appeared normal since none of them demonstrated any morphologic abnormality or rearranged bands (Fig. 2B, lanes 8, 9, 10, and 12). However, hybrid DNA sequences were markedly amplified and thus clearly detected by the PCR technique in samples obtained from patient 1 (Fig. 3C, lanes 4 through 6 and Fig. 4, lanes 5 and 6), indicating the presence of residual neoplastic cells carrying the t(14;18). Even though the hybrid 18q21–J_H DNA sequences were amplifiable in the pretreatment tumor sample obtained from patient 2 (Fig. 3C, lane 7), no hybrid DNA sequences were detected in the remission marrow sample by the PCR technique (Fig. 3C, lane 8). These findings indicate that the concentration of neoplastic cells carrying the t(14;18) was too low to be detected by our current techniques or the patient was completely free of tumor and there were no t(14;18) target DNA sequences present for amplification.

We have shown the feasibility of using the PCR technique to detect minimal numbers of neoplastic cells carrying a chromosomal translocation. Detection of small numbers of circulating monoclonal B cells by flow cytometry or clonal immunoglobulin gene rearrangement in patients with follicular lymphoma in remission has been reported (*1, 16*). The sensitivity of our approach far exceeds the sensitivity limit achieved by conventional Southern blot analysis or the flow cytometric method. Detection of minimal neoplastic cells by means of chromosomal translocation and PCR will make it possible to address the following important biological and clinical questions that could not be answered before. Do patients in long-term remission have quiescent tumor cells with proliferative potential? Can detection of minimal residual tumor cells predict early relapse? Do patients with persistent minimal residual disease after prolonged treatment require non–cross-resistant therapy to prevent relapse? The answers will help in understanding tumor biology and designing strategies for cancer treatment.

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*erb*B-2 Is a Potent Oncogene When Overexpressed in NIH/3T3 Cells

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A wide variety of human tumors contain an amplified or overexpressed *erb*B-2 gene, which encodes a growth factor receptor–like protein. When *erb*B-2 complementary DNA was expressed in NIH/3T3 cells under the control of the SV40 promoter, the gene lacked transforming activity despite expression of detectable levels of the *erb*B-2 protein. A further five- to tenfold increase in its expression under influence of the long terminal repeat of Moloney murine leukemia virus was associated with activation of *erb*B-2 as a potent oncogene. The high levels of the *erb*B-2 product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumor cells that overexpressed this gene. These findings demonstrate a new mechanism for acquisition of oncogenic properties by genes encoding growth factor receptor–like proteins and provide a functional basis for the role of their overexpression in the development of human malignancies.

EVIDENCE THAT RETROVIRAL ONCOgenes can encode proteins that are homologous to either growth factors or growth factor receptors has shed new light on the mechanisms by which cells become malignant. For example, v-*sis* is derived from the gene encoding one chain of the platelet-derived growth factor (*1*). In addition, v-*erb*B and v-*fms* are derived from the genes encoding the epidermal growth factor (EGF) (*2*) and colony-stimulating factor–1 (CSF-1) (*3*) receptors, respectively. A further linkage between growth control and oncogenes is the common tyrosine kinase activity of many growth factor receptors (*4*) and several viral oncogene products (*5*). These connections have motivated a search in nonvirally induced neoplasia for abnormalities of genes involved in the pathways by which growth factors stimulate normal cell growth.

Shih *et al.* identified a transforming gene in ethylnitrosourea (ENU)-induced rat neuroblastomas by means of the NIH/3T3 transfection assay (*6*). This oncogene, termed *neu*, was found to encode a product immunologically related to the *erb*B/EGF receptor (*7*) and to exhibit a cell surface location. Independently, we cloned and partially sequenced an *erb*B-related gene that was detected because of its amplification in a human mammary carcinoma (*8*). This same human sequence, designated *erb*B-2 (*9, 10*) or HER-2 (*11*), was also cloned from normal genomic and complementary DNA (cDNA) libraries, respectively. Sequence analysis has shown that the structure of the predicted *erb*B-2/HER-2 product has significant homology to the EGF receptor (*9, 11*). Several lines of evidence, including sequence analysis and chromosomal mapping, indicate that *neu* is an activated rat homolog of human *erb*B-2/HER-2 (*9, 11–14*).

