with visible light may avoid the usual photodynamic perturbation entirely and facilitate protracted fluorescence observations of living cells.

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9. The number n_a of photons absorbed per fluorophore per pulse depends on τ_p, f_p, p_0, and A, as

$$n_a \approx \frac{p_0^2 \delta}{\tau_p f_p^2} \left(\frac{A^2}{2\hbar c\lambda} \right)^2$$

where c is the speed of light, $\hbar$ is the Planck quantum of action, and δ is the two-photon absorption cross section; saturation is neglected, and the paraxial approximation is assumed.
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13. If certain chemical bonds are photolyzed, an inactive ("caged") derivative of a molecule can be converted into its active form. See (*14, 15*).
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Association of Human Papillomavirus Types 16 and 18 E6 Proteins with p53

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Human papillomavirus type 16 (HPV-16) is a DNA tumor virus that is associated with human anogenital cancers and encodes two transforming proteins, E6 and E7. The E7 protein has been shown to bind to the retinoblastoma tumor suppressor gene product, pRB. This study shows that the E6 protein of HPV-16 is capable of binding to the cellular p53 protein. The ability of the E6 proteins from different human papillomaviruses to form complexes with p53 was assayed and found to correlate with the in vivo clinical behavior and the in vitro transforming activity of these different papillomaviruses. The wild-type p53 protein has tumor suppressor properties and has also been found in association with large T antigen and the E1B 55-kilodalton protein in cells transformed by SV40 and by adenovirus type 5, respectively, providing further evidence that the human papillomaviruses, the adenoviruses, and SV40 may effect similar cellular pathways in transformation.

THE HUMAN PAPILLOMAVIRUSES (HPVs) that infect the anogenital area can be separated on the basis of their clinical associations into two distinct groups. The first group, including HPV-6 and HPV-11, is generally associated with benign anogenital warts that infrequently progress to cancer and have been referred to as "low-risk" viruses. The "high-risk" group, including HPV-16 and HPV-18, is associated with lesions that are at high risk for malignant progression and with almost all cervical carcinomas (*1*). The ability of cloned viral genomes derived from the high-risk but not the low-risk HPVs to transform cells in culture suggests that these papillomavirus types have an etiologic role in these tumors (*2*). In cervical carcinomas and in cell lines derived from cervical carcinomas, the E6 and E7 open reading frames (ORFs) of the high-risk HPVs are regularly found to be intact and actively transcribed, implicating the E6 and E7 genes in the malignant phenotype (*3, 4*). Support for this role is provided by genetic analyses that establish the requirement for both E6 and E7 for the efficient transformation of primary human squamous epithelial cells by HPV-16 (*5*).

DNA tumor viruses appear to exert some of their proliferative and oncogenic effects on the host cell through interactions with cellular proteins. The HPV-16 E7 protein, like SV40 large T antigen (*6*) and adenovirus E1A (*7*), is capable of binding pRB (*8*). The E7 proteins of both high-risk and low-risk genital type HPVs have been shown to bind to pRB (*9*). The E7 proteins of HPV-6 and HPV-11 bind with 20-fold and 5-fold lower affinities, respectively, than the E7 proteins of HPV-16 and HPV-18. Thus, the ability of E7 to bind pRB per se does not alone allow for the qualitative discrimination between the different biologic properties of these viruses. The oncogenic potential of the E6 protein encoded by the high-risk HPVs has been revealed in transformation studies with primary human cells (*5, 10*). Like the E7 protein, which is 98 amino acids in size, the E6 protein is small (158 amino acids), and it is likely that its transforming properties may also result from the ability to form complexes with and potentially modulate the activity of critical cellular proteins that regulate cellular growth and differentiation. Since the large T antigen of SV40 (*11, 12*) and the E1B 55-kD protein of adenovirus 5 (*13*) can form a complex with the p53 protein, we explored the possibility that HPV-16 E6 also encodes a p53 binding protein. Although formerly classified as a dominantly acting oncogene (*14–16*) wild-type p53 has been shown to have tumor suppressor properties (*17–22*).

