The Amino-Terminal 14 Amino Acids of *v-src* Can Functionally Replace the Extracellular and Transmembrane Domains of *v-erbB*

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Received 4 April 1991/Accepted 26 June 1991

The retroviral oncogene *v-erbB* encodes a truncated form of the receptor for epidermal growth factor, an integral membrane protein-tyrosine kinase. By contrast, the oncogene *v-src* encodes a protein-tyrosine kinase that is a peripheral membrane protein. The morphologies and spectra of cells transformed by these two oncogenes differ. In an effort to identify the functional determinant(s) of these differences, we constructed and tested first deletion mutants of *v-erbB* and then chimeras between *v-src* and *v-erbB*. As reported previously, the absence of any membrane anchorage eliminated transformation by *v-erbB*. Anchorage of the cytoplasmic kinase domain of *v-erbB* to membranes with amino-terminal portions of the *v-src* protein permitted transformation. The phenotype and spectrum of transformation were those expected for *v-erbB* rather than for *v-src*. The transforming chimeras lost their biological activity if the signal for myristylation at the amino terminus of *v-src* was compromised by mutation. Biochemical fractionations revealed a correlation between transforming activity and the association of chimeric gene products with the membrane fraction of the cell. For reasons not yet apparent, the combined presence of membrane anchorage domains of *v-src* and the transmembrane domain of *v-erbB* in the same chimera typically (but not inevitably) impeded transformation. Our results suggest that the specificity of transformation by *v-erbB* resides in the selection of substrates by the cytoplasmic domain of the gene product. The protein retains access to those substrates even when anchored to the membrane in the manner of a peripheral rather than a transmembrane protein.

Inoculation of avian erythroblastosis virus (AEV-ES4) into chickens gives rise to erythroblastosis and fibrosarcomas, and the virus transforms corresponding target cells in vitro (13, 14, 41). The genome of AEV-ES4 contains two oncogenes, *v-erbA* and *v-erbB*, both of which were apparently acquired from the chicken genome by transduction (44). The cellular homologs of these genes have been identified: in the case of *v-erbA* the cellular gene encodes the receptor for thyroid hormone (34, 45), whereas the cellular homolog of *v-erbB* encodes the receptor for epidermal growth factor (EGF-R) (8, 43). The *v-erbB* gene is primarily responsible for transformation by AEV-ES4; *v-erbA* is believed to potentiate the transformation of erythroblasts by blocking their differentiation (9, 39, 40, 47, 48).

The protein encoded by the ES4 allele of *v-erbB* differs from the EGF-R in three ways: a large deletion that removes the entire ligand-binding domain of the EGF-R; smaller deletions altering the carboxy-terminal domain of the EGF-R; and various point mutations resulting in conservative changes of amino acids throughout the protein. All of these mutations are believed to play a role in the activation of the protein's transforming activity (21, 32, 42, 43). The product of *v-erbB* is synthesized as a 61-kDa precursor that is subsequently modified by N-linked glycosylation and phosphorylation to 65-, 68-, and 74-kDa species (3, 35, 37). The 65- and 68-kDa processing intermediates are associated primarily with intracellular membranes, whereas the 74-kDa form is found associated with the plasma membrane (3). Studies utilizing a conditional mutant (temperature sensitive) of *v-erbB* suggested that the fully glycosylated form on the

plasma membrane is alone responsible for the transforming activity of *v-erbB* (3, 5, 38).

The product of *v-erbB* exemplifies one of two main genres of protein-tyrosine kinases with transforming activity (12, 21, 48). The other genre is typified by the product of *v-src*, a peripheral membrane protein that is anchored to the cytoplasmic face of the plasma membrane and to other cytoplasmic membranes, a process that requires a combination of myristylation and a protein domain within the *v-src* product (20). Although transformation by the products of both *v-erbB* and *v-src* requires attachment to membranes (2, 19), the morphologies and spectra of cells transformed by the two genes differ. For example, *v-src* transforms mainly fibroblasts (6), whereas *v-erbB* transforms both fibroblasts and immature erythroid cells (14); the morphology of fibroblasts transformed by *v-src* is readily distinguishable from that of fibroblasts transformed by *v-erbB* (see Table 1 and Fig. 2 below); and *v-src* transforms primary cultures of rat embryo fibroblasts, whereas *v-erbB* does not (17).

In an effort to define the structural determinant(s) of these differences, we have constructed chimeric genes that include portions of *v-src* and *v-erbB*. Our results indicate that the character of the biological response to *v-erbB* is determined primarily by the cytoplasmic domain of the gene product rather than by the manner in which the protein is anchored to membranes. In addition, we have confirmed previous studies (1) indicating that the extracellular domain of the *v-erbB* protein is expendable for transformation. In contrast, the transmembrane domain must be either present or replaced by a surrogate—such as the membrane anchor derived from *v-src*.

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MATERIALS AND METHODS

Cells and cell lines were maintained in Dulbecco's modified Eagle minimal medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). The established rodent fibroblast cell lines NIH 3T3 and rat-1 are clonal derivatives of standard cell lines supplied by E. T. Liu (C7 3T3) and A. M. Bruskin (rat-1a) (5). Rat embryo cells (REC) were prepared from 14-day-old Fischer rat embryos as described previously (36). Replication-defective, helper-free virus stocks were produced by transfection of Ψ 2 cells, a retroviral packaging line (4), with the appropriate plasmids and selecting for resistance to G418 (800 μ g/ml; Geneticin; GIBCO). Pooled populations of G418-resistant (G418^R) virus producer cells (500 to 1,000 colonies) were expanded, and virus was collected 18 to 24 h after replacing the media on 80% confluent monolayers. Viral supernatants were centrifuged at 3,000 $\times$ g to remove cellular debris, aliquoted, and frozen at -80°C . Fresh aliquots of virus were used for all experiments.

Plasmids. Plasmid pMLV-*g-erbB* (5) is a replication-defective murine retrovirus vector containing a 1.8-kb *XhoI*-*HindIII* fragment that encodes the entire *gag-erbB* protein and was supplied by A. M. Bruskin. Plasmid pMLV-*VerbNeo3* was constructed by cleaving pMLV-*g-erbB* at its unique *Clal* site and inserting a 2.2-kb *Clal* fragment that encodes resistance to G418 in the same transcriptional orientation as *v-erbB*. Transcription of this gene is promoted by the early region of simian virus 40. This plasmid has previously been referred to as pMLV-*g-erbB*-BN (5).

Construction of *v-erbB* deletion mutants. All constructions were created in pGEM3Zf(+) by standard techniques (27) and were tested for their ability to direct the synthesis of an appropriately sized protein by in vitro transcription followed by in vitro translation of the resulting RNA (data not shown). In addition, the junctions of the deletion mutants were sequenced with in vitro-synthesized RNA by the chain termination method according to the manufacturer's instructions (Promega Biotech).

A 1.8-kb *XhoI*-*HindIII* (XHerbB) fragment containing all of the *v-erbB* coding sequences from pMLV-*VerbNeo3* was isolated from an agarose gel and digested with other enzymes to produce the following fragments: *StuI* (SHerbB), *ThaI* (THerbB), and *FspI* (FHerbB). The positions of these sites in relation to the *v-erbB* coding sequence are indicated in Fig. 1. In order to facilitate the construction and analysis of the various alleles, the *XhoI*-*HindIII* fragment containing *v-erbB* was cloned into the in vitro transcription vector pGEM3Zf(+) (Promega Biotech) to create pGerbB.