Thus far, the established mechanisms that activate genes encoding growth factor receptors to become oncogenes appear to involve structural alterations of their coding sequences. These include truncation or substitutions (*15*) as well as mutational alterations (*14*). While growth factor receptor–like genes with altered coding sequences have been readily detected as transforming genes by DNA transfection analysis, human tumors containing such amplified or overexpressed genes have not yielded transforming genes detectable by this approach (*8, 10*). These findings have suggested that overexpression alone may convert the gene for a normal growth factor receptor into an oncogene. In the present studies, we have investigated the effects of overexpression of the normal coding sequence for the growth

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Table 1. Transfection analysis of *erb*B-2 expression vectors.

DNA clone	Transformed foci/plate with DNA added (μg)*				Specific transforming activity† (FFU/pmol)
	1	0.1	0.01	0.001	
LTR-1/*erb*B-2	TMC	TMC	>100	7,3	4.1×10^4
LTR-2/*erb*B-2	TMC	TMC	30,21	4,1	2.0×10^4
SV40/*erb*B-2	0,0	0,0	0,0	0,0	$<10^6$
LTR/*ras*	TMC	TMC	45,50	6,3	3.6×10^4
pSV2/gpt	0,0	0,0	NT	NT	$<10^6$

*Transfection was performed with 40 μg of calf thymus DNA as carrier by means of the calcium phosphate precipitation technique (*27*). Focus formation on NIH/3T3 cells was scored at 14 to 21 days on duplicate plates. DNA added is shown as micrograms per plate. †Focus-forming units were adjusted to focus-forming units per picomole of cloned DNA added, based on the relative molecular weights of the respective plasmids. NT, not tested; TMC, too many to count.

factor receptor–like human *erb*B-2 gene on growth properties of NIH/3T3 cells. We demonstrate that levels of this gene product comparable to those observed in some human cancers are sufficient to induce neoplastic transformation.

To directly assess the effects of overexpression of the *erb*B-2 gene on cell growth properties, we assembled a full-length normal human *erb*B-2 clone from a series of overlapping clones previously described (*10*). Two nucleotide sequences for *erb*B-2 cDNA have been published (*9, 11*). Our sequence agrees with that of Coussens *et al.* (*11*) with the exception of a neutral change of isoleucine for valine at position 654 and six contiguous amino acid substitutions caused by a difference in reading frame between positions 518 and 523. In this latter region, our nucleotide sequence is identical to that published by Yamamoto *et al.* (*9*). An amino acid substitution of glutamic acid for valine in the putative transmembrane region of the normal rat homolog of *erb*B-2 has been shown to activate the transforming capacity of the *neu* oncogene in ENU-induced rat neuroblastomas (*14*). Our human *erb*B-2 cDNA contained the normal valine at this position.

Expression vectors based on the transcriptional initiation sequences of either the Moloney murine leukemia virus long terminal repeat (Moloney-MuLV LTR) or the SV40 early promoter were constructed in an attempt to express the *erb*B-2 cDNA at different levels in NIH/3T3 cells (Fig. 1) (*16*). Previous studies have indicated different strengths of LTR and the SV40 promoters in these cells (*17*). Upon transfection into NIH/3T3 cells, both LTR-1/*erb*B-2 and LTR-2/*erb*B-2 DNAs induced transformed foci at high efficiencies of 4.1×10^4 and 2.0×10^4 focus-forming units per picomole of DNA (FFU/pmol), respectively (Table 1). In striking contrast, the SV40/*erb*B-2 construct failed to induce any detectable morphological alteration of NIH/3T3 cells transfected under identical assay conditions.

Antiserum prepared against a peptide (anti-peptide) derived from amino acid residues 866 to 880, within the tyrosine kinase domain of the predicted human *erb*B-2 protein (*8, 9, 11*), was utilized to analyze the expression of the human *erb*B-2 protein in the transfectants. As shown in Fig. 2A, LTR-1/*erb*B-2 and LTR-2/*erb*B-2 transfectants each contained a 185-kD protein that was readily recognized by the anti-peptide but not preimmune serum; this recognition was specifically competed out by preabsorption of the anti-peptide with excess peptide. Cells transfected with the SV40/*erb*B-2 vector also produced the 185-kD protein, but at lesser amounts (Fig. 2A). Analysis of several clonally derived cell lines containing either LTR or SV40/*erb*B-2 constructs consistently revealed five- to tenfold higher levels of the 185-kD protein in cells containing the LTR expression vector (Fig. 2B). Since the SV40/*erb*B-2 construct lacked transforming activity, these results demonstrated that the higher levels of *erb*B-2 expression under LTR influence correlated with its ability to exert transforming activity.

