes; error bars represent standard deviation from the mean.

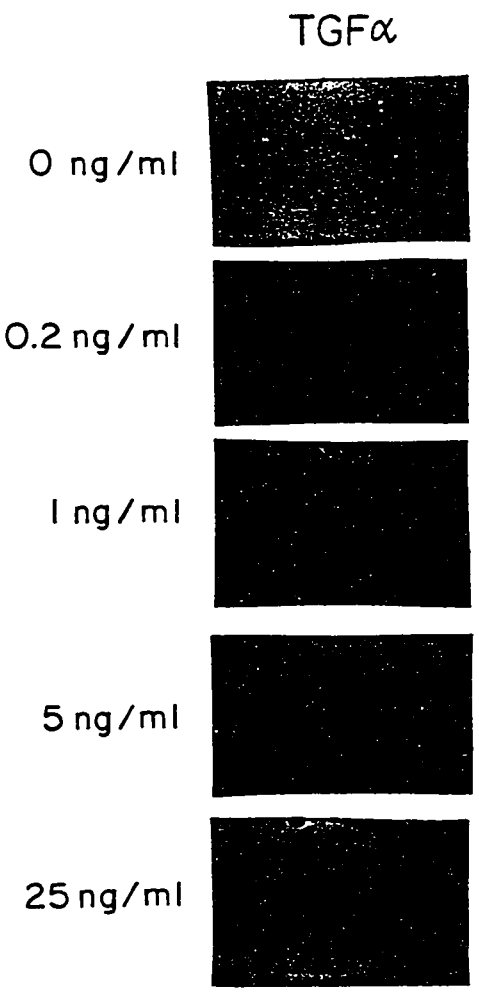

10^5
10^4
10^3
10^2
10^1
Colonies / 10^6 cells
p2
p10
p14
0
2
4
6
8
10
12
Dose of ligand (ng/ml)

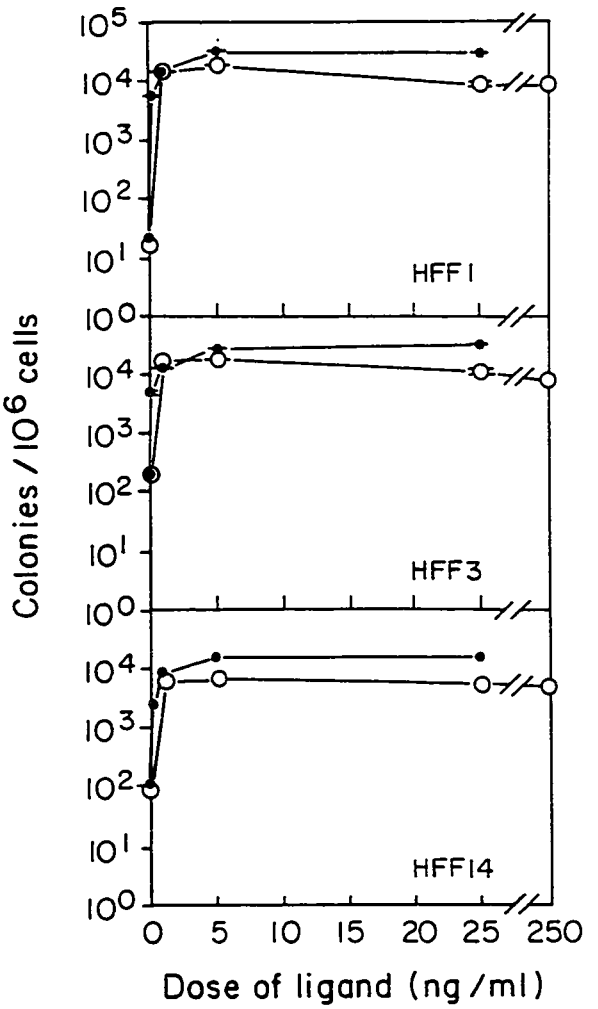
the diminished response of "older" populations of cells would correlate with a decrease in the number of competent cellular receptors. Binding studies were conducted on CEF at their 2nd, 14th and 21st population doublings. Based on ^{125}I-TGFα binding, the number of cell surface, erbB/EGF-receptors remained relatively constant at all population doublings tested (Table 1). This suggested that the reduced ligand responsiveness of the "aging" population was likely due to changes in intracellular signalling and probably not a consequence of changes in the receptors themselves.