The extracellular (EX) domain deletion mutant, Δ STerbB, was created by ligating the THerbB fragment into pGerbB from which the *StuI*-*HindIII* fragment of *v-erbB* had been removed. Similarly, the transmembrane (TM) domain deletion mutant, Δ TFerbB, was created by ligating the FHerbB fragment into pGerbB from which the *ThaI*-*HindIII* fragment of *v-erbB* had been removed. These alleles were then transferred into the murine leukemia virus (MLV) vector plasmid for transfection into Ψ 2 cells.

Construction of *v-src:v-erbB* chimeric alleles. The following plasmids containing portions of *v-src* fused in frame to pyruvate kinase were generously supplied by J. M. Kaplan: *psrc204/PK*, *psrc259/PK*, and *psrcN7259/PK* (19, 20). Plasmids containing *src204*, *src259*, or *N7259/PK* were created by digesting the appropriate starting plasmid with *EcoRI*, which cleaves between the *v-src* and pyruvate kinase-coding sequences. The cleaved end was treated with the Klenow fragment of DNA polymerase to produce a blunt end. The

plasmids were then cleaved with *Sall* to excise the *v-src* coding sequences, and fragments SR204, SR259, and SRN7259 were isolated from an agarose gel. Constructs containing *src14* were created by cleaving *psrc204/pK* with *NaeI* and *Sall*, producing a fragment encoding the amino-terminal 14 amino acids of *v-src* (SN14). Chimeric alleles were produced by ligating the appropriate fragment from *v-src* (SN14, SR204, SR259, or SRN7259) to the appropriate fragment of *v-erbB* (SHerbB or FHerbB), as shown in Fig. 1. In addition, the *StuI*-*HindIII* fragment from the Δ TFerbB allele was used to create chimeras 14 Δ TFerbB, 204 Δ TFerbB, and 259 Δ TFerbB by ligation with SN14, SR204, and SR259, respectively. A total of 12 chimeric alleles were created and tested for biological activity, but only 10 were analyzed in greater detail. All of the chimeric alleles were shuttled into the pMLV-*VerbNeo3* vector which had been cleaved with *XhoI* and *HindIII* to remove the *v-erbB* sequences. pMLV-*srcgpt*, pMLV-*neo*, and pMLV-*srcneo* were gifts of J. M. Kaplan, S. Gerondakis, and M. Verderame, respectively. DNA transfection of Ψ 2 cells (28) was performed by the calcium phosphate technique and was as described elsewhere (15, 46).

Virus infections and titers. All viruses were tested in C7 3T3 and rat-1a cells with essentially the same results. Approximately 2×10^5 cells were seeded into a 60-mm-diameter dish and exposed to various dilutions of virus stocks for 4 h in the presence of 8 μ g of Polybrene (Sigma) per ml. After infection, the monolayers were washed with Tris-buffered saline and fresh medium was replaced in the dishes. After a further 18 to 24 h, the cells were trypsinized: 20% of the cells were placed into fresh medium to measure focus formation; an additional 20% were placed into medium containing 800 μ g of G418 per ml to select for infected cells by virtue of their resistance to G418 (G418^R); the remaining 60% were tested for anchorage-independent growth in low-melting-temperature (LMT) agarose, as described previously (5). Plates for focus formation and G418^R assays were fed every 3 to 4 days. Plates of foci and G418^R colonies were fixed in methanol and stained with 2% (wt/vol) methylene blue, and foci or drug-resistant colonies were counted. Colonies in agarose (>100 cells) were stained with 200 μ g of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) per ml after 21 to 28 days of growth.

Measuring the cloning efficiency of cell populations in LMT agarose. In order to measure the cloning efficiency in LMT agarose of cell populations infected with the various recombinant retrovirus stocks, pools of G418^R cells were established in monolayer culture. Cells were trypsinized and counted with a hemocytometer. Three different cell concentrations (10^6 , 10^4 , and 10^2 cells per dish) were then placed in LMT agarose as described above. After 21 to 28 days, the number of colonies in each dish was counted. The cloning efficiency of each cell population is expressed as a percentage, i.e., the number of colonies in each dish divided by the number of cells placed into the dish at the start of the experiment.

REC transformation assay (24). Approximately 2×10^5 REC were seeded into 60-mm-diameter dishes and infected with 2 ml of undiluted virus as described previously. Forty-eight hours after infection, the cells were trypsinized, and 50% were tested for anchorage-independent growth in LMT agarose; the remainder were tested for resistance to 400 μ g of G418 per ml (G418^R). Colonies in agarose were counted after 21 to 28 days; colonies in G418 were counted after 14 to 21 days.

Analysis of soluble versus particulate localization. C7 3T3

FIG. 1. Schematic representation of the alleles of *v-erbB* and *v-src* and the chimeric alleles described in this paper. The chimeric alleles are ΔSTerbB (deletion of coding sequences between the *StuI* and *ThaI* sites of *v-erbB*); ΔTFerbB (deletion of coding sequences between the *ThaI* and *FspI* sites of *v-erbB*); 14FerbB, 204FerbB, and 259FerbB (fusion of sequences coding for the amino-terminal 14, 204, or 259 amino acids of *v-src* to the *FspI* site of *v-erbB*); N7 259FerbB (fusion of sequences encoding the amino-terminal 259 amino acids of a mutant *v-src* encoding an asparagine residue at position 7 to the *FspI* site of *v-erbB*); 14SerbB, 14ΔTFerbB, 204SerbB, 204ΔTFerbB, 259SerbB, and 259ΔTFerbB (fusion of sequences coding for the amino-terminal 14, 204, or 259 amino acids of *v-erbB* to the *StuI* site of either *v-erbB* or ΔTFerbB). g, EX, TM, and K and R indicate the gag, extracellular, transmembrane, and kinase/regulatory regions of *v-erbB*, respectively. U, SH3/SH2, and K and R indicate the unique region, *src* homology regions 3 and 2, and the kinase/regulatory regions of *v-src*, respectively. Precise details of the construction of these alleles are contained in Materials and Methods. The letter in parentheses after the name of the allele refers to the corresponding photomicrograph in Fig. 2.

cells (60-mm-diameter dish containing approximately 2×10^5 cells) infected with MLVneo alone or MLV containing *v-erbB*, *v-erbB*:*v-src* chimeras, or *v-src* were washed with methionine and serum-free Dulbecco's minimum essential medium. The cells were overlaid with 2 ml of methionine-free medium and preincubated for 30 min at 37°C. [^{35} S]methionine (specific activity, >600 Ci/mmol; Amersham) was added to a concentration of 200 μ Ci/ml, and the cells were incubated for a subsequent 4 h. After this incubation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in a hypotonic buffer consisting of 10 mM Tris-Cl, pH 7.5, containing 2 mM $MgCl_2$. Cells were allowed to swell for 10 min and then lysed with 20 strokes in a cell homogenizer (Bernitech Engineering). Nuclei and intact cells were then removed by low-speed centrifugation (5 min, $1,000 \times g$). Soluble and particulate fractions were prepared from the postnuclear supernatants by high-speed centrifugation ($154,000 \times g$) in a Beckman SW

55.1 rotor for 30 min at 4°C. Membranes from the cells were resuspended in 0.5 ml of 25 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 0.5% Nonidet P-40 and sonicated for 10 s at the lowest setting of a Branson sonicator. To the soluble and solubilized particulate fractions representing 10^5 cells were added anti-C-terminal peptide *v-erbB* protein antibodies (10 μ l) or 20 μ g of mAb327 anti-*v-src* protein monoclonal antibody. Both antibodies were present in excess over antigen. Subsequent immunoprecipitations and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out as described earlier (37).

