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Genetic determinants of neoplastic transformation by the retroviral oncogene v-*erbB*

(protooncogene/tumorigenesis/protein-tyrosine kinase/epidermal growth factor receptor)

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ABSTRACT **The retroviral oncogene v-*erbB* is a mutant version of the gene (c-*erbB* or *ERBB1*) that encodes the cell-surface epidermal growth factor receptor (EGFR). The mutations take three forms: (*i*) a large deletion that removes the entire ligand-binding domain of EGFR, (*ii*) smaller deletions that affect the carboxyl-terminal domain of EGFR, and (*iii*) point mutations that cause conservative substitutions of amino acids. Previous work has shown that, in the absence of the large deletion, *ERBB1* cannot transform cells autonomously. Here we report that when the large deletion is present, no other mutation is required for *ERBB1* to transform established rodent fibroblasts to a tumorigenic phenotype. In particular, there is no need for deletions affecting the carboxyl terminus of the gene product. It appears, therefore, that removal of the ligand-binding domain from the EGFR suffices to create a transforming protein. Deletions at the carboxyl terminus of the EGFR apparently play only a secondary role in transformation by affecting the host range and perhaps the potency of transformation; and there is as yet no evidence to implicate point mutations in the activation of *ERBB1* to an oncogene. Our findings support the view that augmented activity of the EGFR can contribute to tumorigenesis.**

Avian erythroblastosis virus is a retrovirus that induces erythroleukemias and fibrosarcomas in susceptible birds (1, 2). The tumorigenicity of the avian erythroblastosis virus is attributable principally to the oncogene v-*erbB*, which arose by transduction of c-*erbB* (3, 4)—the gene that encodes the epidermal growth factor receptor (EGFR) (5). The human counterpart of c-*erbB* is known as *ERBB1*.

The most widely studied alleles of v-*erbB* are found in the H and ES-4 strains of avian erythroblastosis virus (2, 6, 7). Both alleles differ from the gene encoding EGFR in three ways that may contribute to neoplastic transformation by the viral oncogene (see Fig. 1). (*i*) A large deletion has removed virtually the entire extracellular domain of the EGFR (8) and is thought to confer constitutive activity on the protein-tyrosine kinase of the receptor (9–11). (*ii*) A smaller deletion has removed a portion of the carboxyl terminus of the receptor (8, 12); this deletion influences the host range of transformation (13, 14) and has been reported to be essential for transformation of avian fibroblasts by the product of v-*erbB* when the protein is fused to the ligand-binding domain of the EGFR (15). The ES-4 allele also has an internal deletion near the carboxyl terminus of the protein, the effect of which remains uncertain (refs. 7, 12, 16, and 17 and M. Dolan, J. Jackson, and J.M.B., unpublished data). (*iii*) Point mutations have introduced amino acid substitutions into the protein encoded by v-*erbB* (7, 12, 16, 17); the physiological effects of these mutations have not been explored.

In an effort to elucidate the role of genetic damage in the biological activity of v-*erbB*, we have created and characterized chimeric genes in which the bulk of v-*erbB* has been replaced by corresponding domains from *ERBB1*. Our results show that these chimeras can transform established lines of rodent fibroblasts to a tumorigenic phenotype. We conclude that the normal kinase domain of EGFR can mediate neoplastic transformation, as shown previously by other experimental strategies (18–20), and that deletions of the carboxyl-terminal domain of the EGFR are not a requirement for transformation—although they can affect the host range and perhaps the potency of transformation (refs. 14 and 20 and see below).

MATERIALS AND METHODS

Hybrid alleles were constructed from cDNAs representing v-*erbB* (ES-4) and human *ERBB1* (A. Bruskin, A.W., and J.M.B., unpublished data). The cDNAs were fused at a shared *Bam*HI site near the amino terminus of the kinase domain of *ERBB1* (Fig. 1*A*), corresponding to nucleotide 2378 of the human cDNA (8). Portions of the carboxyl terminus of the coding domain for *ERBB1* were deleted by inserting a termination codon (purchased from Pharmacia) at nucleotides 3625 (a *Hin*cII site), 3758 (a *Pvu* II site), or 4206 (a *Hin*dIII site) to give alleles designated *erb-HR-Hc*, *erb-HR-Pv*, and *erb-HR-H3*, respectively (see Fig. 1).