The normal ligand for the *erb*B-2 receptor–like protein has yet to be identified. If present in serum, this ligand might be responsible for stimulating the overexpressed *erb*B-2 product and triggering its transforming ability. Thus, we investigated whether *erb*B-2–transformed cells maintained their altered phenotype when cultured in medium lacking serum (*18*). In this medium, LTR-1/*erb*B-2–transfected cells continued to exhibit a stable, transformed phenotype by growing as foci of densely packed cells.

To directly establish that a ligand-receptor interaction was not required for transformation by the *erb*B-2 protein, we engineered both LTR- and SV40-driven constructs such that *erb*B-2 sequences encoding the NH_2-terminal 621 amino acids were deleted (*19*). When the biological activities of these constructs were analyzed, the LTR-driven truncated *erb*B-2 cDNA, designated LTR-1/ΔN*erb*B-2, exhibited a transforming activity of 3.5×10^5 FFU/pmol, almost tenfold greater than that of LTR-1/*erb*B-2. The transforming efficiencies of the *erb*B-2 and truncated *erb*B-2 coding sequences under LTR influence were at least comparable to those of known oncogenes such as v-H-*ras* and avian v-*erb*B (*20*). Moreover, the truncated *erb*B-2 sequence under the control of an SV40 promoter demonstrated a readily detectable transforming ability of around

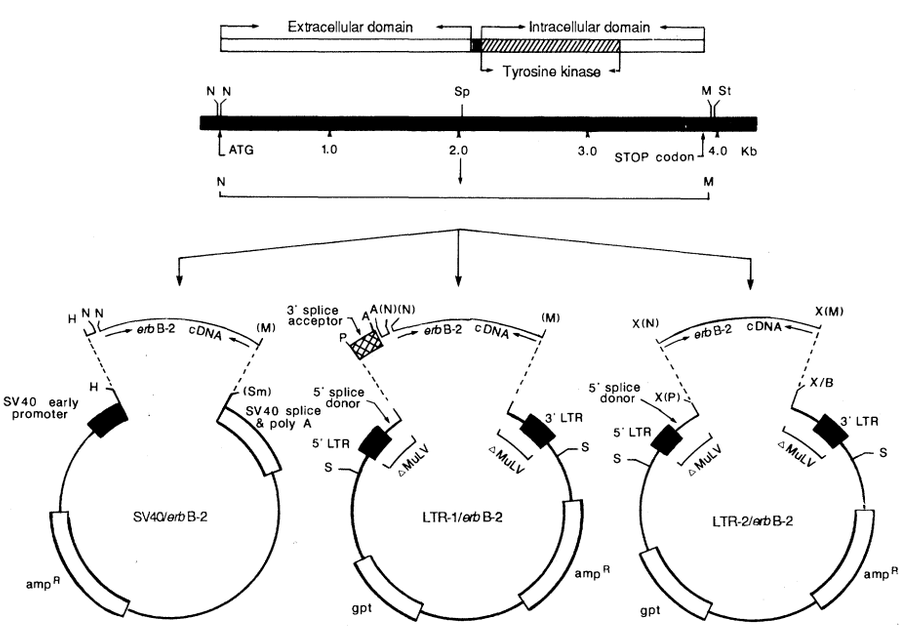

Fig. 1. Construction of expression vectors for the human *erb*B-2 cDNA. A Nco I–Mst II fragment encompassing the entire *erb*B-2 open reading frame was cloned under the transcriptional control of either the SV40 early promoter of Moloney-MuLV LTR. Crosshatched box (LTR-1/*erb*B-2), *erb*A–*erb*B intergenic region of pAEII containing the 3′ splice acceptor site. Abbreviations: N, Nco I; Sp, Sph I; M, Mst II; St, Stu I; H, Hind III; Sm, Sma I; P, Pst I; B, Bam HI; and X, Xho I. Sites indicated in parenthesis were not reconstituted after the cloning procedures.

Table 2. Anchorage-independent growth and tumorigenicity of *erb*B-2-transformed cells.

DNA transfectant*	Colony-forming efficiency in agar (%)†	Cell number for 50% tumor incidence‡
LTR-1/*erb*B-2	45	10^3
LTR-1/Δ*N*erbB-2	70	$<10^3$
SV40/*erb*B-2	<0.01	$>10^6$
LTR/*erb*B	20	5×10^4
LTR/*ras*	35	10^3
pSV2/gpt	<0.01	$>10^6$

*All transfectants were isolated from plates that received 1 μg of cloned DNA and were selected by their ability to grow in the presence of killer HAT medium (*28*). †Cells were plated at tenfold serial dilutions in 0.33% soft agar medium containing 10% calf serum. Visible colonies comprising >100 cells were scored at 14 days. ‡NFR nude mice were inoculated subcutaneously with each cell line. Ten mice were tested at cell concentrations ranging from 10^6 to 10^3 cells per mouse. Tumor formation was monitored at least twice weekly for up to 30 days.