The possibility that HPV-16 E6 or E7 bound to or interacted with p53 was assessed by an in vitro binding assay similar to that used to show E7 complex formation with pRB (*8*). For these experiments, labeled HPV-16 E6 and E7 proteins synthesized in rabbit reticulocyte lysates were mixed with lysates of unlabeled mouse F9 cells. The F9 cells contain wild-type p53 protein that, unlike mutant p53 protein, binds efficiently to SV40 large T antigen (*19, 23*). The mixture was incubated with antibodies directed against p53, and the immunoprecipitate was analyzed for the

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presence of labeled E6 or E7 protein. The HPV-16 E6 protein was coprecipitated by either PAb421 (*24*) or PAb246 (*25*), two monoclonal antibodies that recognize distinct epitopes on the murine p53 protein (Fig. 1). An irrelevant monoclonal antibody [PAb419 (*24*)], specific for SV40 large T antigen, did not immunoprecipitate E6 from the mixtures, and in lysis buffer with no p53, PAb421 was unable to immunoprecipitate E6 protein. Similar experiments with HPV-16 E7 proteins revealed no binding to p53 (*26*). Use of either 50 or 200 μl of PAb421 precipitated the same amount of labeled HPV-16 E6, confirming that the experiments were carried out in antibody excess.

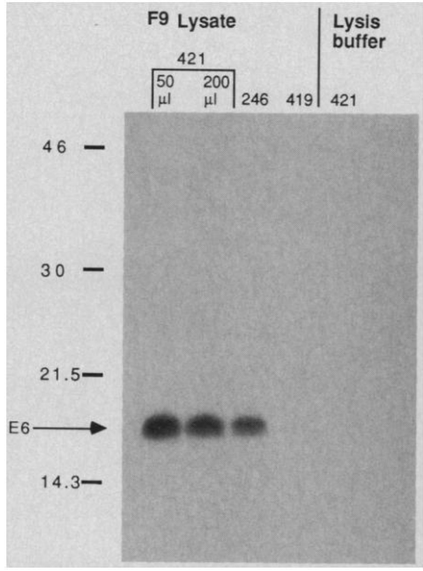

Fig. 1. Coimmunoprecipitation of labeled HPV-16 E6 protein with wild-type murine p53 (mu-p53). The [^{35}S]cysteine-labeled, in vitro–translated E6 proteins were incubated with unlabeled F9 cell lysates or cell lysis buffer, and immunoprecipitated with either PAb421 (a species cross-reactive, p53-specific monoclonal antibody recognizing amino acid residues 370 to 378 of the mu-p53 protein) (*24, 25*), or PAb246 (a monoclonal antibody recognizing a conformation-dependent epitope spanning residues 88 to 109 and present exclusively on wild-type mu-p53) (*25*). PAb419, a SV40 large T–specific monoclonal antibody, was used as a control (*24*). The numbers at the left of the figure indicate molecular size standards (in kilodaltons). The mixing experiments are a modification of a previously published method (*9*). Subconfluent F9 cell cultures were lysed on ice for 1/2 hour in 0.5 ml per 10-cm plate of cell lysis buffer [1% NP-40, 100 m*M* tris-HCl (*p*H 8.0), and 100 m*M* NaCl]. The HPV-16 E6 ORF from nucleotides 79 to 559 was cloned from the previously described plasmid p1224 (*35*) into the polylinker of the prokaryotic expression vector pGEM-2. The plasmid was linearized with Eco RI 3′ to the E6 ORF and complementary RNA (cRNA) transcribed from the T7 promoter was used to direct synthesis of [^{35}S]cysteine-labeled E6 proteins in rabbit reticulocyte lysates (total cRNA from 1.5 μg of template DNA for 100 μl of reaction). The reticulocyte lysates were cleared with PAb421 after translation to remove any endogenous p53. After dilution with an equal volume of cell lysis buffer, the labeled E6 proteins (3×10^5 cpm) were incubated for 3 to 12 hours at 4°C with 200 μl of either a lysate of F9 cells (4×10^6 cells) or cell lysis buffer. The mixtures were immunoprecipitated with either 50 or 200 μl of PAb421 or 200 μl of PAb246, and the proteins were separated on a 14% polyacrylamide gel (*36*) and visualized by fluorography (*37*).