E) Transformation of HFF by TGFα and EGF. Following the success of TGFα/EGF-induced CEF cell transformation, we extended our analyses to diploid human fibroblasts. Six independent HFF preps were analyzed for their responses to treatment with various doses of TGFα and EGF. Numerous HFF cultures were examined to ensure the representative nature of the result. The cells were plated into agar, treated with ligand and assayed for their ability to display anchorage-independent growth. The quantitative results from 3 of 6 HFF cultures are shown in Fig. 5, a related result from a 4th appears in a slightly different form in Fig. 6. Also shown in Fig. 5 are qualitative results from HFF 1, following graded doses of TGFα. All HFF cultures assayed were effectively transformed by both TGFα and EGF, though there were variations in the absolute magnitudes of

Fig. 5. TGFα/EGF-induced transformation in HFF.

Quantitative responses of three individual HFF strains, 1, 3 and 14, are shown following approximately 21 days growth in the presence of 0.0, 0.2, 1, 5 and 25 ng/ml TGFα and 1, 5, 25, and 250 ng/ml EGF. A qualitative representation of the TGFα results from strain HFF 1 is also shown(see photographs). For quantitative results, each experimental point reflects the results from four to five individual dishes; error bars represent standard deviation from the mean.


TGFα
0 ng/ml
0.2 ng/ml
1 ng/ml
5 ng/ml
25 ng/ml


Colonies / 10^6 cells
HFF1
HFF3
HFF14
0 5 10 15 20 25 250
Dose of ligand (ng/ml)

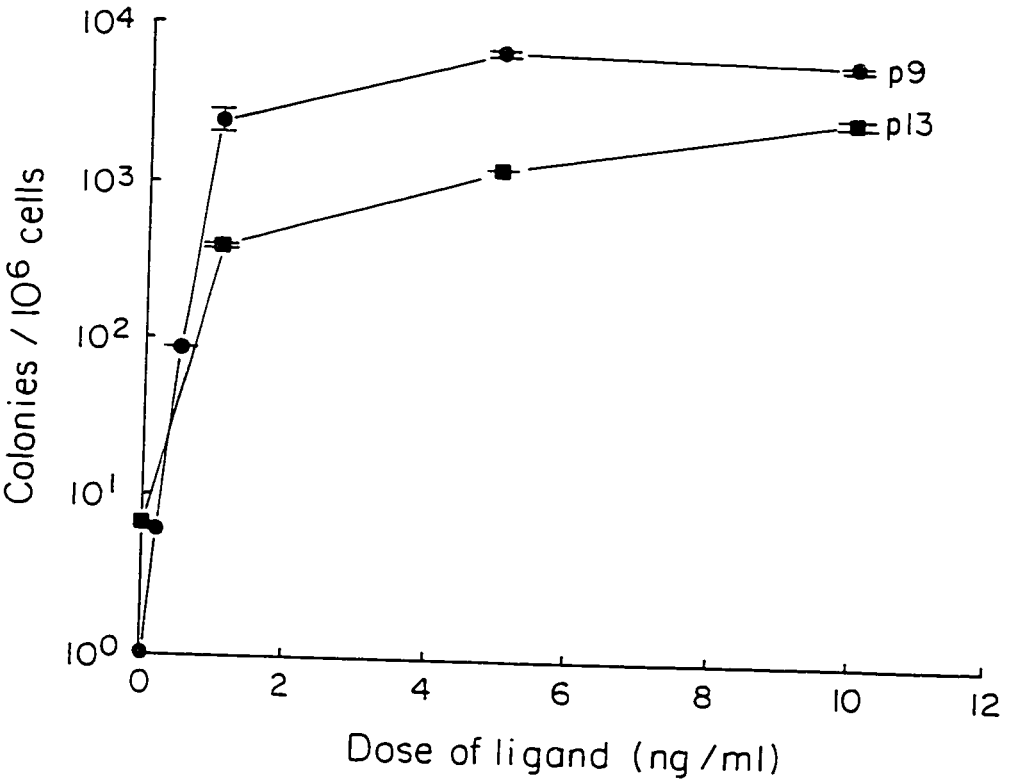
the individual responses. In virtually all cases, maximal colony development, approximately 1% colony formation, occurred at a ligand dose of 5 ng/ml and, in general, the cells responded similarly to both TGFα and EGF, unlike line 0 CEF. This similarity in biological response correlates nicely with the nearly equivalent binding affinity of the human EGF-receptor for TGFα and EGF (27). As was the case with CEF, a single treatment with either TGFα or EGF was sufficient to induce transformation, additional treatments resulted in larger-sized colonies. Interestingly, higher doses of either ligand above a level of 5 ng/ml, i.e. 250 ng/ml, either had little or no effect or gave rise to a slight decrease in transformation frequency.