Immune complex kinase assay. The immune complex kinase assay was performed essentially as described previously (5). Briefly, 10^6 infected fibroblasts were washed twice with ice-cold PBS and then lysed in 1 ml of a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 150 mM NaCl, 10% glycerol, and 1% digitonin (Wako). The solubilized cells were centri-

fused at $100,000 \times g$ for 10 min at 4°C. Cell lysates were then assayed for total cell protein by the method of Markwell et al. (29), and equal amounts of protein (100 µg) were immunoprecipitated by incubation for 30 min with excess antibody raised to a peptide corresponding to the C-terminal 12 amino acids of the *v-erbB* gene product. The antibody was pre-bound to protein A-Sepharose for 30 min at 4°C. The immunoprecipitates were washed three times with a mixture containing 50 mM HEPES (pH 7.5), 2 mM $MnCl_2$, 20 µM sodium orthovanadate, and 10 µM ATP and then incubated on ice in the same buffer containing 5 µCi of [γ - ^{32}P]ATP (NEN) for 20 min. The reaction was stopped by addition of Laemmli sample buffer and heating to 100°C for 5 min. Subsequent SDS-polyacrylamide gel electrophoresis and autoradiography were as described above for [^{35}S]methionine labeling.

Western immunoblotting. Dishes (100-mm diameter) of infected fibroblasts were washed twice with ice-cold PBS and solubilized in 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 1% Nonidet P-40. The cell lysates were then cleared by centrifugation at $100,000 \times g$. Laemmli sample buffer was added to the cleared lysate and heated to 100°C for 5 min. Equal amounts of total cell protein (100 µg) were loaded onto each lane of a 10% polyacrylamide gel. Following electrophoresis, proteins were blotted onto an Immobilon-P membrane by using a Milliblot-SDE semidry blotter for 40 min at 2.5 MA/cm². The Immobilon-P membrane was blocked for 30 min with a solution of PBS containing 5% (wt/vol) nonfat dry milk. The blocked membrane was then incubated overnight at room temperature in an anti-*erbB* monoclonal antibody ascitic fluid (E1.21.4) (37) diluted 1:1,000 in TMT buffer (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5% nonfat dry milk, and 0.2% Tween 20). The blot was then washed three times for 20 min each time in TMT buffer and then incubated for 30 min with ^{125}I -protein A (ICN) at 0.1 µCi/ml in TMT buffer. Finally, the blot was washed three times for 20 min each time with TMT buffer, wrapped in Saran Wrap, and exposed to X-ray film.

Cell labeling with [^3H]myristic acid. Dishes (60-mm diameter) containing approximately 10^6 infected fibroblasts were overlaid with 2 ml of Dulbecco's modified Eagle medium, and [^3H]myristic acid (Amersham; 50 mCi/ml in dimethyl sulfoxide) was added to a concentration of 1 mCi/ml. The cells were incubated for 18 h at 37°C, washed twice with PBS, and solubilized in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1% Nonidet P-40. The lysates were subsequently cleared, and portions representing equal amounts of total cell protein were immunoprecipitated and analyzed as described under "Analysis of soluble versus particulate localization."

Protein assay. The assay for total cell protein was performed by a modification of the method of Lowry as described by Markwell et al. (29).

RESULTS

Testing the transforming activity of *v-erbB* in mammalian cells. Mammalian cells offered both convenience and useful phenotypic distinctions for our purposes. We employed an MLV vector carrying the highly active ES4 allele of *v-erbB*. In previous work, we demonstrated that this vector can transform established lines of rodent fibroblasts (5) but not primary cultures of REC (11, 17). Others have found that a similar vector carrying *v-erbB* transforms murine pre-B cells (33) and immature erythroid cells (30) and causes B-cell tumors in mice (33).

TABLE 1. Measurement of the transforming efficiency of the recombinant retrovirus stocks containing *v-erbB*, *v-src*, and the chimeric alleles^a

Allele contained by MLV stock ^b	No. of transformed foci/ml	No. of G418 ^R cells/ml	CFU/ml	Cloning efficiency (%) of G418 ^R cells ^c
neo (A)	0	10^5	0	0
<i>v-erbB</i> (B)	$\sim 10^4$	$\sim 10^4$	$\sim 10^3$	10–15
<i>v-src</i> (C)	$\sim 10^4$	$\sim 10^{4d}$	$\sim 5 \times 10^3$	60–70
Δ STerbB (D and E)	$\sim 5 \times 10^3$	$\sim 10^4$	$\sim 5 \times 10^2$	10–15
Δ TFerbB (F)	0	$\sim 10^4$	0	0
14FerbB (M)	$\sim 10^4$	$\sim 10^4$	$\sim 4 \times 10^3$	50–60
204FerbB	$\sim 10^4$	$\sim 10^4$	$\sim 5 \times 10^3$	50–60
259FerbB (N)	$\sim 10^4$	$\sim 10^4$	$\sim 4 \times 10^3$	50–60
N7259FerbB (O)	0	$\sim 10^4$	0	0
14SerbB (J)	$\sim 5 \times 10^2$	$\sim 10^4$	$\sim 10^2$	10–15
14 Δ TFerbB (G)	$\sim 10^4$	$\sim 10^4$	$\sim 2 \times 10^3$	50–60
204SerbB (K)	0	$\sim 10^4$	0	0
204 Δ TFerbB (H)	$\sim 10^4$	$\sim 10^4$	$\sim 3 \times 10^3$	50–60
259SerbB (L)	0	$\sim 10^4$	0	0
259 Δ TFerbB (I)	$\sim 10^4$	$\sim 10^4$	$\sim 2 \times 10^3$	60–70

^a C7 3T3 cells were infected with various dilutions of the recombinant retrovirus stocks. The ability of the viruses to yield transformed foci, colonies of drug-resistant cells, and colonies (CFU) in semisolid media was assayed as described in Materials and Methods.

^b All virus stocks except that with *v-src* contain the neomycin resistance gene. The letter in parentheses refers to the corresponding photomicrograph in Fig. 2.

^c Established cultures of drug-resistant cells were also tested for their ability to form colonies in semisolid media, and this is expressed as a percentage, i.e., the number of cells that form colonies in LMT agarose divided by the total number of cells tested as described in Materials and Methods.

^d Cells selected for resistance to mycophenolic acid.