3.9×10^2 FFU/pmol. The largest and most actively growing foci were observed with the truncated *erb*B-2 coding sequence under LTR influence (Fig. 3). All of these findings suggested that NH_2-terminal truncation, if anything, increased the transforming activity of the *erb*B-2 protein.

To investigate expression of the truncated *erb*B-2 protein under SV40 and LTR influence, mass cell populations transformed by either construct were subjected to immunoprecipitation analysis with the anti–*erb*B-2 peptide serum. This antiserum specifically detected protein(s) migrating in the range of 80 to 90 kD (Fig. 2C). Both full-length and truncated *erb*B-2 proteins were larger than their predicted amino acid lengths by about 45 and 15 to 20 kD, respectively. This is likely due to post-translational modification by glycosylation, which has been reported for the rat homolog of *erb*B-2 (*21*). Cells transformed by the SV40/Δ*N*erbB-2 construct expressed five- to tenfold lower levels of the truncated protein than did LTR/Δ*N*erbB-2 transformants (Fig. 2C). These results further implied that NH_2-terminal truncation constitutively activated transforming activity of the *erb*B-2 gene at a lower level of expression. Both full-length and truncated *erb*B-2 proteins segregated with the subcellular membrane fraction, suggesting that their sites of action were localized to the cell membrane (*22*).

To compare the growth properties of NIH/3T3 cells transfected by these genes, we analyzed the transfectants for anchorage-independent growth in culture. The colony-forming efficiencies of both LTR-1/*erb*B-2 and LTR-1/Δ*N*erbB-2 transfectants were very high and comparable to those of LTR-driven v-H-*ras* and v-*erb*B transfectants (Table 2). Moreover, the LTR-1/*erb*B-2 transfectants were as malignant in vivo as cells transformed by the highly potent v-H-*ras* oncogene and 50-fold more tumorigenic than cells transfected with v-*erb*B. The LTR-1/Δ*N*erbB-2–transfected cells were the most potent of all, inducing tumors within 3 weeks in all mice inoculated with 10^3 cells. In contrast, the SV40/*erb*B-2 transfectant failed to display anchorage-independent growth in vitro and did not grow as tumors in nude mice even when 10^6 cells were injected (Table 2).

The *erb*B-2 gene has been reported to be amplified or overexpressed (or both) in a wide variety of human malignancies (*8, 10, 13, 23*). We sought to compare the level of overexpression of the *erb*B-2–encoded 185-kD protein in human mammary tumor cell lines possessing amplified *erb*B-2 genes with that of NIH/3T3 cells transformed by the *erb*B-2 coding sequence. Human mammary tumor cell lines, SK-BR-3 and MBA-MB361, each exhibit *erb*B-2 gene amplification and overexpress a single normal-sized *erb*B-2 transcript (*10*). The antiserum against the *erb*B-2 product detected several discrete protein species ranging in size from 150 to 185 kD in extracts of MDA-MB361 and SK-BR-3 mammary tumor cell lines, as well as in LTR/*erb*B-2 NIH/3T3 transformants (Fig. 4). The relative levels of the 185-kD *erb*B-2 product were similar in each cell line and markedly elevated over that expressed by MCF-7 cells, a mammary adenocarcinoma cell line displaying no *erb*B-2 gene amplification or overexpression (Fig. 4). Thus, human mammary tumor cells that overexpressed the *erb*B-2 gene had levels of the *erb*B-2 gene product capable of inducing malignant transformation in a model system.

Our present studies demonstrate that the human *erb*B-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The level of the *erb*B-2 product was shown to be critical in determining its trans-