F) The effect of culture age on TGFα/EGF-induced transformation in HFF. In light of our CEF results, we were eager to determine whether successive rounds of cell division would also diminish the response of HFF to ligand-induced transformation. If so, this might help solve the apparent paradox involving the relative ease with which HFF can be transformed by TGFα/EGF exposure in spite of the difficulty encountered in transforming these cells by oncogene transfection. In the latter case, selection for a cotransfected drug resistance gene and extensive expansion of a small number of resistant clones has been the approach used most frequently (40). To address this question we analyzed the responses of

several strains of HFF cells at various "ages" as measured by the number of population doublings the cultures had undergone. An example of this effect is shown in Fig. 6. In this HFF strain, strain 5, roughly a 10-fold decrease in responsiveness was detected, at doses of 1 and 5 ng/ml, after an increase of only 4 population doublings, from the 9th to the 13th. At a higher ligand dose, 10 ng/ml, the effect of increasing "age" was less pronounced. In an attempt to explain this phenomenon we thought it might be possible that HFF cultures consisted of subpopulations of cells with mixed sensitivities to TGFα/EGF. We hypothesized that upon culturing in the absence of TGFα or EGF the less responsive subpopulations might experience a growth advantage, and thus become the predominant population. Such an event could explain the observed loss of responsiveness of these cultures with increasing cell passage. If this were the case, then growth of the cultures in the presence of ligand should reverse the phenomenon. To address this possibility, HFF cultures, strain 5, were divided into two halves: one half was grown under standard conditions, while the other was grown in standard growth medium supplemented with 25 ng/ml EGF. The cultures were maintained accordingly for a period spanning approximately 15 population doublings, and portions of the cultures were frozen at intermediate stages. Once the desired number of doublings was achieved, cells at lower numbers of population doublings were thawed and all

Fig. 6. The effect of increasing population doublings on TGFα/EGF-induced transformation in HFF.

Cells from HFF strain 5, at their 9th and 13th population doublings, were plated into agar and treated with various doses of EGF (at passage 9, doses of 0.0, 0.2, 1.0, 5.0 and 10.0 ng/ml, and at passage 13, doses of 0.0, 1.0, 5.0, and 10.0 ng/ml) over a period of approximately 21 days. Fresh applications of EGF were administered at weekly intervals. Each experimental point reflects the results from four to five individual dishes; error bars represent standard deviation from the mean.


104
103
102
101
100
Colonies / 10⁶ cells
0
2
4
6
8
10
12
Dose of ligand (ng/ml)
p9
p13

cultures were grown for 48 hrs in standard grown medium to normalize conditions prior to soft agar analysis. The cells were then plated into soft agar and examined for their responsiveness to ligand-induced transformation following treatment with various doses of EGF. At the end of the experiment there was basically no difference in the responsiveness of cells grown in the presence or absence of EGF; all showed a similar decline in EGF-induced transformation with increasing "age". From these results we conclude that this phenomenon is likely an intrinsic feature of HFF, CEF cells and perhaps cells derived from primary cultures in general and not simply an artifact of a particular culture regimen. We also tested the cloning efficiency of cultures at population doublings which displayed decreased sensitivity to ligand-induced transformation and found basically no difference in this measure of intrinsic growth potential (data not shown). It would appear unlikely, therefore, that approaching culture senescence was in any way responsible for the observed phenomenon.