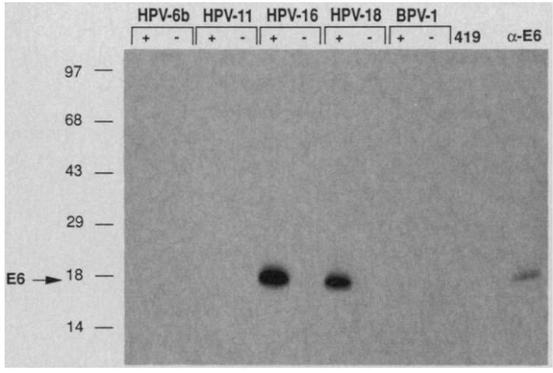

Fig. 2. In vitro association of papillomavirus E6 proteins with wild-type hu-p53. Reticulocyte lysates containing labeled E6 proteins of the indicated papillomaviruses were mixed with either unlabeled reticulocyte lysates programmed with hu-p53 (+) or no cRNA (−), and immunoprecipitated with PAb421, a monoclonal antibody to p53 (*32*). These mixed lysates were immunoprecipitated with either PAb419 (419) or antibody to HPV-16 E6 (α-E6) as controls, on the mixtures of lysates containing labeled HPV-16 E6 and unlabeled hu-p53. The numbers at the left of the figure indicate molecular size standards (in kilodaltons). A cDNA encoding wild-type hu-p53 (*28*) and the E6 ORFs of HPV-6b, HPV-11, HPV-16, HPV-18, and BPV-1 were each cloned into pGEM-1 or pGEM-2 prokaryotic expression vectors. The HPV-16 E6 plasmid has been described (*35*). The E6 ORFs of HPV types 6b, 11, and 18, and of BPV-1 were cloned by polymerase chain reaction (PCR) amplification from cloned full-length viral genomes. Restriction sites Sal I and Hind III were incorporated into the 5′ sense and 3′ antisense oligonucleotide primers, respectively, to facilitate cloning into the pGEM polylinker (*38*). Each of the clones was verified by DNA sequence analysis. The complete hu-p53 cDNA sequence derived from the original clone php53c-1 (*27*) was cloned into the Bam HI site of the pGEM-2 polylinker. The mixing experiment was modified from a previously published method (*8*). The plasmids were linearized 3′ to their respective termination codons, and cRNA transcribed from the SP6 or T7 promoters was used to direct synthesis of [^{35}S]cysteine labeled E6 proteins and unlabeled hu-p53 in rabbit reticulocyte lysate. After dilution with an equal volume of cell lysis buffer, equal volumes of p53 proteins were incubated for 3 to 12 hours at 4°C with amounts (3×10^5 cpm) of each of the different E6 proteins. Incorporation of label into full-length E6 proteins was verified by gel electrophoresis and fluorography. Volumes were adjusted with a 1:1 mix of rabbit reticulocyte lysate and cell lysis buffer. Immunoprecipitated proteins were separated on a 12% polyacrylamide gel (*36*) and visualized by fluorography (*37*). The proportion of labeled E6 proteins coimmunoprecipitated by PAb421 was determined by comparing input levels of the labeled E6 proteins as determined by trichloroacetic acid (TCA) precipitation with the amounts of coimmunoprecipitated E6 proteins in the excised gel bands. These measurements indicate that 12% of the input HPV-16 E6 and 6.8% of the input HPV-18 E6 proteins were coimmunoprecipitated in complex with hu-p53.

The binding of HPV-16 E6 to p53 protein prompted us to investigate whether the E6 proteins of other papillomaviruses associated with genital tract lesions could also complex with p53 protein. The E6 proteins of all genital type HPVs are highly conserved [45 to 70% amino acid similarity to HPV-16 E6 (*26*)] and may be expected to have similar biological functions. Although the bovine papillomavirus type 1 (BPV-1) E6 protein sequence is only 25% similar to HPV-16 E6, it was also assayed for p53 protein binding because it has also been shown to have transforming properties (*27*). In this experiment, mixed extracts were used with in vitro–translated wild-type human p53 (hu-p53) (*28*) (Fig. 2). The E6 proteins of both HPV-16 and HPV-18 could be coprecipitated by the PAb421 antibody only in the lysates containing hu-p53. No labeled HPV-6, HPV-11, or BPV-1 E6 protein was detected in complex with p53 protein. Densitometry showed the E6-specific signal from HPV-18 to be 50% that of HPV-16 E6.