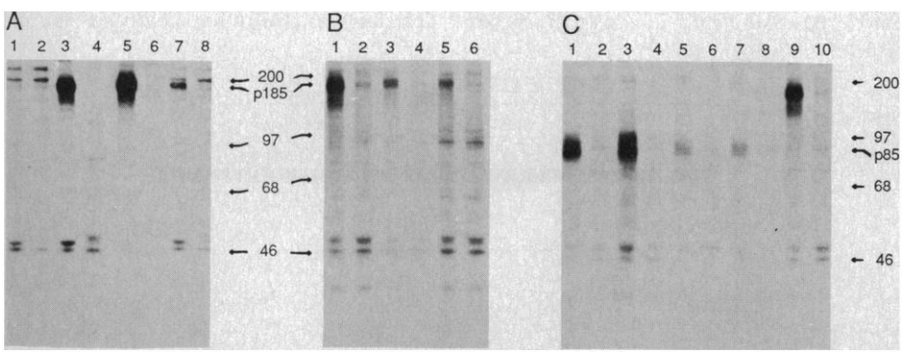

Fig. 2. Immunoprecipitation and electrophoretic analysis of *erb*B-2–specific products in *erb*B-2–transfected NIH/3T3 cells under LTR or SV40 promoter control. LTR-1/*erb*B-2, LTR-2/*erb*B-2, and LTR/Δ*N*erbB-2 transfectants were isolated from plates that received 1 μg of cloned DNA and were selected by their ability to grow in the presence of killer HAT medium (*28*). SV40/*erb*B-2 and SV40/Δ*N*erbB-2 transfectants were obtained by cotransfecting the cloned DNA (1 μg) with pSV2/gpt at a molar ratio of 20:1 (SV40/*erb*B-2:pSV2/gpt) and selecting for growth in killer HAT medium (*28*). (**A**) Cell extracts from [^{35}S]methionine-labeled mass populations of LTR- or SV40-driven *erb*B-2 transfectants were treated with rabbit anti–*erb*B-2 peptide serum directed against amino acid residues 866 to 880 (lanes 1, 3, 5, and 7) or preimmune serum (lanes 2, 4, 6, and 8). Lanes 1 and 2, NIH/3T3; lanes 3 and 4, LTR-1/*erb*B-2; lanes 5 and 6, LTR-2/*erb*B-2; lanes 7 and 8, SV40/*erb*B-2. (**B**) [^{35}S]methionine-labeled extracts from clonally derived LTR- or SV40-driven *erb*B-2 cultures were immunoprecipitated with the above-mentioned antibody alone (lanes 1, 3, and 5) or with antibody that had been preadsorbed for 30 minutes with the specific competing peptide (lanes 2, 4, and 6). Lanes 1 and 2, LTR-1/*erb*B-2 cl1; lanes 3 and 4, SV40/*erb*B-2 cl1; lanes 5 and 6, SV40/*erb*B-2 cl2. (**C**) [^{35}S]methionine extracts of mass populations of cells transfected with the NH_2-truncated *erb*B-2 under either LTR or SV40 influence and marker-selected, as indicated above, were immunoprecipitated with antibody alone (lanes 1, 3, 5, 7, and 9) or antibody-preadsorbed with the specific competing peptide (lanes 2, 4, 6, 8, and 10). Lanes 1 and 2, LTR-1/Δ*N*erbB-2 mass population #1; lanes 3 and 4, LTR-1/Δ*N*erbB-2 mass population #2; lanes 5 and 6, SV40/Δ*N*erbB-2 mass population #1; lanes 7 and 8, SV40/Δ*N*erbB-2 mass population #2; lanes 9 and 10, LTR-1/*erb*B-2. Sizes are shown in kilodaltons.

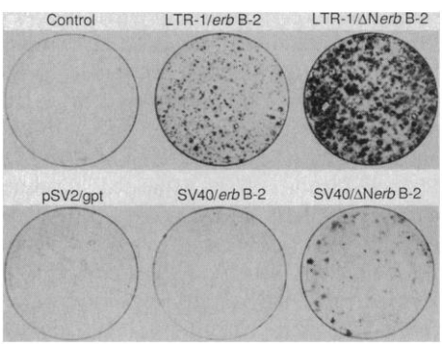

Fig. 3. Comparison of transforming activities of *erb*B-2 or Δ*N*erbB-2 under LTR or SV40 control. NIH/3T3 cells were transfected with 1 μg of DNA from each construct by means of the calcium phosphate precipitation technique (*27*). Plates were stained with hematoxylin at 14 days after transfection. Control, calf thymus DNA. pSV2/gpt was also used as a negative control.

forming ability. An SV40-driven *erb*B-2 cDNA construct lacked detectable focus-forming ability despite the fact that NIH/3T3 cells containing this construct exhibited readily detectable levels of the *erb*B-2 protein. When the same *erb*B-2 cDNA was placed under LTR control, a further five- to tenfold increase in expression of the *erb*B-2 product was associated with the acquisition of transforming properties by the gene. Previous studies have indicated that a 50-fold amplification of the normal rat homolog of *erb*B-2 under SV40 promoter influence was insufficient to induce the malignant phenotype (*24*). Whether its gene product was expressed at as high a level as that of our transforming LTR-*erb*B-2 construct or whether normal human *erb*B-2 is inherently more active as a transforming gene than its rat homolog when overexpressed remains to be elucidated.