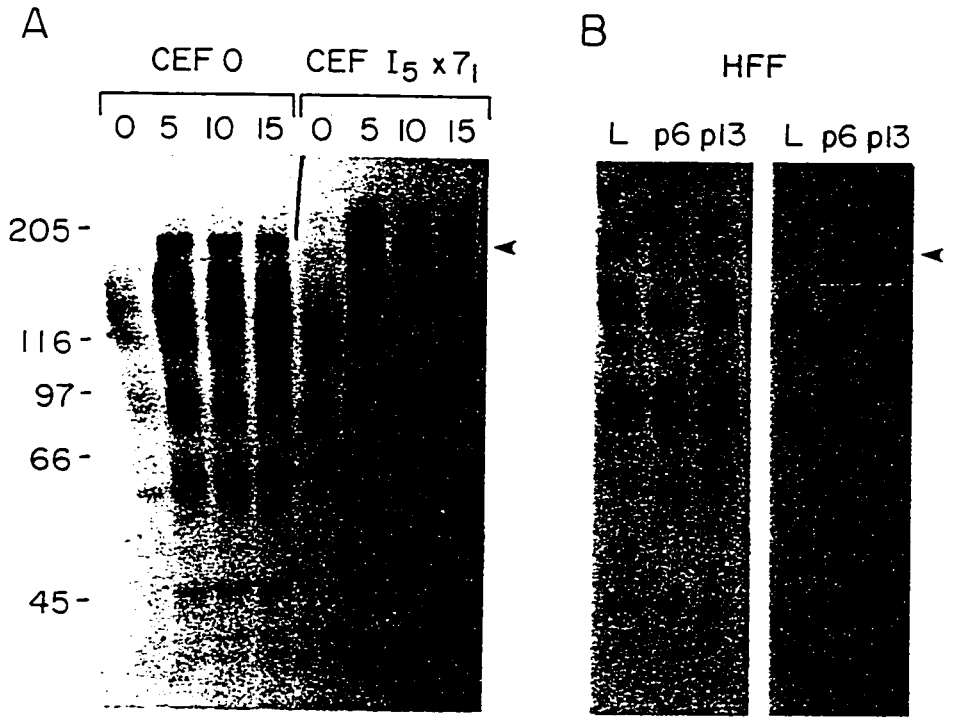
G) Analysis of ligand-induced phosphotyrosine activity in CEF and HFF. We next examined phosphotyrosine activity by western blotting to gain some sense of the biochemical consequences of ligand stimulation in CEF and HFF. Time course analyses were performed on all three CEF strains, lines 0, 151XI4 and 151I5X71, following exposure to TGFα and EGF.

Results from CEF strains 0 and 1515X71, following 0.0, 5.0, 10.0 and 15.0 min. exposure to 5 ng/ml TGFα, are shown in Fig. 7A. A very similar response was obtained with CEF strain 151XI4 (data not shown). Maximal phosphotyrosine activity was detected 5 to 15 minutes after ligand treatment and was manifest by an increase in band intensity, the appearance of novel bands, and a shift in the size of bands, in comparison to the untreated control. EGF treatment elicited an identical series of events, though it did so considerably less effectively; band intensities were considerably reduced in comparison to those resulting from TGFα treatment (data not shown). As shown in Fig. 7A, we detected very little difference in ligand-induced tyrosine phosphorylation among the three CEF strains examined. Thus, the unique biological responses seen among the different CEF strains are unlikely to be due to the relative abilities of their receptors to affect phosphorylation of abundant substrates.

Results obtained upon TGFα treatment of an HFF culture, strain 5, at its 6th, 13th and over its 20th population doubling, at 5 and 15 min. time points, are shown in Fig. 7B. Lane L is a result derived from a $G418^r$ culture which we estimate to have been somewhere above its 20th population doubling, as a result of G418 selection. The $G418^r$ population represented in lane L responded extrememly poorly to ligand-induced transformation. Despite considerable differences in the biological responses of the HFF cultures at the passages examined, we were unable to

Fig. 7 Phosphotyrosine analysis of CEF strains 0 and $15I_5X7_1$ and of HFF strain 5, at its 6th, 13th and over its 20th population doubling.

A) CEF cultures from strains 0 and $15I_5X7_1$ were treated with 5 ng/ml TGFα and, following an exposure time of 0.0, 5.0, 10.0 or 15.0 mins., were assayed for induced tyrosine phosphorylation. For each lane within a strain, equal amounts of protein were loaded; between the two strains, the amount of protein analyzed per lane was variable. B) Cultures of HFF 5 at their 6th, 13th and over their 20th population doubling were treated with 5 ng/ml TGFα and analyzed for induced tyrosine phosphorylation following 5.0 min. (left) or 15 min. (right) exposure. Lane L represents results from the culture over its 20th population doubling. Each lane was loaded with equivalent amounts of protein. For each set of results, arrowheads are used to mark the location of the endogeneous EGF receptor. Approximate size markers are provided.