The various alleles were inserted into a murine leukemia virus (MuLV) vector that also carried the gene for neomycin resistance, driven by the early promoter of simian virus 40 and permitting selection for resistance to the antibiotic G418 (Fig. 1*A*). The vector was a gift from M. Scott (University of California, San Francisco) and has been described elsewhere (21). We generated virus stocks from ψ-2 cells (22), pooling in excess of 200 individual clones to avoid use of a single spontaneous variant. The titers of virus stocks were obtained by enumerating the formation of G418-resistant colonies in infected NIH 3T3 cells. Transforming potential was evaluated with two separate viral stocks for each allele, without appreciable discrepancies.

Rat-1 or NIH 3T3 cells were infected in the presence of Polybrene (2 μg/ml; Sigma). The cells were propagated for 36 hr in Dulbecco's modified Eagle's medium, then trypsinized and divided into four equal parts for propagation as follows: (*i*) in standard growth medium, for detection of transformed foci; (*ii*) in growth medium containing 0.45% agarose (FMC, Rockland, ME) to assess anchorage-independent growth; (*iii*) in growth medium containing G418 (300 μg/ml; GIBCO) to enumerate cells resistant to killing by the antibiotic; and (*iv*) in growth medium containing G418, to prepare populations of cells for evaluation of morphology, tumorigenicity, expres-

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Abbreviations: EGFR, epidermal growth factor receptor; MuLV, murine leukemia virus.

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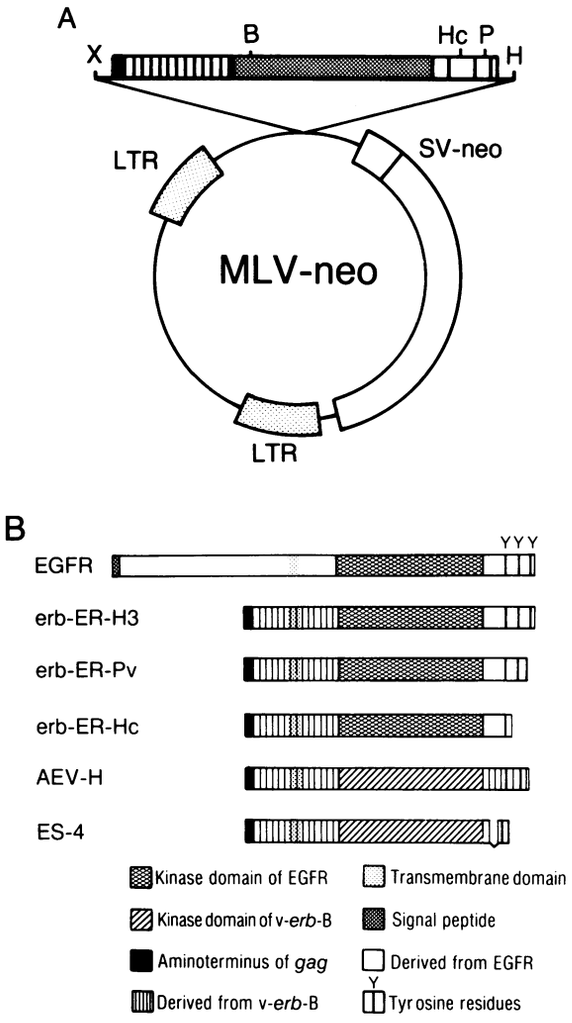

FIG. 1. Chimeric alleles of v-*erbB* and *ERBB1*. Chimeric alleles were constructed from cDNA clones of v-*erbB* and *ERBB1*. (*A*) MuLV vector employed to express the chimeras in rodent cells. (*B*) Topography of the chimeras and representative alleles of v-*erbB*. X, *Xho* I; B, *Bam*HI; Hc, *Hin*cIII; P, *Pvu* II; H, *Hin*dIII; LTR, long terminal repeat; and SV-*neo*, the bacterial gene for neomycin resistance driven by the early transcriptional promoter of simian virus 40.

sion of genes carried by the viral vector, and proviruses arising from the vector.