An increased number of growth factor receptors may cause transformation by raising the level of constitutive tyrosine kinase activity (*4*) to a threshold required for growth stimulation or by facilitating receptor-receptor interactions that may be a prerequisite for their activation. As the ligand for the *erb*B-2 protein has yet to be identified, it is possible that *erb*B-2 transformed cells themselves might produce this ligand; thus, the receptor might be chronically stimulated. Since NH_2-terminal truncation of the *erb*B-2 coding sequence removed its extracellular ligand-binding domain, the need for a ligand for the truncated protein can be excluded. This protein was shown to transform when expressed at significantly lower level than that required for activity of the full-length *erb*B-2 product. Investigation of the tyrosine kinase activity and processing of the truncated as compared to the normal *erb*B-2 receptor protein should provide insight into the basis for its enhanced transforming activity.

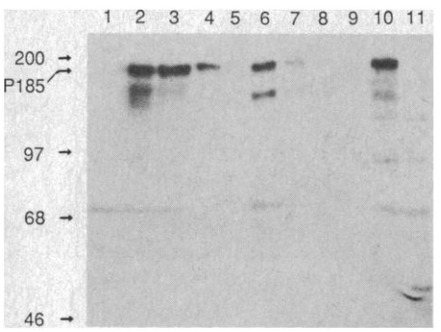

Fig. 4. Comparison of the levels of *erb*B-2 proteins in LTR-1/*erb*B-2–transformed NIH/3T3 cells and human mammary tumor lines by immunoblot analysis. Varying amounts of total cellular protein were separated by electrophoresis and transferred to nitrocellulose filters. The *erb*B-2 protein was detected with rabbit anti–*erb*B-2 peptide serum coupled to ^{125}I-labeled protein A as described (*10*). Lane 1, NIH/3T3 (40 μg); lane 2, LTR-1/*erb*B-2 (40 μg); lane 3, LTR-2/*erb*B-2 (40 μg); lane 4, LTR-2/*erb*B-2 (13 μg); lane 5, LTR-2/*erb*B-2 (4.5 μg); lane 6, SK-BR-3 (40 μg); lane 7, SK-BR-3 (13 μg); lane 8, SK-BR-3 (4.5 μg); lane 9, SK-BR-3 (1.5 μg); lane 10, MDA-MB361 (40 μg); and lane 11, MCF-7 (40 μg). Sizes are shown in kilodaltons.

The v-*erb*B oncogene, which represents a truncated version of the avian EGF receptor, was found to be considerably less effective than either full-length or truncated *erb*B-2 coding sequences in inducing the neoplastic phenotype. Preliminary evidence indicates that the human EGF receptor coding sequence is also much less active as a transforming gene when overexpressed in NIH/3T3 cells (*22*). Thus, the growth signals generated by these two related receptors appear to differ in some way. Investigation of recombinants between the EGF receptor and *erb*B-2 coding sequences should make it possible to identify those regions that confer more potent transforming activity to the *erb*B-2 protein.

There is considerable evidence that genes encoding growth factor receptors can be activated as transforming genes by structural alterations in their coding sequences (*3, 13, 15*). In contrast, abnormalities involving growth factor receptors in human malignancies appear most commonly to involve their overexpression in the absence of structural alterations (*8, 10, 13, 23, 25, 26*). In addition, amplification of *erb*B-2 has been reported to be an important prognostic indicator of a more aggressive phenotype in mammary adenocarcinomas (*26*). Our results demonstrate that human tumors with *erb*B-2 gene amplification can express the *erb*B-2 gene product at very high levels comparable to LTR/*erb*B-2–transformed NIH/3T3 cells. Thus, our studies establish a mechanistic basis for amplification of the growth factor receptor gene as representing a causal driving force in the clonal evolution of a tumor cell rather than being an incidental consequence of tumorigenesis.