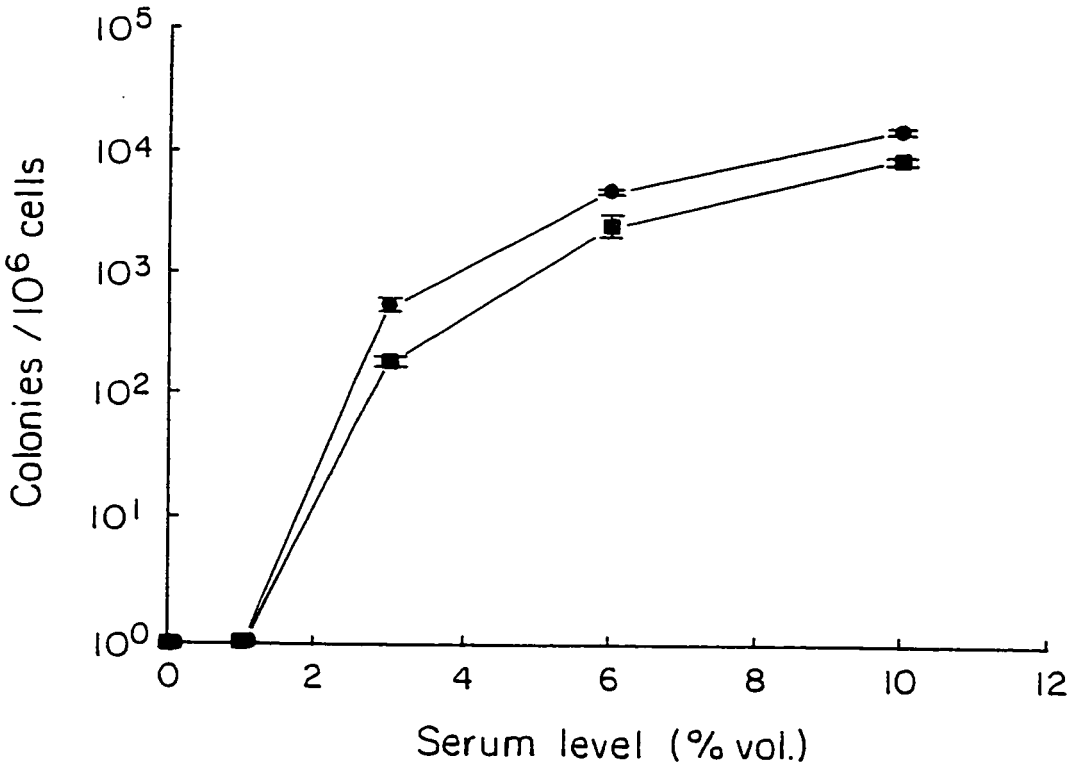

A
CEF 0
CEF I5 x7I
0 5 10 15 0 5 10 15
205-
116-
97-
66-
45-
B
HFF
L p6 p13 L p6 p13

detect an obvious correlation between reduced sensitivity to ligand-induced transformation and obvious changes in induced patterns of tyrosine phosphorylation. An identical result was obtained following similar analysis in CEF (data not shown).

H) The effect of serum concentration on TGFα- and EGF- induced transformation in HFF. During the course of our analyses we observed an effect of serum on the efficiency of TGFα/EGF-induced transformation. To address this issue more completely, the following efforts were undertaken. Experiments were conducted in which the level of ligand, TGFα, was maintained at a constant level (5ng/ml) while serum concentration (v/v) was varied (0, 1, 3, 6, and 10%). The results from two of these experiments are shown in Fig. 8. No colony development was detected at 0 or 1% serum. An appreciable cellular response was seen at 3% serum (~200 to 600 colonies per 10^6 cells), and a doubling of the serum concentration to 6% resulted in approximately a 10-fold increase in colony yield (~2000 to 6000 colonies per 10^6 cells). A final increase in serum concentration to 10%, the level at which our HFF experiments are normally conducted, led to only a 3 to 4 fold increase in colony yield (~9000 to 20,000 colonies per 10^6 cells), and the slope of this line suggested the effects of serum were nearing a plateau phase. A typical background response of these cells, at 10% serum in the absence of ligand stimulation, was ~20 colonies

Fig. 8 The effect of serum concentration on ligand-induced transformation in HFF.

Cells from HFF strain 1 were plated into agar containing serum concentrations of 0.0, 1.0, 3.0, 6.0, and 10.0% (v/v). The agar cultures were then treated with 5 ng/ml TGFα and allowed to grow for approximately 2 1 days, with weekly additions of fresh ligand. The results from two independent experiments are shown. Filled circles represent results from experiment #1; filled squares represent results from experiment #2. Each experimental point reflects the results from four to five individual dishes; error bars represent standard deviation from the mean.