Attempts to coimmunoprecipitate labeled hu-p53 protein from similar mixtures in which hu-p53 was labeled and the HPV-16 E6 protein was unlabeled with HPV-16 E6-specific antisera were not successful (*26*), suggesting that the E6 antibody might be interfering with p53-E6 complex formation. To circumvent this problem, the coding sequence for an 11–amino acid peptide corresponding to an antigenic determinant of the influenza hemagglutinin protein (HA1) (*29*) was added in frame to the 5′ ends of E6 ORFs of HPV types 11, 16, and 18. An antibody to this epitope could then be used to immunoprecipitate the E6 fusion proteins. An analogous experiment to that presented in Fig. 2 was carried out in which rabbit reticulocyte lysates containing the individual labeled E6 fusion proteins were mixed with lysates containing labeled hu-p53 protein or lysates derived from extracts in the absence of translated protein (Fig. 3). The antibody (12CA5) directed against the epitope efficiently precipitated each of the E6 fusion proteins (Fig. 3). Furthermore, when this lysate was mixed with labeled hu-p53 extracts, the p53 protein could readily be identified in complex with the modified

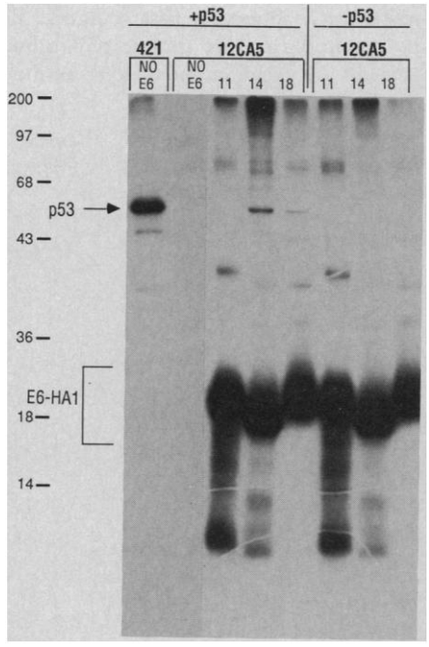

Fig. 3. Coprecipitation of E6-p53 complexes from mixtures of hu-p53 and E6 proteins bearing the HA1 epitope (E6-HA1). Labeled E6-HA1 proteins were mixed with either labeled hu-p53 or reticulocyte lysate and immunoprecipitated by PAb421 (*24*) or 12CA5 (*29*), a monoclonal antibody specific for the HA1 epitope. The numbers at the left of the figure indicate molecular size standards (in kilodaltons). The method of in vitro transcription and translation of [^{35}S]cysteine-labeled proteins, and mixing and immunoprecipitation were described in Fig. 2. The HA1 epitope derived from the influenza virus hemagglutinin protein has been previously described in detail (Y-P-Y-D-V-P-D-Y-A-S-L) (*29*). A sequence coding for this epitope was cloned downstream of a methionine initiation codon and was fused via a P-G spacer to amino acid 2 of E6. The coding region for the 11–amino acid epitope and spacer was incorporated into the 5′ oligonucleotide primers used for PCR amplification of the E6 ORFs before cloning into pGEM. PCR amplification and cloning was carried out as described in Fig. 2. Sal I and Hind III sites were incorporated into the 5′ and 3′ oligonucleotide primers, respectively, to facilitate cloning into the pGEM polylinker (*39*). All clones were sequenced to confirm the addition of the HA1 coding region in frame with codon 2 of the respective wild-type E6 sequence. The proportion of input p53 in complex with the E6 protein and coimmunoprecipitated by the 12CA5 antibody was determined by first measuring the efficiency of E6 immunoprecipitation by this antibody by comparing the amount of input E6, as determined by TCA precipitation, with the amount of immunoprecipitated E6 present in the excised gel bands. The amount of coimmunoprecipitating p53 in the excised gel bands was measured and the amount in complex with E6 was determined taking into account the efficiency of E6 immunoprecipitation by 12CA5. The amount of input p53 was determined by TCA precipitation, and from these measurements one could determine that 12% of the input hu-p53 was associated with HPV-16 E6 and 7.3% of input hu-p53 was associated with HPV-18 E6.

E6 proteins of HPV-16 and HPV-18, but not HPV-11.

These experiments show that the E6 proteins of HPV-16 and HPV-18 can associate with wild-type hu-p53 in vitro. Although there is no evidence for the association of E6 proteins of HPV-6 or HPV-11 with p53 protein, it is possible that they do associate but with an affinity below that necessary for detection in the in vitro assay used in this study.