Cells were labeled with [^{35}S]methionine and analyzed by immunoprecipitation or immunoblots, all as described (23, 24). The analyses were performed with two monoclonal antibodies (291-3 and 291-4) raised against the cytoplasmic domain of EGFR (R. Schatzman, A.W., and J.M.B., unpublished data) and a polyclonal antiserum raised against a peptide located at the amino terminus of v-*erbB* (23).

RESULTS

Chimeric Alleles of v-*erbB* and *ERBB1*. Hybrid alleles were constructed by fusing cDNAs for v-*erbB* and *ERBB1* at a shared *Bam*HI site in the amino-terminal portion of the kinase domain (Fig. 1*A*). The resulting chimeras were congenic with v-*erbB*, except where substituted with regions from *ERBB1* (Fig. 1*B*). Translation initiated from the AUG codon normally used to express the wild-type allele of v-*erbB*.

The three chimeras were designed to contain one (*erb-ER-Hc*), two (*erb-ER-Pv*), or all three (*erb-ER-H3*) of the tyrosine

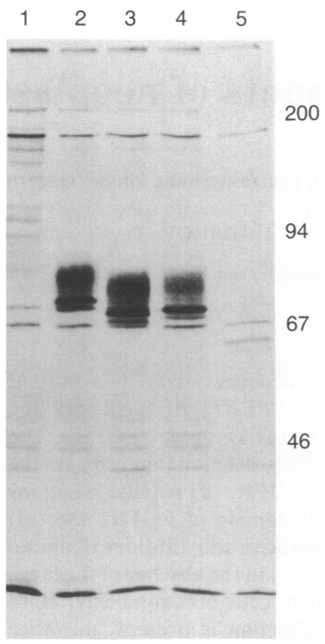

FIG. 2. Proteins expressed from chimeric alleles in rat cells. Rat-1 cells expressing various alleles of *erbB* carried in an MuLV vector were labeled with [^{35}S]methionine and analyzed by immunoprecipitation. Samples (two million cells) included cells carrying an unexpressed version of *ERBB1* (lane 1) and cells transformed by *erb-ER-H3* (lane 2), *erb-ER-Hc* (lane 3), *erb-ER-Pv* (lane 4), or v-*erbB* (lane 5). Molecular masses of marker proteins in kDa are indicated.

residues whose phosphorylation may regulate the activity of EGFR (25, 26). The *erb-HR-Hc* and *erb-HR-Pv* chimeras resemble (but are not identical to) the ES-4 and H alleles of v-*erbB*, respectively (see Fig. 1*B*), whereas the *erb-HR-H3* chimera resembles an allele of chicken c-*erbB* that was mutated by insertion of retroviral DNA and that appears to transform only erythroid cells (20). Two additional alleles were employed as controls: (*i*) a complete cDNA for v-*erbB* (ES-4) and (*ii*) an *ERBB1* cDNA that could not give rise to a functional protein because the region encoding the amino-terminal domain of the EGFR (including the signal sequence and initiation codon) had been deleted (leaving nucleotides 479–4206). All cDNAs were inserted into an MuLV vector that also carried the gene for neomycin resistance, encoding resistance to the antibiotic G418 (Fig. 1*A*).

Expression of Chimeric Alleles in Infected Cells. The efficacy of the viral vectors was documented by evaluating the synthesis of protein encoded by the chimeric alleles. Infected cells were labeled with [^{35}S]methionine and analyzed by immunoprecipitation (Fig. 2). Lacking a single antiserum that reacted effectively with all of the gene products under study, we used a combination of two monoclonal antibodies that react well with human EGFR, but poorly with the product of

Table 1. Effects of *erbB* alleles on Rat-1 cells

Allele	Morph. trans., %	Colonies in soft agar, %	Tumorigenicity, latency in days	
			10^6 cells	10^5 cells
None*	0	0	NO	NO
Unexpressed *ERBB1*	0	0	NO	NO
erb-ER-H3	17	0	13–14	26–27
erb-ER-Hc	16	0	12–14	21–26
erb-ER-Pv	20	0	13–14	26–29
v-*erbB* (ES-4)	47	35	9–10	19–21

To establish morphological transformation (Morph. trans.) and growth in soft agar, at least 1000 colonies were tested. Data are presented as percent of total G418-resistant colonies. Tumorigenicity was scored as the number of days until palpable tumors (diameter, ≈2 cm) appeared after either 10^6 or 10^5 cells were injected. All tumors progressed to a severity that required sacrifice of the animals. NO, none observed over a period of 75 days.
*MuLV vector alone.