Colonies / 10^6 cells
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
10
12
Serum level (% vol.)

per 10^6 cells. From these data it is clear that serum concentration does influence the response of cells following ligand stimulation. The exact reasons for this, however, are not readily apparent.

I) DISCUSSION

Chicken embryo fibroblasts have been frequently used to study oncogene transformation because they can be efficiently transformed by many acute retroviruses including those which carry single oncogenes. Recently, the utility of this assay system was significantly enhanced by the development of a transfection protocol which allows single oncogenes to be assayed in CEF without the need for complementing oncogenes (1). CEF cells offer unique advantages for the study of nuclear oncogenes such as *myc* and *jun*, genes which cannot be easily studied in immortalized rodent cell lines. We undertook the present study to determine whether CEF would be sufficiently sensitive to detect transforming activities following exogeneous application or autocrine release of growth factors. If so, this would provide a more "natural" setting in which to study events in TGFα- and EGF-induced transformation (see below). We have also been interested in the differential effects of TGFα and EGF on the chicken erbB/EGF-R. The chicken EGF-R binds TGFα with an affinity 100-200 fold higher than that of EGF. However, until

now the biological consequeces of this differential affinity have not been examined. In this study we have shown: 1) CEF can be readily transformed by exogeneous application or autocrine release of TGFα and EGF. The response following autocrine ligand production was much more potent. 2) The chicken EGF-R, on all CEF strains tested, displayed a 100-fold higher affinity towards TGFα than EGF, consistent with previous reports based on a mouse cell line overexpressing a chicken EGF-R cDNA (27). 3) Though all CEF strains examined were transformed by ligand treatment, as defined by anchorage-independent growth, the transforming potential of either ligand at a given dose was found to be dependent upon the genetic background of the CEF strains. Quantitatively, at higher ligand doses (>= 5ng/ml), lines 151x14 and 1515x71 responded quite similarly to either ligand, whereas line 0 was much more responsive to TGFα. At doses <= 1ng/ml a differential biological response was detected in all CEF strains. At these doses, TGFα was considerably more efficient than EGF in inducing cellular transformation. This effect was particulary pronounced in the case of line 0 CEF. Qualitatively, all three CEF strains displayed more vigorous colony formation in response to TGFα.

We assayed the CEF strains to determine their receptor numbers, ligand-binding affinities, and patterns of ligand-induced tyrosine phosphorylation. In each instance, these characteristics were found to be quite similar despite the unique

biological responses among the three strains. These data suggest that the rate-limiting steps in receptor signal transduction do not occur at the level of receptor-ligand interaction, but rather further downstream. The data suggest the presence of important differences in the signalling pathways of CEF derived from line 151xI4 and 1515x71 birds. These differences may account for their inability to discriminate between low and high affinity ligands, after a particular point, and their enhanced growth potentials at given ligand concentrations, as compared to line 0 CEF. 151xI4 and 1515x71 birds are bred for their susceptibility to retrovirally-induced lymphoma and leukemia. Whether there is a direct link between sensitivity to in vitro transformation and in vivo disease susceptibility remains to be investigated. Currently, experiments are underway to determine whether there are differences in the phosphorylation of several of the known signal substrates (PLCc, GAP, PI3-kinase) among the three CEF strains.

Secretion of TGFα is a fairly common feature of human carcinomas (12). This observation and our CEF cell results compelled us to extended our analyses to normal, diploid, human fibroblasts. We found that HFF could also be transformed by exogeneous EGF and TGFα treatment. In this case, EGF and TGFα bound the EGF-receptor with equal affinities and induced cellular transformation with comparable efficiencies in each of the six independent HFF preparations we

tested. The transformation frequency was affected by both age and serum concentration. The relative ease with which we were able to transform human diploid fibroblasts, while echoing results from our CEF studies, were initially suprising to us. Human fibroblasts are believed to be particularly resistant to oncogene transformation (16, 23, 26, 40). Furthermore, earlier attempts to transform immortalized rodent cell lines by simple exogeneous EGF treatments failed. These immortalized lines, considered more easily transformed than normal fibroblasts, could not be transformed by TGFα and EGF unless: 1) additional copies of the EGF-R gene were introduced to establish higher receptor levels (10^5 to 10^6 molecules per cell) or 2) an expressible EGF gene was introduced to establish an autocrine loop. We wish to note, however, that the approach taken here differs from many other human oncogene transformation studies in one important aspect - the "age" of the cultures under analyses. Human oncogene studies have typically been conducted by way of cotransfection with and protracted selection for a selectable marker gene. As shown here, the transforming responses of normal fibroblasts may have an important age-related component. Cultures which have undergone strenuous selection regimens, containing cells which have undergone extensive rounds of cell division, may exhibit reduced susceptibility to certain transforming events. Immortalized cell lines by their very nature consist of aged cell