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16. The *erb*B-2 cDNA was cloned either under the transcriptional control of the SV40 promoter (SV40/*erb*B-2) or under the Moloney-MuLV LTR (LTR-1/*erb*B-2 and LTR-2/*erb*B-2). To obtain SV40/*erb*B-2, pSV2/*neo* was first digested with Hind III and Sma I. The Mst II site of the *erb*B-2 fragment was rendered blunt-ended and cloned into the Sma I site; the Hind III site and the Nco I site were joined by use of an oligonucleotide with the following structure: 5′-AGCTTGGCCGGAGCCG-CAGTGAGCAC-3′. This oligonucleotide restored the first 20 nucleotides upstream of the initiating ATG codon of *erb*B-2 cDNA. To engineer LTR-driven constructions, a multifunctional cloning vector was constructed from an integrated form of Moloney-MuLV and pSV2/gpt. The resulting molecule was designated pSV2/gpt/MuLV. Digestion of pSV2/gpt/MuLV with Pst I and Bam HI yielded a molecule depleted of most of the MuLV coding regions as well as the initiation codon for the *gag* polypeptide, but retaining the putative 5′ splice donor site of MuLV. Therefore, LTR-1/*erb*B-2 was constructed with the 463-bp Pst I–Apa I fragment from pAEVII, which contains a 3′ splice acceptor site used for the expression of the v-*erb*B gene in avian erythroleukemia virus. The Mst II site of the *erb*B-2 cDNA was cloned in the Bam HI site of pSV2/gpt/MuLV after both sites were rendered blunt-ended. Joining of the Nco I site of the *erb*B-2 cDNA and the Apa I site was achieved with the following adapter oligonucleotide: 5′-CAGAC-CACTG-3′. To engineer LTR-2/*erb*B-2, the Nco I–Mst II sites of *erb*B-2 cDNA were converted to Xho I and cloned into the pSV2/gpt/MuLV vector between Bam HI and Pst I sites converted to Xho I. The LTR-2/*erb*B-2 construction was entirely sequenced in the *erb*B-2 open reading frame to ensure that no alterations were introduced following the cloning procedure. The remaining constructions were sequenced in the junction regions to verify that the predicted structure was achieved after the recombination procedures.
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19. To construct expression vectors for 5′-truncated *erb*B-2 cDNA, the Sph I–Stu I fragment of the *erb*B-2 cDNA was cloned into the LTR-1 or SV40-based expression vectors previously described (Fig. 1) (*16*). The translation of the truncated *erb*B-2 protein begins at a methionine 32 amino acids proximal to the transmembrane region. The Sph I–Stu I fragment was cloned into the Hind III–Sma I cut pSV2/*neo* vector (described in the legend to Fig. 1) to obtain SV40/Δ*Nerb*B-2. Joining between the Hind III and Sph I sites at the 5′ end of the coding sequence was obtained with an oligonucleotide of the following structure: 5′-AGCTTCAGGGCCCA-GACCACTGCATGGCATG-3′. This oligonucleotide contained an in-frame ATG to initiate the translation of the truncated *erb*B-2 protein. LTR-1/Δ*Nerb*B-2 was engineered essentially following the same strategy outlined in Fig. 1 for construction of LTR-1/*erb*B-2. The joining between the Apa I site and the 5′ Sph I site of the *erb*B-2 cDNA was obtained using the following adapter oligonucleotide: 5′-CAGACCACTGCATGGCATG-3′. This oligonucleotide contained an in-frame ATG to direct the synthesis of an *erb*B-2 protein. All of the above constructions were sequenced in the junction regions to verify that the predicted structure was achieved after the recombination procedures.
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Variable Occurrence of the *nrd*B Intron in the T-Even Phages Suggests Intron Mobility

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The bacteriophage T4 *nrd*B gene, encoding nucleoside diphosphate reductase subunit B, contains a self-splicing group I intervening sequence. The *nrd*B intron was shown to be absent from the genomes of the closely related T-even phages T2 and T6. Evidence for variable intron distribution was provided by autocatalytic ^{32}P–guanosine 5′-triphosphate labeling of T-even RNAs, DNA and RNA hybridization analyses, and DNA sequencing studies. The results indicate the nonessential nature of the intron in *nrd*B expression and phage viability. Furthermore, they suggest that either precise intron loss from T2 and T6 or lateral intron acquisition by T4 occurred since the evolution of these phages from a common ancestor. Intron movement in the course of T-even phage divergence raises provocative questions about the origin of these self-splicing elements in prokaryotes.

WHILE SELF-SPLICING RNAs ARE remarkable for their autocatalytic properties (*1*), there is mounting evidence implicating these introns as mobile elements. There are several examples where the presence of eukaryotic group I introns is not uniform in equivalent genes of closely related species [for example, the large ribosomal RNA (rRNA) of *Tetrahymena* (*2*)] or even in different strains of the same species [for example, the large rRNA of *Saccharomyces cerevisiae* mitochondria (*3*)]. In the latter case site-specific transposition of the intron from intron$^+$ to intron$^-$ copies of the gene, with the aid of an intron-encoded endonuclease, has been demonstrated (*3*). Furthermore, there is evidence (based on conserved features of sequence and structure) for the transfer of group I introns between different fungal mitochondrial genomes (*4*) and between fungal mitochondrial genes and the *Tetrahymena* nucleus (*5*). Surprisingly, the protein potentially encoded by the intron open reading frame (ORF) of gene *td* in bacteriophage T4 (*6*) shares homology with intron ORFs of three group I mitochondrial intervening sequences of filamentous fungi (*7*). This observation led to speculations on a recent genetic exchange between these fungi and the genome of T4 (*7*).