Table 2. Effects of *erbB* alleles on NIH 3T3 cells

Allele	Morph. trans., %	Colonies in soft agar, %	Tumorigenicity, latency in days	
			10^6 cells	10^5 cells
None*	0	0	NO	NO
Unexpressed *ERBB1*	0	0	NO	NO
erb-ER-H3	12	0	20–22	34–37
erb-ER-Hc	20	0	20–20	36–37
erb-ER-Pv	20	0	20–22	34–35
v-*erbB* (ES-4)	90	40	14–16	20–22

To establish morphological transformation (Morph. trans.) and growth in soft agar, at least 1000 colonies were tested. Data are presented as percent of total G418-resistant colonies. Tumorigenicity was scored as the number of days until palpable tumors (diameter, ≈2 cm) appeared after either 10^6 or 10^5 cells were injected. All tumors progressed to a severity that required sacrifice of the animals. NO, none observed over a period of 75 days.
*MuLV vector alone.

v-*erbB* (R. Schatzman, A.W., and J.M.B., unpublished data). Each of the three chimeric alleles gave rise to three recognizable products—two that appeared as sharp bands in polyacrylamide gels and represented partially glycosylated intracellular forms of erbB protein, and a third that migrated diffusely and represented the fully glycosylated cell-surface form of the gene product (27–30). The apparent molecular weights for the three proteins ranged from 70,000 to 85,000, as anticipated from the sizes of the wild-type proteins. The analogous products of v-*erbB* were detected weakly by immunoprecipitation with the monoclonal antibodies (not visible in Fig. 2, lane 5). The specificity of the detections was documented by analyzing cells infected with a vector carrying the unexpressed form of *ERBB1* (Fig. 2, lane 1). Similar results were obtained with all the alleles by immunoblotting with the same antisera.

Immunoprecipitations were also performed with an antiserum raised against an epitope in the extracellular domain of the v-*erbB* product (23). The antiserum displayed equivalent but weak affinity for the products of the three chimeras and v-*erbB* (R. Schatzman, A.W., and J.M.B., unpublished data). Analysis with this antiserum revealed that all of the alleles under test gave rise to roughly equal amounts of protein when carried in the MuLV vector (data not shown).

Transformation by Chimeric Alleles in Cell Culture. Colonies of G418-resistant cells were selected from infected cultures of Rat-1 and NIH 3T3 cells and then evaluated for morphological transformation. Between 15 and 20% of the colonies carrying chimeric alleles appeared transformed, whereas v-*erbB* elicited transformation in ≈50% of Rat-1 colonies and ≈90% of NIH 3T3 colonies (Tables 1 and 2).

Pools of colonies carrying each of the alleles were propagated until transformed cells predominated and then were evaluated by photomicroscopy. The changes elicited by all of the alleles in Rat-1 cells were subtle, although transformation by v-*erbB* was marginally more apparent than that by any of the chimeric alleles (Fig. 3). Transformation of NIH 3T3 cells was more obvious and was equally apparent with v-*erbB* and the chimeric alleles (Fig. 4).

Antibiotic-resistant colonies were also tested in soft agar for anchorage-independent growth (Tables 1 and 2). Both Rat-1 and NIH 3T3 cells carrying v-*erbB* grew in soft agar with an efficiency of 35–40%. By contrast, the chimeric alleles elicited no detectable growth in soft agar (efficiencies were <0.1%).