populations. Perhaps this is in part the explanation why some of these lines are resistant to simple growth factor-induced transformation. We hypothesize that "young" fibroblasts carry a complementary function that is transiently expressed and is responsible for their heightened sensitivity to cellular transformation. Based on ligand binding and kinase activation studies, this complementary function cannot be centered on the receptor molecules themselves. Curiously, the decrease in ligand-induced transformation we detected upon increasing numbers of population doublings in HFF was not accompanied by a corresponding decrease in mitogenic resonsiveness.

While Palmer et al. previously demonstrated the transforming potential of PDGF in HFF, their data on EGF-induced transformation was inconclusive (33). At serum levels we found necessary for efficient EGF-induced soft agar colony formation, these investigators observed a high frequency of ligand-independent colonies. This high background may have impaired their ability to detect EGF-induced transformation. Under the conditions of our analyses, background frequencies were on the order of 0.001 to 0.01%, at least 100-fold less than the frequencies of ligand-induced colonies. Once again, the issue of culture age may be an important consideration in explaining the apparent discrepancy between the two results. Our analyses were done with young HFF cell cultures. We do not believe our findings are based on a chance discovery of an atypical HFF

result since we have tested HFF's from six independent preparations and have obtained consistent results in each case. These two studies nevertheless both support the conclusion that exogeneous application of growth factors can lead to HFF transformation and that serum concentrations are important in the development of EGF-induced transformation. The exact reasons for the importance of serum are not at all clear. Whether serum is important because 1) it provides essential nutritional components or 2) it provides ancillary factors which support EGF-induced transformation are issues which remain unanswered.

The acquisition of an anchorage-independent phenotype has been shown to be one of the best in vitro correlates with in vivo tumorigenesis (18, 25, 30, 41). We have tested the tumorigenicity of the TGFα virus-infected CEF and were able to detect nodule formation at the site of injection. However, these nodules later regressed, an occurrence we occasionally experienced during our previous studies with oncogene-transfected CEF. It is important to note, in this regard, that the birds used for these studies were immunocompetent and were from a chicken line not yet sufficiently inbred to be histocompatible. Our attempts to transfect the TGFα gene into HFF for tumorigenicity testing in nude mice were confounded by the effects of increasing culture age. The G418 selection used to enrich the population for successfully transfected cells resulted

in a fairly aged cell culture which displayed only a low frequency of soft agar colony formation on its own and even in the presence of exogeneously added ligand. An alternative approach would be to use an amphotropic retrovirus carrying the TGFα gene to efficiently infect young HFF cultures. However, biosafety concerns have dampened our enthusiasm for pursuing this line of research.

Finally, studies of TGFα-induced transformation in NRK cells demonstrated the need for an accompanying factor, TGFβ, in the induction of transformation by exogeneously added TGFα (51). In similar studies with CEF and HFF cells, we detected no appreciable effect of TGFβ on TGFα-induced transformation at TGFβ levels of 1 or 10 ng/ml.

In summary, we have shown that simple, exogeneous treatment with either TGFα or EGF is sufficient to induce transformation in chicken embryo fibroblasts and normal, diploid, human fibroblasts. In both instances, standard growth conditions supported the transforming potentials of TGFα and EGF, no complimenting genes or additional factors were required. Furthermore, we have identified parameters which are important determinants of the magnitudes of the ligand-induced responses. We believe our results reflect a natural and normal response of cells to an otherwise aberrant scenario: sustained and persistent receptor interactions with activating ligands. The systems examined here did not require receptor

amplification or immortalizing functions to support their biological responses. As a result they may provide more meaningful and physiologically relevant environments for the study of substrates involved in EGF- or TGFα-induced transformation. These systems may also prove invaluable in defining the role(s), if any, played by these molecules in human neoplasia.