The transforming activity of MLV carrying various alleles of *v-erbB* was tested by infection of both C7 3T3 and rat-1a cells. Transformation was determined by the focus formation assay, the ability of infected cells to grow in an anchorage-independent fashion in LMT agarose, and, finally, by assessment of the morphology of colonies of G418-resistant cells derived by infection with the various virus stocks. In these studies we utilized pMLV**erbB**neo as a starting vector. This vector allows the expression of the ES4 allele of *v-erbB* as a fusion protein with the amino-terminal 6 amino acids of the viral structural gene *gag*, which serves to greatly enhance the transforming ability of *v-erbB* (5). The results of the experiments with the mutant alleles are summarized in Table 1 and Fig. 2. The parental allele of *v-erbB* produced a profound morphological alteration of C7 3T3 cells and distinct foci of transformed cells (Fig. 2, compare panels A and B). In addition, close to 100% of the G418^R colonies of C7 3T3 cells carrying *v-erbB* were obviously morphologically transformed. When *v-erbB* was expressed in rat-1a cells, the morphological alteration was more subtle and the foci were less distinct (data not shown; for an example, see reference 5). For reasons that are unclear, rat-1a cells expressing *v-erbB* formed colonies with higher efficiency in LMT agarose than their C7 3T3 counterparts, but both types of cells formed tumors in the appropriate animal host (5).

Role of an extracellular and transmembrane domain in transformation by *v-erbB*. In an effort to explore the role that certain structural features of the *v-erbB* protein play in transformation, we made two deletion mutants, one in which 70 amino acids from the extracellular domain of the protein

FIG. 2. Morphological characteristics of retrovirus-infected C7 3T3 cells. C7 3T3 cells infected with replication-defective retrovirus stocks were selected for drug resistance as described in Materials and Methods. Pools of drug-resistant colonies were trypsinized and established in monolayer culture. Cells were cultured until they were 70 to 95% confluent and then photographed with a Nikon TMS photomicroscope using Polaroid Type 57 film. Magnification is $\times 79$. Cells were infected with the following retrovirus stocks: neo (A), *erbB*neo (B), *srcgpt* (C), Δ *STerbB*neo (transformed) (D), Δ *STerbB*neo (not transformed) (E), Δ *TFerbB*neo (F), 14 Δ *TFerbB*neo (G), 204 Δ *TFerbB*neo (H), 259 Δ *TFerbB*neo (I), 14*SerbB*neo (J), 204*SerbB*neo (K), 259*SerbB*neo (L), 14*FerbB*neo (M), 259*FerbB*neo (N), N7 259*FerbB*neo (O).

have been removed (Δ STerbB; Fig. 1) and another in which the hydrophobic transmembrane region has been deleted (Δ TFerbB; Fig. 1). The Δ STerbB allele was almost as potent as the wild-type allele in transforming activity (Table 1 and Fig. 2). The major difference was that approximately 50% of the G418^R colonies expressing Δ STerbB was flat and had a nontransformed morphology (Fig. 2, compare panels D and E). Preliminary evidence suggests that these flat colonies expressed less of the Δ STerbB protein than the colonies that appeared to be morphologically transformed (29a). By contrast, the Δ TFerbB allele from which coding sequences for the transmembrane domain were deleted was transformation defective. G418^R cells infected with a virus carrying this allele were flat and nonrefractile, resembled cells derived from infection with MLVneo alone (Fig. 2, compare panels A, B, and F), and were unable to grow in LMT agarose (Table 1).

We concluded that most of the external domain of *v-erbB* is dispensable for the transforming activity of the protein; it follows that posttranslational modification of the *v-erbB* protein by N-linked glycosylation is of little consequence to transformation by this molecule. Furthermore, from the results with the Δ TFerbB allele, we concluded that the transmembrane domain of the protein is required for transformation, possibly by acting as a noncleavable signal sequence and for stable membrane insertion.

Fusion of 14, 204, or 259 amino acids from the amino terminus of *v-src* can reactivate the transforming potential of the kinase domain of *v-erbB*. The association of the gene product of *v-src* with the cytoplasmic face of the plasma membrane requires the cotranslational removal of the amino-terminal methionine and the addition of myristic acid to the glycine residue that now constitutes the amino terminus of the protein (6, 19). In addition, there are specific interactions between the amino-terminal half of the *src* protein and one or more components of the plasma membrane (20). In order to determine whether the membrane localization signal of *v-src* could function in the context of *v-erbB* and perhaps reactivate the transformation-negative mutant of *v-erbB* (Δ TFerbB), several chimeric alleles were created (Fig. 1). First, portions of *v-src* coding for 14, 204, or 259 amino acids were fused to FHerbB, which codes for the kinase domain and carboxy-terminal regulatory portions of the protein (14FerbB, 204FerbB, and 259FerbB). The same portions of *v-src* were fused to SHerbB, which contains nearly all of the *v-erbB* coding sequence (14SerbB, 204SerbB, and 259SerbB); thus, this second set of chimeras contained membrane anchorage domains from both *v-src* and *v-erbB*. Finally, the same portions of *v-src* were fused to the *StuI* site of the allele from which the transmembrane domain had previously been removed (Δ TFerbB) to generate a third set of chimeras (14 Δ TFerbB, 204 Δ TFerbB, and 259 Δ TFerbB). All of these alleles were tested for their transforming potential. The results are summarized in Table 1 and Fig. 1 and 2.

The chimeras 14FerbB, 204FerbB (not shown in Fig. 2), and 259FerbB were highly transforming, as measured by anchorage-independent growth and by focus assay (Table 1). In addition, the morphology of 95 to 100% of G418^R colonies of C7 3T3 cells expressing these alleles was rounded, refractile, and highly reminiscent of transformed cells (Fig. 2, compare panel A with panels M and N). Interestingly, there was no obvious qualitative or quantitative difference in the phenotypes elicited by alleles coding for 14, 204, or 259 amino acids of the *v-src* protein. The results presented here suggest that the SH3/SH2 region of *v-src* located between residues 60 and 260 of the protein had little or no effect on

the transformation properties of the *v-erbB* kinase domain present in the chimeric proteins (see Discussion).

In the set of alleles in which the point of fusion between *v-src* and *v-erbB* was upstream of the transmembrane domain (i.e., at the *StuI* or *SalI* sites [latter data not shown]), only the 14SerbB allele displayed any transforming activity, and that was weak (Table 1 and Fig. 2, compare panel A with panels J to L). In contrast, if the transmembrane domain was subsequently removed (14 Δ TFerbB, 204 Δ TFerbB, and 259 Δ TFerbB), the resulting alleles were highly transforming (Table 1 and Fig. 2, compare panels J and G, K and H, and L and I). This suggested that the transmembrane domain of *v-erbB* was acting in a negative fashion to inhibit the transforming activity of the fusion proteins: its subsequent removal reactivated the transforming potential of the protein.

The transforming activity of the chimeras appeared more potent than that of wild-type *v-erbB*: morphological conversion of both C7 3T3 and rat-1a cells was more blatant in response to the chimeras (Fig. 2, compare panels B and C with panels G to J, M, and N). Similar results were obtained with rat-1a cells (not illustrated). Furthermore, C7 3T3 cells expressing the chimeric proteins formed colonies with higher efficiency in LMT agarose (Table 1) than cells containing *v-erbB*.