Tumorigenicity of Chimeric Alleles. The ability of infected cells to form tumors in experimental animals was evaluated in syngeneic rats with Rat-1 cells (Table 1) and in nude mice with NIH 3T3 cells (Table 2). Polyclonal pools of cells transformed by each of the chimeric alleles formed tumors that had a slightly greater latency than did the tumors elicited

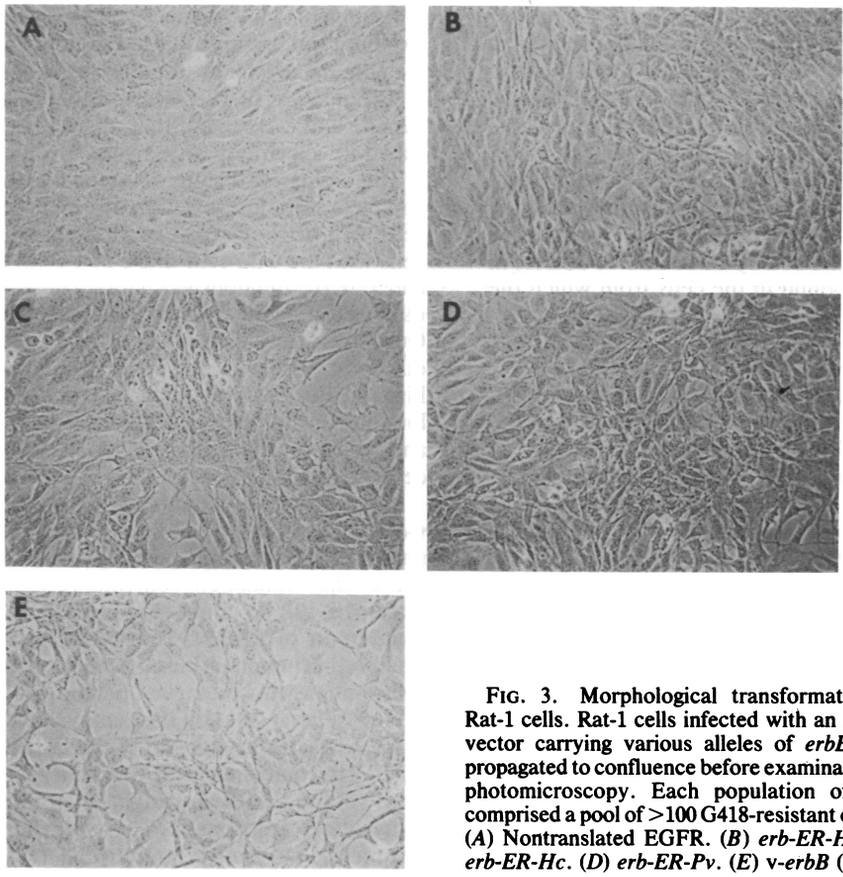

FIG. 3. Morphological transformation of Rat-1 cells. Rat-1 cells infected with an MuLV vector carrying various alleles of *erbB* were propagated to confluence before examination by photomicroscopy. Each population of cells comprised a pool of >100 G418-resistant clones. (*A*) Nontranslated EGFR. (*B*) *erb-ER-H3*. (*C*) *erb-ER-Hc*. (*D*) *erb-ER-Pv*. (*E*) v-*erbB* (ES-4).