In the face of these provocative observations that argue in favor of intron mobility, we have investigated the distribution of the group I *nrd*B intron of phage T4 (*8, 9*) in the T-even phage family. We provide evidence that the intron in the T4 *nrd*B gene, which encodes the B subunit of ribonucleoside diphosphate reductase, is absent from the genomes of T2 and T6. These results indicate the dispensability of the *nrd*B intron for T-even phage viability and imply movement of the T4 *nrd*B intron since the divergence of the closely related T-even phages from their common ancestor.

The existence of multiple T4 genes encoding group I self-splicing RNAs was inferred by labeling T4 RNA with ^{32}P–guanosine 5-triphosphate (GTP) under self-splicing conditions (*8*). In addition to a labeled band corresponding to the previously identified *td* intron (*6, 10*), several other labeled species were seen. The most intense of these other bands corresponds to the 0.6-kb *nrd*B intron (*8*). The profile of labeled bands was strikingly different in a similar autocatalytic ^{32}P-GTP labeling experiment with T2 and T6 RNAs (Fig. 1). While the *td* band appeared to be present in all three phages, the 0.6-kb *nrd*B species was apparently absent from T2 and T6 RNA. The *td* intron is indeed homologous throughout the T-even phages, as demonstrated by hybridization and dideoxy primer-extension analysis of pre–messenger RNA (mRNA) and splice products (*11*). In contrast, similar experiments argue in favor of the variable occurrence of the *nrd*B intron in the T-even phage family.

Dot-blot analysis revealed that several *nrd*B intron–specific probes that gave a strong hybridization signal with T4 DNA or RNA did not hybridize to T2 or T6 DNA or RNA (Fig. 2, probes 3 and 4). This was true for end-labeled oligonucleotide probes directed against the intron ORF (probe 4) as well as for probes that hybridized to noncoding sequences believed to form part of the active splicing conformation (probe 3). Similar results were obtained with a nick-translated intron probe (see legend to Fig. 2C). The *nrd*B intervening sequence thus appears to be absent from the genomes of T2 and T6. In contrast, the *td* intron-specific control probe hybridized throughout, except to a T4 phage construct that has a *td* deletion (Fig. 2A, probe *td*i). These data, suggesting the absence of the *nrd*B intron from T2 and T6, are in agreement with the results obtained with a synthetic

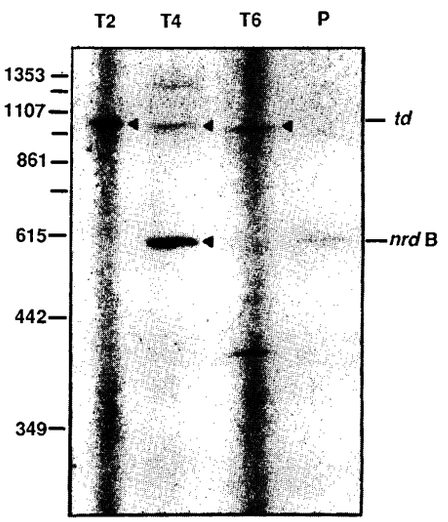

Fig. 1. Labeling of T2, T4, and T6 RNAs with ^{32}P-GTP. RNA was extracted from T2-, T4- or T6-infected *Escherichia coli* B 12 minutes after infection or from cells transformed with plasmid pJSK6 after transcriptional activation of the intron-containing *nrd*B gene (*8*). After incubating the deproteinized RNA with α-[^{32}P]GTP at 42°C for 1 hour under self-splicing conditions (*8, 18*), samples were separated on a 5% acrylamide gel alongside denatured DNA size standards (a 123-bp ladder) used to infer species lengths (left). The position of the *td* intron was indicated by a *td* control clone and that of the *nrd*B intron from the band in lane P, which contains labeled RNA from pJSK6. The absence of an *nrd*B band of equivalent intensity or mobility from the T2 and T6 lanes should be noted. Unmarked bands in the T4 and T6 lanes have not been identified and may well represent other group I introns.

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