The small DNA tumor viruses with their limited coding capacity may principally affect cellular pathways by modulating the activities of key cellular proteins. The E6 and E7 ORFs are well conserved among human papillomaviruses regardless of their associated risk for malignant progression and it is likely that E6 and E7 contribute to the proliferation of basal cells of the squamous epithelium infected by these viruses. The putative roles of pRB and p53 in cell-cycle regulation (*30*) suggests that inactivation or modulation of the activities of these proteins may result in cellular proliferation and altered differentiation, thus permitting the expansion of a pool of squamous epithelial cells for the replication of viral particles. Intrinsic differences in the E6 and E7 proteins of high-risk and low-risk genital papillomaviruses may account for the differences in transforming potential of these viruses, since these functions map to these genes. It is tempting to speculate that the observed differences in the carcinogenic potential of these viruses may relate to differences in the interactions with cellular proteins such as pRB and p53. The ability of the transforming proteins encoded by the human papillomaviruses, the adenoviruses, and SV40 to bind to the same cellular proteins suggests that they all transform by perturbing the same cellular pathways (Fig. 4).

The p53 gene product appears to be a negative regulator of cell proliferation (*30*). The gene has been found to be mutated in Friend virus–induced mouse leukemias (*31*), and mutations have been noted in a high percentage of colon carcinomas (*22*) and in human lung carcinomas (*32*). Mutant forms of the murine p53 clones can immortalize primary rat embryo fibroblast cells (*14, 20*) and cooperate with an activated *ras* oncogene to transform cells (*14, 15, 17–19*). There is evidence that mutated forms of p53 that are transforming are trans-dominant over wild-type p53 (*20, 22*). These activating mutations result in conformational changes in the protein enabling it to bind to the heat shock protein hsp70. This change in conformation is associated with enhanced protein stability (*17*). The trans-dominant phenotype of the mutated p53 protein may be explained by its ability to oligimerize with wild-type p53, drawing it into this complex and effectively inactivating it (*16, 19*). The SV40 large T antigen and adenovirus E1B 55-kD proteins form complexes with p53 protein resulting in increased half-life (*33*), presumably also inactivating its normal function as a negative regulator of cellular growth. The consequence of the interaction of E6 with p53 is likely to be different. Levels of E6 in cervical carcinoma cell lines and in HPV-16– or HPV-18–transformed cell lines are low (*4*). Levels of p53 are undetectable in HeLa cells despite the presence of translatable mRNA (*34*), and our analysis of p53 in a series of additional carcinoma cell lines indicates that several contain low levels of this protein (*26*). Furthermore, we have found that the levels of p53 in human keratinocytes transformed by HPV-16 are low when compared to the levels in primary keratinocytes or in SV40-transformed keratinocytes (*26*). This raises the possibility that E6 may facilitate the degradation of p53. The rapid degrada-

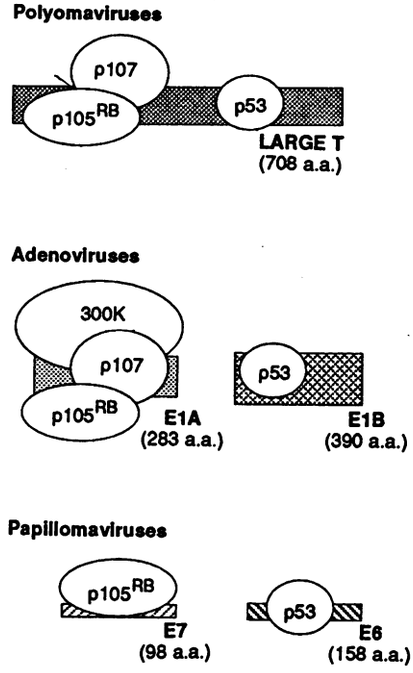

Fig. 4. Schematic representation of the common cellular protein-binding properties shared by the transforming proteins from the different DNA tumor viruses. Both pRB and p53 bind to large T antigen of SV40 (*6, 11*), whereas each binds to separate adenovirus- (*7, 12*) and HPV-encoded proteins. Sequences required for cellular transformation are also required for binding of SV40 large T antigen to pRB and p53 (*6, 40*), and for binding of E1A and E7 to pRB (*7, 9*). Although the sequences required for E1B 55-kD and E6 binding to p53 have not been defined, each of these proteins is required for full transformation of primary cells (*5, 10*).

tion of the protein in the cell would have the same functional result as SV40 large T antigen, adenovirus E1B 55-kD protein, or a mutated form of the p53 protein in eliminating wild-type p53 and thus preventing it from its function as a regulator of cell growth.