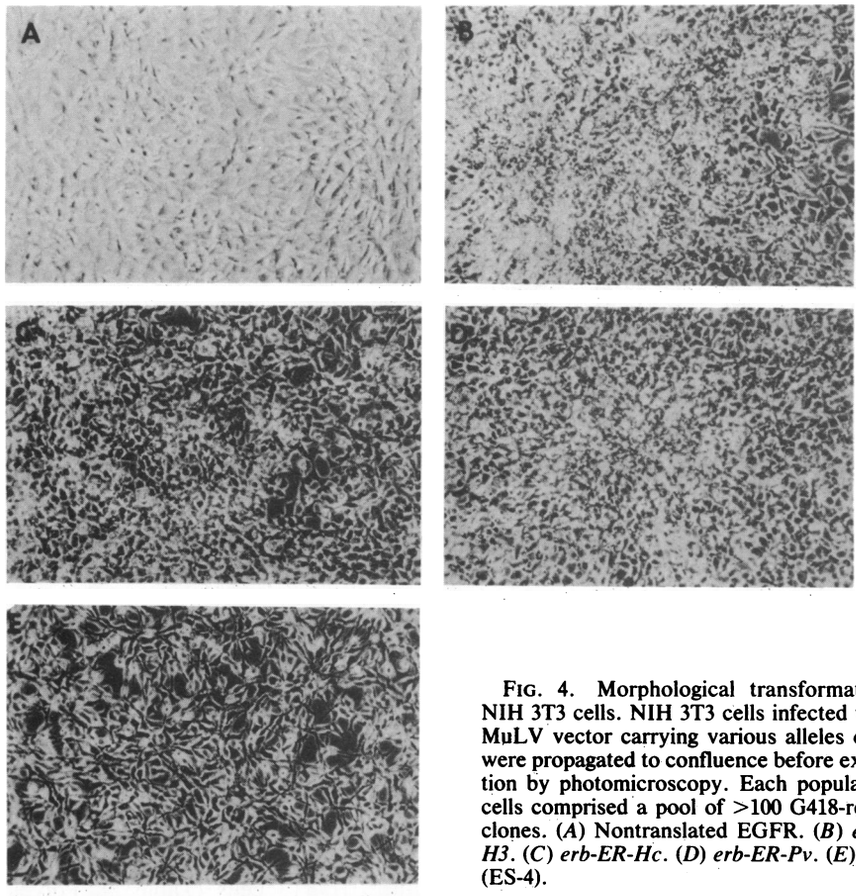

FIG. 4. Morphological transformation of NIH 3T3 cells. NIH 3T3 cells infected with an MuLV vector carrying various alleles of *erbB* were propagated to confluence before examination by photomicroscopy. Each population of cells comprised a pool of >100 G418-resistant clones. (*A*) Nontranslated EGFR. (*B*) *erb-ER-H3*. (*C*) *erb-ER-Hc*. (*D*) *erb-ER-Pv*. (*E*) v-*erbB* (ES-4).

by v-*erbB*. The genesis of the tumors was evaluated in two ways.

First, restriction mapping was used to evaluate the number and configuration of vector proviruses in DNA obtained from explanted tumor cells. The results indicated that all of the tumors were composed of numerous clones of infected cells, as anticipated if the tumors arose from many of the inoculated clones rather than from rare variants (data not shown).

Second, tumors arising from two pools of cells were explanted into cell culture and examined by immunoblotting for the presence of ERBB protein. The analysis documented the presence of proteins encoded by the chimeric alleles, in abundance similar to that found in the cells from which the tumors arose (data not shown).

DISCUSSION

Chimeras Between v-*erbB* and *ERBB1*. Our principal objective was to determine whether mutations affecting the cytoplasmic domain of EGFR are required to create an oncogene from *ERBB1*. To achieve this objective, we incorporated appropriate portions of *ERBB1* into chimeric genes that were otherwise congenic with v-*erbB*. Expression of v-*erbB* is accomplished by splicing that joins the first six codons of the viral gene *gag* to the transduced portion of c-*erbB*. The *gag* component of this fusion provides the initiation site for translation. In addition, the presence of the *gag* gene amino acids at the amino terminus of the v-*erbB* product substantially enhances the ability of the protein to transform cells in culture—for reasons that are not yet known (A. Bruskin, R. Schatzman, and J.M.B., unpublished data). The chimeric genes that we tested included these crucial features.

Transforming Activity of the Chimeric Alleles. Although the chimeric alleles elicited morphological transformation and tumorigenic growth, they proved less potent than v-*erbB* in either of these properties, and they failed to elicit anchorage-independent growth (see Tables 1 and 2). We have not explored the origin of these differences by experiment. But we note the existence of point mutations that distinguish the kinase domain of v-*erbB* from the kinase domains of both *ERBB1* and c-*erbB* and that might alter the potency and/or substrate specificity of the gene product (8, 16, 17).

The biological testing of oncogenes is conventionally not complete without assays performed in normal embryonic cells. We were unable to transform cultures of rat embryo cells with the wild-type allele of v-*erbB* (A. Bruskin, E. Liu, and J.M.B., unpublished data). These findings discouraged us from testing the chimeric alleles in embryonic rodent cells. Our failure to transform embryonic rodent cells with v-*erbB* does not reflect a universal property of protein-tyrosine kinases. For example, morphological transformation of diploid human fibroblasts has been reported with v-*erbB* (31), and the retroviral oncogene v-*src* transforms embryonic rat cells with great efficacy (32).