Myristylation is required for the transforming potential of the chimeric proteins. Myristylation is required for both the membrane localization and transforming potential of *v-src* (6, 19, 20). The amino acid residue at position 7 is important to the process of myristylation; both lysine and arginine at this position allow efficient myristylation, whereas asparagine at this position leads to a 5- to 10-fold reduction in myristylation. Mutants of *v-src* carrying an asparagine residue at position 7 were found to be nontransforming and no longer associated with cellular membranes (19). In order to test whether myristylation was important for the biological activity of the *v-src*:*v-erbB* chimeras, a mutant was constructed in which sequences coding for 259 amino acids of a mutant *v-src* were fused to FHerbB (N7 259FerbB). This allele contained an asparagine codon at position 7 and would therefore be expected to encode a poorly myristylated protein. When tested, this virus proved to be transformation defective (Table 1) and colonies of infected cells appeared flat and nontransformed and were incapable of forming colonies in agarose (Fig. 2, compare panel O with panel N). Subsequent experiments clearly indicated that this allele was expressed at similar levels as its transforming counterpart, 259FerbB, showing that the difference in transforming potential did not merely reflect the level of expression of the two different alleles (data not shown).

Chimeric *v-src*:*v-erbB* alleles do not transform REC. Murine retroviruses carrying *v-src* are capable of transforming REC without the assistance of another oncogene, whereas similar retroviruses carrying *v-erbB* do not transform such cells (17). Since the chimeric alleles appeared more potent in transformation assays than *v-erbB*, we speculated that the chimeras may have acquired some or all of the transforming properties of *v-src*. To test this hypothesis, REC were infected with viruses containing *v-erbB*, *v-src*, and the various chimeras. The results shown in Table 2 reveal that only *v-src* was capable of transforming REC. Furthermore, it was possible to isolate G418-resistant colonies of REC containing the chimeric alleles. In all cases, the cells were flat and nontransformed, whereas 20% of the mycophenolic acid-resistant colonies obtained after infection with MLVsrcgpt were highly transformed (data not shown).

TABLE 2. Measurement of the transforming efficiency of the recombinant retrovirus stocks in primary cultures of REC^a

Allele contained by MLV stock	CFU/ml	No. of G418 ^R cells/ml
MLVneo	0	10 ⁴
MLVsrcgpt	200	2 × 10 ^{3b}
MLV Δ erbBneo and all of the viruses carrying a <i>v-src:v-erbB</i> chimera	0	10 ³ –10 ⁴

^a Primary cultures of REC were infected with 2 ml of each of the undiluted recombinant retrovirus stocks. The ability of the viruses to yield colonies in LMT agarose and colonies of drug-resistant cells was assayed as described in Materials and Methods.

^b Cells selected for resistance to mycophenolic acid.

Expression and enzymatic activity of *v-erbB* and fusion proteins in C7 3T3 cells. In order to investigate the biochemical properties of the various chimeric proteins, we conducted a series of experiments on pools of infected C7 3T3 colonies that had been selected in G418. By using an antiserum that recognizes the kinase domain of *v-erbB*, the proteins expressed in each cell population were analyzed by Western blotting (Fig. 3A). A second antiserum raised to a peptide corresponding to the C-terminal 12 amino acids encoded by *v-erbB* was used to determine the enzymatic activity of the proteins by an immune complex kinase assay (Fig. 3B).

We found that cells infected with a virus that expresses *v-erbB* contained three forms of *erbB* proteins of 65, 68, and 74 kDa (Fig. 3A, lane 2), all of which became labeled in the kinase assay (Fig. 3B, lane 2). The nontransforming Δ TF Δ erbB allele was expressed as a single polypeptide species (Fig. 3A, lane 3). In work not shown, inclusion of tunicamycin (an inhibitor of N-linked glycosylation) had no effect on the expression pattern of Δ TF Δ erbB protein observed when cells expressing this molecule were labeled with [³⁵S]methionine and then analyzed by immunoprecipitation.

These results suggested that the Δ TF Δ erbB protein was not posttranslationally glycosylated, in spite of the fact that it contains the three putative sites of N-linked glycosylation that are believed to be modified in *v-erbB*. The Δ TF Δ erbB protein displayed an intrinsic protein kinase activity (Fig. 3B, lane 3) that was roughly equivalent to that of the *v-erbB* protein.

Fusion sequences coding for 14 or 259 amino acids of *v-src* to the *StuI* site of Δ TF Δ erbB restored the transforming activity of this molecule, as discussed earlier. These alleles were expressed as single polypeptide species (Fig. 3A, lanes 4 and 5) which had kinase activity (Fig. 3B, lanes 4 and 5). Similarly, if the fusion junction was at the *Fsp* site in *v-erbB*, the molecule was transforming as mentioned earlier, migrated as a single polypeptide species (Fig. 3A, lane 6), and retained kinase activity (Fig. 3B, lane 6). Finally, if the first 25 amino acids encoded by *v-erbB* containing the important *gag* amino acids were replaced by the first 14 amino acids encoded by *v-src*, the resulting protein appeared to be expressed in a manner analogous to that of *v-erbB*: similar patterns of polypeptide species were detected in both the Western blot and kinase assays (Fig. 3A, lane 7, and Fig. 3B, lane 7, respectively). The 14 Δ erbB gene product was apparently posttranslationally modified by N-linked glycosylation, similar to that of *v-erbB*. Thus, the amino-terminal 14 amino acids encoded by *v-src* can replace the *gag* amino acids encoded by *v-erbB* to produce a transforming protein; however, this allele is somewhat less transforming than its parental counterpart (Table 1) but more transforming than the allele from which the *gag* sequences have been deleted (5).

Myristylation of *v-src*, *v-erbB*, and various chimeric proteins. To investigate the possibility that the portions of *v-src* fused to *v-erbB* were directing the myristylation of the resulting chimeric proteins, we labeled cells expressing these alleles with [³H]myristic acid. *v-erbB*, *v-src*, and chimeric proteins were subsequently immunoprecipitated and ana-

FIG. 3. Expression (analyzed by Western blotting) (A) and immune complex kinase assay (B) of *v-erbB/v-src* chimeras. C7 3T3 fibroblasts infected with MLVneo alone (lanes 1), MLVneo containing *v-erbB* (lanes 2), or various *v-erbB* mutants and *v-erbB:v-src* chimeras as noted in the figure (lanes 3 through 7) were lysed. (A) Equal amounts of total cell protein (100 µg) were loaded onto each lane of an SDS–10% polyacrylamide gel. Following electrophoresis the proteins were blotted onto Immobilon-P and probed with a monoclonal antibody to *v-erbB* protein, and the antibody was detected with ¹²⁵I-protein A as described in Materials and Methods. Arrowheads indicate the locations of *v-erbB* proteins; the protein running in each lane at approximately 70 kDa is a cross-reactive protein not related to *v-erbB* protein, as determined by V8 protease mapping (36a). The autoradiogram was exposed for 18 h at –80°C. (B) The lysed material was immunoprecipitated with an anti-*v-erbB* protein antibody, and the immune complex was assayed and analyzed for protein kinase activity as described in Materials and Methods. Equal amounts of total cell protein (100 µg) were used in each immunoprecipitation. Phosphorylated *v-erbB:v-src* proteins are indicated with arrowheads. Electrophoresis was in an SDS–7.5% polyacrylamide gel. The autoradiogram was exposed for 6 h at –80°C.