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38. Sequences of the oligonucleotide primers used for PCR amplification were: HPV-6b 5′ sense, CAGTCGACACCATGGAAAGTGCAAATGCCTCC and 3′ antisense, GTAAGCTTTAGGGTAACATGTCTTCC; HPV-11 5′ sense, GCGTCGACCACCATGGAAAGTAAAGATG and 3′ antisense, GCAAGCTTAGGGTAACAAGTCTTC; HPV-18 5′ sense, GCGTCGACCACCATGGCGCGCTTTGAG and 3′ antisense, GCAAGCTTATACTTGTGTTTCTC; BPV-1 5′ sense, GCGTCGACCACCATGGACCTGAAACCTTTTG and 3′ antisense, GCAAGCTTCTATGGGTATTTGGACCTTG.
39. The 3′ antisense primers for HPV-11 and HPV-18 were the same as those used to clone the wild-type E6 ORFs. For HPV-16, the E7 3′ antisense oligonucleotide primer 5′-GGTACCTGCAGGATCAGCCATG-3′ was used. The 5′ oligonucleotide primer sequences used were (5′–3′): HPV-11 E6, GCGTCGACCACCATGTACCCGTACGACGTGCCGGACTACGCGAGCCTGCCGGGCCTGGAAAGTAAAGATGCCTCCAC; HPV-16 E6, GCGTCGACCACCATGTACCCGTACGACGTGCCGGACTACGCGAGCCTGCCGGGCCTGTTTCAGGACCCACAGGAGCG; HPV-18-E6, GCGTCGACCACCATGTACCCGTACGACGTGCCGGACTACGCGAGCCTGCCGGGCCTGGCGCGCTTTGAGGATCCAAC.
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EGF Receptor and *erb*B-2 Tyrosine Kinase Domains Confer Cell Specificity for Mitogenic Signaling

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The epidermal growth factor (EGF) receptor (EGFR) can efficiently couple with mitogenic signaling pathways when it is transfected into interleukin-3 (IL-3)–dependent 32D hematopoietic cells. When expression vectors for *erb*B-2, which is structurally related to EGFR, or its truncated counterpart, Δ*Nerb*B-2, were introduced into 32D cells, neither was capable of inducing proliferation. This was despite overexpression and constitutive tyrosine kinase activity of their products at levels associated with potent transformation of fibroblast target cells. Thus, EGFR and *erb*B-2 couple with distinct mitogenic signaling pathways. The region responsible for the specificity of intracellular signal transduction was localized to a 270–amino acid stretch encompassing their respective tyrosine kinase domains. Thus, tissue- or cell-specific regulation of growth factor receptor signaling can occur at a point after the initial interaction of growth factor with receptor. Such specificity in signal transduction may account for the selection of certain oncogenes in some malignancies.

THE INTERACTION OF GROWTH FACTORS with specific membrane receptors triggers a series of intracellular events that are of critical importance in the regulation of normal cell proliferation. Subversion of these mitogen-responsive pathways plays a determinant role in the neoplastic process (*1*). Little is known about the nature of such signaling pathways and the specificity of receptor-pathway coupling. The cDNA of a foreign receptor introduced into a naïve cell can confer responsiveness to its ligand, indicating that regulation of cell proliferation involves growth factor receptor expression and ligand availability (*2, 3*). Intracellular specificity in signaling pathways may also exist as indicated by findings that different subsets of cellular proteins are phosphorylated in response to various growth factors (*4*).

EGFR and *erb*B-2 genes differ in their efficiency of transformation for NIH 3T3 fibroblasts, suggesting that they may couple with different efficiency to one or more intracellular signal transduction pathways (*5, 6*). We initially sought to compare mitogenic signaling by these two genes in the hematopoietic line 32D, which lacks either receptor and is normally dependent on interleukin-3 (IL-3) for proliferation (*7*). For this purpose the eukaryotic expression vectors, LTR-EGFR (*6*) and LTR–*erb*B-2 (*5*), were transfected into 32D cells by electroporation (*8*). These vectors contained the transcriptional initiation sequences of the Moloney murine leukemia virus (M-MuLV) long terminal repeat, along with the *Ecogpt* selectable marker (*9*), which confers resistance to mycophenolic acid (*9*). After transfection and marker selection, viable cell lines designated 32D-EGFR and 32D–*erb*B-2

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