Molecular Determinants of Neoplastic Transformation by v-*erbB*. The wild-type allele of *ERBB1* cannot transform cells unless the gene product is expressed in exceptional abundance and stimulated constitutively by epidermal growth factor (18, 19). By contrast, v-*erbB* (derived from the chicken counterpart of *ERBB1*) transforms cells autonomously and elicits lethal tumors in birds. Several structural features might contribute to this biological potency.

First, a large deletion has removed all but a small remnant of the extracellular domain of the EGFR (8). The deletion is reputed to confer constitutive activity on the protein-tyrosine kinase of the EGFR and might, therefore, be responsible for neoplastic transformation by v-*erbB* (9–11). Our results sustain this suggestion by showing that neither point muta-

tions nor deletions in the cytoplasmic domain of EGFR are required for neoplastic transformation.

Second, a small carboxyl-terminal deletion removes a domain of the EGFR that may exert an allosteric effect on the kinase activity of the receptor (8, 12, 26). Phosphorylation of tyrosine residues within this domain accompanies activation of the EGFR and may be required to achieve or sustain the activation (25, 26). Alleles of v-*erbB* that retain the domain transform avian erythroid cells but not avian fibroblasts (14, 20). These last findings are nominally in conflict with the results reported here, but they were obtained with primary cultures of avian cells rather than with established cultures of mammalian cells and with the chicken rather than the human version of the EGFR. Either of these variables might contribute to the discordance among the results.

Other workers have argued that the deletion at the carboxyl terminus of v-*erbB* can be "predominantly responsible for" transformation of rodent fibroblasts (15). This conclusion was reached with a chimeric gene composed of the entire extracellular domain of *ERBB1* and the intracellular domain of v-*erbB*. Transformation by this chimera was dependent at least in part on stimulation by epidermal growth factor. We have not tested alleles of *ERBB1* that retain the ligand-binding domain for epidermal growth factor, but our results show that, in the absence of that domain, deletions affecting the carboxyl terminus of the EGFR have no perceptible effect on the transformation of rodent fibroblasts.

Third, several point mutations distinguish the cytoplasmic domain of the v-*erbB* product from its cellular counterpart. These mutations may contribute to the relative potency of the viral oncogene (see above), but they are not necessities: our results demonstrate that the cytoplasmic domain of the normal EGFR can mediate transformation of cells to a tumorigenic phenotype, and other workers have shown (20) that a truncated but otherwise normal allele of chicken c-*erbB* can elicit erythroblastosis when carried in a retroviral vector.

Fourth, v-*erbB* includes an efficient initiation codon for translation and five other codons acquired from the retroviral *gag* gene during transduction (A. Bruskin and J.M.B., unpublished data). We have included these features in all of the chimeric alleles with *ERBB1*, thus compensating for their effects. In any event, these features enhance but are not essential for transformation by v-*erbB* (A. Bruskin, R. Schatzman, and J.M.B., unpublished data).

We conclude that the potency of v-*erbB* as an oncogene arises from the combinatorial effect of several genetic lesions. Preeminent among these is the deletion that removes the ligand-binding domain of the EGFR and that appears to be essential for autonomous transforming activity. The other lesions may augment the efficacy of v-*erbB* and expand its host range, but they do not of themselves create an oncogene.

The Role of *ERBB1* in Tumorigenesis. The expression of *ERBB1* is frequently (but not inevitably) augmented by gene amplification in at least two types of human tumors—glioblastoma (33, 34) and squamous cell carcinoma (35). These findings have engendered the inference that amplification of *ERBB1* may contribute to tumorigenesis. Results reported here and elsewhere sustain this inference by showing that cells in culture can be transformed to neoplastic growth by abundant expression of either ligand-dependent (18, 19) or ligand-independent (present data) versions of the human EGFR. In both instances, cellular transformation is presumably mediated by sustained activity of the protein-tyrosine kinase carried in the EGFR. Amplification of *ERBB1* may achieve the same circumstance.

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