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CHAPTER 4

SIGNIFICANCE AND FUTURE STUDIES

Chapter 2 of this thesis details a series of studies which examine the utility of direct DNA transfection into CEF cells. At the time these studies were initiated it was felt that CEF cells were refractory to direct DNA transfection. For such experiments to be efficient it was felt that an associated helper virus was required to spread the gene of interest throughout the culture by way of the generation of infectious hybrid virus particles. It is important to note that this impression developed at a time when it was fairly common to transfect cells with genomic tumor DNA as opposed to more highly purified cloned DNA. In addition, these early studies did not include the use of selectable marker genes to positively identify successfully transfected cells and eliminate the unsuccesfully transfected population. The studies outlined in chapter 2 clearly demonstrate that CEF cells can be successfully transfected by direct DNA transfection without the need for an associated helper virus and that these transfections can routinely achieve efficiencies comparable to those of immortalized rodent cell lines. Further, it is shown that: 1) cotransfection with a selectable marker gene is an essential component for successful CEF transfection studies, 2) transfected CEF cells can reliably descriminate between oncogenic and nononcogenic forms of

genes and 3) CEF cells transfected with various oncogenes or oncogene combinations can score positively in tumorigenesis assays when injected into the wing-webs of young chicks, under the proper conditions.

There is at least one additionally important finding from the CEF cell transfection studies which is perhaps not so readily apparent, namely, the exquisite sensitivity of these cells in detecting transforming potentials. Our grasp of this sensitivity led us to the pivotal experiments of chapter 3 where we examined the transforming potential of TGFα and EGF in CEF cells and HFF. The potential role(s) of TGFα/EGF in transformation and tumorigenesis is an important issue as many forms of human carcinomas are known to secrete TGFα. In chapter 3 it is shown that both TGFα and EGF are capable of transforming CEF and HFF to an anchorage-independent phenotype. Also shown in chapter 3 are results from three independent strains of CEF which despite very similar Kd's, receptor numbers, and patterns of tyrosine phosporylation respond quite uniquely to TGFα- and EGF-induced transformation.

The data from chapter 3 establishes a base for a continiung series of experiments to more extensively address the role(s) of TGFα and EGF in cellular transformation and tumorigenesis. The unique biological responses of CEF strains 15_1XI_4 and $15I_5X7_1$ versus line 0 in response to EGF-induced transformation may be

an important clue to critical events downstream of receptor/ligand interactions important in the transformation process. Though crude western analysis of TGFα/EGF-induced tyrosine phosphorylation could basically detect no differences among the three strains of CEF examined, the sensitivity of these analyses are such that only the phosphorylation of abundant proteins would be readily detected. Less abundant yet strategically important tyrosine-phosphorylated proteins might go undetected in these analyses. An alternative, more sensitive, approach would be to analyze individual proteins via immunoprecipitation. Among the first proteins to be analyzed in this fashion should be PLCχ, GAP, the *raf* protein, PI-3 kinase, MAP kinase and P53.

While the acquisition of an anchorage-independent phenotype is one of the best transformation parameters in terms of correlations with tumorigenesis it is still only a single measure of transformation. It would be useful and would build a stronger case if additional transformation assays were performed on TGFα/EGF-treated CEF and HFF cell populations. Among these assays might be an immunofluorescent examination of actin filament rearangements in response to ligand treatments. A very powerful additional transformation assay would be focus formation or the ability to overcome contact-inhibition. However, because of the nature of the inducing event it will be difficult if not impossible to design a

scenario to make this approach feasible.

Additional CEF cell studies should readdress the issue of TGFα-induced tumorigenesis. The relevant tumorigenesis data presented in chapter 3 refers to quickly-forming nodules at the site of injection which then rapidly regress but these data are the results of only a single experiment. Perhaps tumors would develop and grow progressively if greater numbers of cells were introduced initially, or if the cells were introduced into newly hatched chicks instead of chicks several days old. The best candidates for these studies will be CEF cells infected or multiply infected with the one-round TGFα virus.

The most powerful and defining experiment will be to establish an HFF culture which constitutively expresses EGF or TGFα, via viral infection, and to test the tumorigenic character of these cells by injecting them into immune-compromised mice. Perhaps here more than anywhere else it's important to keep things in perspective. Cancer is believed to involve a multi-step process. At best, with the development of an HFF culture constitutively expressing TGFα we would simply be providing a continuous supply of activating ligand for a single signal pathway. We will not have mutated any tumor suppressor genes or other genes frequently altered in human cancers. As an investigator it would be terribly intriguing if constitutive expression of TGFα would confer a tumorigenic phenotype to an otherwise normal HFF cell population.

CHAPTER 5

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