FIG. 4. Myristylation of proteins encoded by *v-erbB*:*v-src* chimeras. C7 3T3 fibroblasts infected with MLVneo alone (lane 1), MLVneo containing *v-erbB* (lane 2), or various *v-erbB* mutants and *v-erbB*:*v-src* chimeras as noted in the figure (lanes 3 through 8) were radiolabeled with [^{3} H]myristic acid for 18 h, immunoprecipitated, and analyzed as described in Materials and Methods. Tritiated *v-erbB*:*v-src* or *v-src* proteins are indicated with arrowheads. Electrophoresis was in an SDS-7.5% polyacrylamide gel. The autoradiogram was exposed for 6 weeks at -80°C .

lyzed by polyacrylamide gel electrophoresis. We found that, in contrast to *v-src*, neither *v-erbB* nor its nontransforming counterpart, ΔTFerbB , encodes proteins that are detectably myristylated (Fig. 4, lanes 2 and 3). However, all of the *v-erbB*:*v-src* chimeras are myristylated (Fig. 4, lanes 4 through 8), as was the *v-src* protein (Fig. 4, lane 8); the stoichiometry of myristylation of each of the chimeras is unknown. It is worthy of note that the transformation-positive 14*SerbB* allele appeared to be both myristylated and glycosylated, as evidenced by the presence of myristic acid in the processed forms of the protein (Fig. 4, lane 7). This result would suggest that the cotranslational addition of myristic acid to the amino terminus of the protein does not preclude transport through the rough endoplasmic reticulum and the Golgi apparatus with the concomitant addition of sugar residues to the protein.

Biochemical fractionation of cells expressing *v-erbB*, *v-src*, and the various chimeric proteins. It is possible to separate

the proteins expressed in a cell into a soluble fraction and a particulate fraction by Dounce homogenization in hypotonic buffer followed by high-speed centrifugation. Generally, proteins that partition into the particulate fraction are membrane associated, whereas those that partition in the soluble fraction are not. To investigate the subcellular location of proteins encoded by *v-erbB*, *v-src*, and the various chimeras, cells expressing these alleles were labeled with [^{35} S]methionine and fractionated as described above and in further detail in Materials and Methods. Appropriate antisera were then used to immunoprecipitate the proteins and analyze their distribution in the cell.

The products of *v-erbB* and *v-src* clearly partitioned in the particulate fraction of the cell (Fig. 5, lanes 4 and 16), as had been reported previously (19, 55). The large majority of the nontransforming ΔTFerbB protein was found in the soluble fraction (Fig. 5, lane 5), suggesting that it was either no longer or only weakly associated with cellular membranes. A substantial percentage, if not all, of the protein products of the chimeric alleles that were analyzed was found in the particulate fractions (Fig. 5, lanes 7 through 14), suggesting that these molecules are membrane associated. It is of interest to note that while most of the 14*ΔTF* protein was found in the particulate fraction, approximately 10 to 15% was in the soluble fraction (Fig. 5, compare lanes 5 and 6); in contrast, 100% of the 259*ΔTFerbB* protein was particulate (Fig. 5, lanes 9 and 10). There is a clear indication from other studies that although 14 amino acids encoded by *v-src* are sufficient to direct membrane localization of a normally cytoplasmic protein, there are domains of the *src* protein between residues 15 and 259 that serve to stabilize association with membranes and perhaps to direct the association of the proteins to other cellular membranes or organelles (20).

DISCUSSION

Requirement for membrane attachment in transformation by *v-erbB*. The data reported here demonstrate that transformation by *v-erbB* requires the transmembrane domain of the protein but not an appreciable portion of the extracellular domain. Thus, it appears that the product of *v-erbB* must be attached to membranes in order to exert its biological

FIG. 5. Localization of proteins encoded by *v-erbB*:*v-src* chimeras. C7 3T3 cells infected with MLVneo alone (lanes 1 and 2), MLVneo containing *v-erbB* (lanes 3 and 4), and various *v-erbB* mutants and *v-erbB*:*v-src* chimeras (lanes 5 through 14) or *v-src* (lanes 15 and 16) were radiolabeled with [^{35} S]methionine for 4 h, lysed with a cell homogenizer, and separated into soluble (S) or particulate (P) fractions by ultracentrifugation at $154,000 \times g$. Samples representing equal numbers of cells were immunoprecipitated with *v-erbB* or *v-src* protein antibodies and analyzed as described in Materials and Methods. Arrowheads indicate the positions of *v-erbB*:*v-src* proteins. Electrophoresis was in an SDS-10% polyacrylamide gel. The autoradiogram was exposed for 8 h at -80°C .

effects. This conclusion is in general accord with results obtained previously (1, 2, 25, 31). There is one discrepancy, however: in the previous work, an allele of *v-erbB* lacking the transmembrane domain elicited microcolonies in agar, whereas the analogous allele was biologically inert in our hands (2). We can offer two possible explanations for this discrepancy. First, the previous testing was performed with an avian retroviral vector in primary cultures of chick embryo cells, whereas the present work employed a murine retroviral vector in established lines of rodent cells. Second, the avian vector retained *v-erbA*, which some investigators claim has a limited capacity of its own to elicit growth of chick fibroblasts in agar or to complement a partially defective *v-erbB* gene (10, 18). The main conclusion seems secure; the product of *v-erbB* loses most if not all of its transforming potential if it is not attached to cellular membranes.

A surrogate membrane anchor for the product of *v-erbB*. The amino-terminal 111 amino acids of the *v-erbB* protein include a multiply glycosylated extracellular domain and a domain that spans the plasma membrane. Deletion of both of these regions leaves a protein that is now cytoplasmic and retains undiminished protein-tyrosine kinase activity but cannot transform cells. Transforming activity can be restored by adding no more than 14 amino acids from the amino terminus of the *v-src* protein, which attaches proteins to the cytoplasmic face of the plasma membrane by a combination of myristylation and protein-protein interactions. These results indicate that the *v-erbB* kinase domain retains access to its crucial substrates even when anchored to the membrane in the manner of a peripheral rather than a transmembrane protein.

Two of the domains from *v-src* used in the chimeras (those with residues 1 to 204 and 1 to 259) contain either two-thirds or all, respectively, of a region known as SH3/SH2, which apparently exerts regulatory influences on the kinase activity of the *v-src* protein (16). We had speculated that the presence of these domains in the chimeras might either inhibit or enhance the transforming potential of the kinase domain of *v-erbB*. It is clear, however, from the results presented here that these regulatory domains in the chimeras had no appreciable effect on either the enzymological activity of the gene products or the spectrum and potency of transforming activity.

The *v-erbB* protein does not possess the cleavable signal sequence by which the intact EGF-R crosses the plasma membrane during translation. Instead, previous evidence has suggested that the transmembrane domain of the *v-erbB* protein does double duty by providing an uncleaved signal sequence. Our evidence sustains this suggestion. First, translation of *v-erbB* mRNA in vitro gives rise to a protein that crosses membranes and becomes glycosylated without reduction in size by proteolytic cleavage (data not shown). Second, deletion of the transmembrane domain causes the *v-erbB* protein to remain in the cytoplasm in an unglycosylated form (Fig. 5, lane 5).

Three of the chimeras that we studied contain both the transmembrane domain of *v-erbB* and the membrane attachment domain of the *v-src* protein. Two of these chimeras; 204*SerbB* and 259*SerbB*, are biologically inactive. We cannot presently explain why the combined presence of two distinct devices for membrane attachment interferes with transformation, when either of the two alone facilitates biological activity. The third of these chimeras, 14*SerbB*, contains both the first 14 amino acids encoded by *v-src* (as an amino-terminal domain) and most of the extracellular do-

main and the transmembrane and cytoplasmic domains of *v-erbB*. This allele is at least weakly transforming, and the gene product is both myristylated and glycosylated (the latter implying a transmembrane configuration). The reduced transformation potential may simply reflect a lower level of expression of the protein (Fig. 3A). These results suggest that the cotranslational addition of myristic acid does not preclude transit across the membrane, glycosylation, and transport through the endoplasmic reticulum and Golgi apparatus to the plasma membrane. Nonetheless, subsequent deletion of the transmembrane domain of *v-erbB* from the 14*SerbB* allele (14Δ*TFerbB*) significantly increases its transforming activity.

Attachment to the plasma membrane and transformation by *v-erbB*. Previous analysis of a temperature-sensitive allele of *v-erbB* suggested that attachment to the plasma membrane (as opposed to other cytoplasmic membranes) is required for transformation (3). But a chimera which contains only the first 14 amino acids from the amino terminus of the *v-src* protein may violate that tenet. According to previous results, the portion of *v-src* present in this chimera directs proteins principally to an unidentified form of cytoplasmic vesicle rather than to the plasma membrane. The large portions of *v-src* used in other chimeras can direct the gene product to perinuclear membranes and the plasma membrane (20), but these domains had no additional effect on the biological activity of the chimeras. We presume that the rules of localization previously defined also apply to the chimeric proteins studied here. We have been unable to test this presumption, however, because immunofluorescence microscopy failed to detect any of the chimeric proteins in infected cells (data not shown), even though the proteins were readily detectable by other means. It remains intuitively likely to us that the crucial substrates for the *v-erbB* protein are located on or near the plasma membrane, because that is the location from which the EGF-R itself appears to act.

Determinants of specificity in transformation by protein-tyrosine kinases. Although the potency of transformation by the chimeric genes was moderately greater than that by *v-erbB* itself, the phenotype and spectrum of transformation were those expected for *v-erbB* rather than for *v-src*. We conclude that the specificity of transformation by *v-erbB* probably resides in the selection of substrates by the cytoplasmic domain of the gene product. If the membrane anchorage provided by *v-src* is targeted to specific locations on cytoplasmic membranes (20), these locations do not preclude access of the *v-erbB* protein to its crucial substrates.

A variety of chimeric protein-tyrosine kinases have been constructed, by using both transmembrane and peripheral membrane varieties of the enzymes (7, 23, 26, 34). Most of these have given similar results. For example, the character of the biological response to a growth factor is determined not by the protein domain to which the ligand binds, but by the catalytic domain that the binding activates. Furthermore the protein encoded by *v-ras*, which is normally anchored to the plasma membrane by carboxy-terminal lipid modifications, retains its characteristic transforming activity even when it is anchored to the membrane by an amino-terminal domain derived from *v-src* (22). Thus, our conclusion for *v-erbB* reflects what may be a general principle. The specificity of biological response to protein-tyrosine kinases resides mainly in the cytoplasmic domain, rather than elsewhere in the molecule.

ACKNOWLEDGMENTS

We thank Joshua Kaplan, Arthur Bruskin, Alan Rogers, and Alan Wells for providing reagents and for helpful discussion throughout this work and Monica Vetter for critical reading of this manuscript. (M.M. is especially indebted to Karyl Nakamura, Lynn Vogel, and Karen DeVaney for their invaluable assistance during the production of this manuscript.)

Work reported here was supported by the National Institutes of Health (grant no. CA 44338) and by funds from the G. W. Hooper Research Foundation. M.M. was supported by fellowships from the Imperial Cancer Research Fund and the American Cancer Society (California Division).

ADDENDUM IN PROOF

Crowe and Hayman have recently demonstrated that the amino-terminal 14 amino acids of *v-src* can replace the viral envelope component of the transmembrane protein tyrosine kinase oncogene *v-sea* (A. Crowe and M. Y. Hayman, A myristylated form of the sea oncoprotein can transform chicken embryo fibroblasts. *J. Virol* 65:2533–2538, 1991).

REFERENCES

1. Bassiri, M., and M. L. Privalsky. 1986. Mutagenesis of the avian erythroblastosis virus *erbB* coding region: an intact extracellular domain is not required for oncogenic transformation. *J. Virol.* 59:525–530.
2. Bassiri, M., and M. L. Privalsky. 1987. Transmembrane domain of the AEV *erb-B* oncogene protein is not required for partial manifestation of the transformed phenotype. *Virology* 159:20–30.
3. Beug, H., and M. J. Hayman. 1984. Temperature-sensitive mutants of avian erythroblastosis virus: surface expression of the *erbB* product correlates with transformation. *Cell* 36:963–972.
4. Brown, A. M. C., and M. R. D. Scott. 1987. Retroviral vectors, p. 189–212. In D. M. Glover (ed.), *DNA cloning, a practical approach*, vol. III. IRL Press, Oxford.
5. Bruskin, A., J. Jackson, J. M. Bishop, D. J. McCarley, and R. C. Schatzman. 1990. Six amino acids from the retroviral gene *gag* greatly enhance the transforming potential of the oncogene *v-erb-B*. *Oncogene* 5:15–24.
6. Cooper, J. A. 1990. The *src* family of protein-tyrosine kinases, p. 85–113. In B. Kemp (ed.), *Peptides and protein phosphorylation*. CRC Press, Boca Raton, Fla.
7. Di Fiore, P. P., O. Segatto, W. G. Taylor, S. A. Aaronson, and J. H. Pierce. 1990. EGF receptor and *erbB-2* tyrosine kinase domains confer cell specificity for mitogenic signaling. *Science* 248:79–83.
8. Downward, J., Y. Yarden, E. Mayes, G. Scrase, N. Totty, P. Stockwell, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and *v-erb-B* oncogene protein sequences. *Nature (London)* 307:521–527.
9. Frykberg, L., S. Palmieri, H. Beug, T. Graf, M. J. Hayman, and B. Vennstrom. 1983. Transforming capacities of avian erythroblastosis virus mutants deleted in the *erbA* or *erbB* oncogenes. *Cell* 32:227–238.
10. Gandrillon, O., P. Jurdic, M. Benchaibi, J. H. Xiao, et al. 1987. Expression of the *v-erbA* oncogene in chicken embryo fibroblasts stimulates their proliferation *in vitro* and enhances tumor growth *in vivo*. *Cell* 49:687–697.
11. Gazit, A., J. H. Pierce, M. H. Kraus, P. P. Di Fiore, C. Y. Pennington, and S. A. Aaronson. 1986. Mammalian cell transformation by a murine retrovirus vector containing the avian erythroblastosis virus *erbB* gene. *J. Virol.* 60:19–28.
12. Gilmore, T., J. E. DeClue, and G. S. Martin. 1985. Protein phosphorylation at tyrosine is induced by the *v-erbB* gene product *in vivo* and *in vitro*. *Cell* 40:609–618.
13. Graf, T., and H. Beug. 1983. Role of the *v-erbA* and *v-erbB* oncogenes of avian erythroblastosis virus in erythroid cell transformation. *Cell* 34:7–9.
14. Graf, T., B. Royer-Pokora, G. E. Schubert, and H. Beug. 1976. Evidence for the multiple oncogenic potential of cloned leukemia virus: *in vivo* and *in vitro* studies with avian erythroblastosis virus. *Virology* 71:423–433.
15. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467.
16. Hirai, H., and H. E. Varmus. 1990. Site-directed mutagenesis of the SH2- and SH3-coding domains of *c-src* produces varied phenotypes, including oncogenic activation of *p60^{c-src}*. *Mol. Cell. Biol.* 10:1307–1318.
17. Hjelle, B., E. Liu, and J. M. Bishop. 1988. Oncogene *v-src* transforms and establishes embryonic rodent fibroblasts but not diploid human fibroblasts. *Proc. Natl. Acad. Sci. USA* 85:4355–4359.
18. Jansson, M., H. Beug, C. Gray, T. Graf, and B. Vennstrom. 1987. Defective *v-erbB* genes can be complemented by the *v-src* *onco* in erythroblast and fibroblast transformation. *Oncogene* 1:167–173.
19. Kaplan, J. M., G. Mardon, J. M. Bishop, and H. E. Varmus. 1988. The first seven amino acids encoded by the *v-src* oncogene act as a myristylation signal: lysine 7 is a critical determinant. *Mol. Cell. Biol.* 8:2435–2441.
20. Kaplan, J. M., H. E. Varmus, and J. M. Bishop. 1990. The *src* protein contains multiple domains for specific attachment to membranes. *Mol. Cell. Biol.* 10:1000–1009.
21. Kris, R. M., I. Lax, W. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin, and J. Schlessinger. 1985. Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and *v-erbB* protein. *Cell* 40:619–625.
22. Lacal, P. M., C. Y. Pennington, and J. C. Lacal. 1988. Transforming activity of *ras* proteins translocated to the plasma membrane by a myristylation sequence from the *src* gene product. *Oncogene* 2:533–537.
23. Lammers, R., A. Gray, J. Schlessinger, and A. Ullrich. 1989. Differential signalling potential of insulin- and IGF-1-receptor cytoplasmic domains. *EMBO J.* 8:1369–1375.
24. Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* 304:596–602.
25. Lee, E. G., and M. J. Hayman. 1990. Analysis of the role of the extracellular domain of the *v-erbB* oncogene in cellular transformation. *Oncogene* 5:1165–1172.
26. Lee, J., T. J. Dull, I. Lax, J. Schlessinger, and A. Ullrich. 1989. HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. *EMBO J.* 8:167–173.
27. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33:153–159.
29. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87:206–210.
- 29a. McMahon, M., and R. C. Schatzman. Unpublished observations.
30. Miller, M., A. Kennewell, Y. Takayama, A. Bruskin, J. M. Bishop, G. Johnson, and G. Symonds. 1990. Transformation of early erythroid precursor cells (BFU-E) by a recombinant murine retrovirus containing *v-erb-B*. *Oncogene* 5:1125–1131.
31. Ng, M., and M. L. Privalsky. 1986. Structural domains of the avian erythroblastosis virus *erbB* protein required for fibroblast transformation: dissection by in-frame insertional mutagenesis. *J. Virol.* 58:542–553.
32. Pelley, R. J., N. J. Maihle, C. Boerkoel, H.-K. Shu, T. H. Carter, C. Moscovici, and H.-J. Kung. 1989. Disease tropism of *c-erbB*: effects of carboxyl-terminal tyrosine and internal mutations on tissue-specific transformation. *Proc. Natl. Acad. Sci. USA* 86:7164–7168.
33. Pierce, H. J., A. Gazit, P. O. Di Fiore, M. Kraus, C. Y. Pennington, K. L. Holmes, W. F. Davidson, H. C. Morse III, and

- S. A. Aaronson. 1986. Mammalian cell transformation by a recombinant murine retrovirus containing the avian erythroblastosis virus *erbB* gene. *Curr. Top. Microbiol. Immunol.* **132**:55–61.
34. Privalsky, M. L. 1987. Creation of a chimeric oncogene: analysis of the biochemical and biological properties of *v-erbB/src* fusion polypeptide. *J. Virol.* **61**:1938–1948.
35. Privalsky, M. L., L. Sealy, J. M. Bishop, J. P. McGrath, and A. D. Levinson. 1983. The product of the avian erythroblastosis virus *erbB* locus is a glycoprotein. *Cell* **32**:1257–1267.
36. Sap, J., A. Munoz, K. Damm, Y. Goldberg, et al. 1986. The *c-erb-A* protein is a high-affinity receptor for thyroid hormone. *Nature (London)* **324**:635–640.
- 36a. Schatzman, R. Unpublished observations.
37. Schatzman, R. C., G. I. Evan, M. L. Privalsky, and J. M. Bishop. 1986. Orientation of the *v-erb-B* gene product in the plasma membrane. *Mol. Cell. Biol.* **6**:1329–1333.
38. Scotting, P., B. Vennstrom, M. Jansen, T. Graf, et al. 1987. Common site of mutation in the *erbB* gene of avian erythroblastosis virus mutants that are temperature sensitive for transformation. *Oncogene Res.* **1**:265–278.
39. Sealy, L., G. Moscovici, C. Moscovici, and J. M. Bishop. 1983. Site-specific mutagenesis of avian erythroblastosis virus: *v-erb-A* is not required for transformation of fibroblasts. *Virology* **130**:179–194.
40. Sealy, L., M. L. Privalsky, G. Moscovici, C. Moscovici, and J. M. Bishop. 1983. Site-specific mutagenesis of avian erythroblastosis virus: *erb-B* is required for oncogenicity. *Virology* **130**:155–178.
41. Sheiness, D., B. Vennstrom, and J. M. Bishop. 1981. Virus-specific RNAs in cells infected by avian myelocytomatosis virus and avian erythroblastosis virus: modes of oncogene expression. *Cell* **23**:291–300.
42. Shu, H.-K. G., R. J. Pelley, and H.-J. Kung. 1990. Tissue-specific transformation by epidermal growth factor receptor: a single point mutation within the ATP-binding pocket of the *erbB* product increases its intrinsic kinase activity and activates its sarcomagenic potential. *Proc. Natl. Acad. Sci. USA* **87**:9103–9107.
43. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Liberman, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. Seeburg. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature (London)* **309**:418–425.
44. Vennstrom, B., and J. M. Bishop. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell* **28**:135–143.
45. Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, et al. 1986. The *c-erb-A* gene encodes a thyroid hormone receptor. *Nature (London)* **324**:641–646.
46. Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* **14**:725–731.
47. Yamamoto, T., H. Hihara, T. Nishida, S. Kawai, and K. Toyoshima. 1983. A new avian erythroblastosis virus, AEV-H, carries *erbB* gene responsible for the induction of both erythroblastosis and sarcomas. *Cell* **34**:225–232.
48. Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Oi, and K. Toyoshima. 1983. The *erbB* gene of avian erythroblastosis virus is a member of the *src* gene family. *Cell* **35**:71–78.