JOURNAL OF VIROLOGY, June 1987, p. 1938–1948
0022-538X/87/061938-11$02.00/0

Vol. 61, No. 6

# Creation of a Chimeric Oncogene: Analysis of the Biochemical and Biological Properties of a v-*erbB*/*src* Fusion Polypeptide

MARTIN L. PRIVALSKY

*Department of Bacteriology, University of California at Davis, Davis, California 95616*

Received 19 November 1986/Accepted 16 March 1987

**A novel gene was created that linked complementary portions of two different tyrosine kinase oncogenes: v-*erbB* and v-*src*. The v-*erbB*/*src* chimera encoded a glycoprotein exhibiting the subcellular distribution of the v-*erbB* protein but containing the kinase catalytic domain of the v-*src* parent. Fibroblasts expressing the v-*erbB*/*src* gene product became transformed to an oncogenic state and closely resembled cells expressing the v-*erbB* parent oncogene. Our results indicated that v-*erbB* sequences can be functionally replaced by sequences derived from a different oncogene, v-*src*, and that important determinants of the transformed phenotype appear to be encoded in oncogene sequences distinct from those defining the kinase catalytic domain itself.**

Over 80 distinct oncogenic retroviruses have been isolated in the years since 1908 (49). The genetic loci responsible for oncogenesis by many of these viruses have been identified and characterized (6). Many of these retroviral oncogenes, although distinct from one another, appear to be classifiable into interrelated families. The largest of these oncogene families is that represented by the loci that encode tyrosine-specific protein kinases (6, 25). Members of this group include the v-*src* gene of the Rous sarcoma viruses (RSV), the v-*erbB* gene of avian erythroblastosis virus (AEV), the v-*abl* gene of Abelson leukemia virus, the v-*yes* gene of Y-73 virus, the v-*fps* genes of the Fujinami and PRC-II viruses, the v-*fms* and v-*fes* genes of two strains of feline sarcoma virus, and the v-*ros* gene of UR-2 virus (reviewed in reference 25).

The tyrosine-specific protein kinase family of oncogenes encodes enzymes that phosphorylate tyrosine residues in specific substrate (target) polypeptides, an activity apparently involved in their mechanism of action (25). Many of these oncogene kinases recognize overlapping sets of the same in vitro and in vivo target polypeptides (25). Members of the tyrosine kinase family share a number of additional properties. All share a segment of conserved coding sequence, termed the kinase domain, which appears to define a portion of the enzyme active site (3, 6, 25, 30). With certain exceptions, many of the tyrosine kinase oncogene proteins are membrane associated, although the exact nature of the membrane association varies from oncogene to oncogene (6). Virtually all of the tyrosine-specific protein kinase oncogenes are capable of oncogenic transformation of fibroblasts in vitro, and many also induce fibrosarcomas in animals (6).

Despite these similarities, different members of the tyrosine kinase family, such as the AEV v-*erbB* and RSV v-*src* oncogenes, also demonstrate many divergent characteristics. Although both possess an archetypic kinase domain, most of the AEV v-*erbB* protein is unrelated in amino acid sequence to the RSV v-*src* polypeptide. Even within the relatively conserved kinase domain itself, there is a 64% divergence of amino acid sequence between v-*erbB* and v-*src*. Reflecting these structural differences, v-*erbB* and v-*src* also exhibit divergent biochemical and oncogenic properties. The v-*src* oncogene protein is synthesized on free polysomes and associates with the inner surface of the host cell plasma membrane because of posttranslational addition of myristic acid to its N terminus (8, 17, 33, 42, 45; reviewed in reference 32). The v-*src* protein is not glycosylated, is not exposed on the surface of a transformed cell, and induces primarily fibrosarcomas in susceptible host animals (6). In contrast, the v-*erbB* oncogene polypeptide is a transmembrane glycoprotein that is synthesized on rough endoplasmic reticulum and subsequently transported to the host cell plasma membrane (4, 23, 24, 40, 41). AEV principally induces a rapidly lethal erythroleukemia, although fibrosarcomas can also be detected (14, 22).

Our ultimate goal is a better understanding of the mechanism of action of the v-*erbB* oncogene. We reasoned that, since the v-*erbB* and v-*src* oncogenes display both related and unrelated structural and functional motifs, an analysis of chimeric oncogene constructs would permit us to better understand the relationship of structure to function in both oncogenes. We report here the construction of a chimeric v-*erbB*/*src* oncogene that links the transmembrane glycosylated domain of the v-*erbB* gene to the kinase domain of the v-*src* gene. This chimeric gene, when transfected into avian cells, gave rise to a glycoprotein fully capable of oncogenic transformation of fibroblasts, demonstrating that the transmembrane N terminus of the v-*erbB* protein can functionally replace the myristylated membrane-association domain of the v-*src* polypeptide. The biological properties of the chimeric oncogene most closely resemble those of the v-*erbB* parent and are distinct from those of the v-*src* oncogene. Our results indicate that certain of the differences in transformation phenotype manifested by RSV- and AEV-infected fibroblasts are due to (i) differences in N-terminal sequences, perhaps affecting target protein specificity or accessibility, rather than (ii) divergences within the kinase active site itself.

## MATERIALS AND METHODS

**Virus, cells, and molecular clones.** Chicken embryo secondary cells consisting largely of fibroblasts were obtained from SPAFAS flock C/O or C/E embryos. All fibroblast cell cultures were maintained and propagated in DME 8+1 (Dulbecco modified Eagle medium supplemented with 10% tryptose phosphate broth, 8% fetal bovine serum, 1% heat-inactivated chicken serum, 1 mg of streptomycin per ml, 100 U of penicillin per ml, and 2.5 μg of amphotericin per ml; components obtained from GIBCO Laboratories).

Stocks of the Schmidt-Ruppin A strain of RSV were generously provided by Kathryn Radke. Molecular clones of the ES-4 strain of AEV were provided by Bjorn Vennstrom (50). A molecular clone of the v-*src* sequences of the Schmidt-Ruppin A strain of RSV, representing the *Eco*RI B DNA fragment from the RSV genome subcloned into pBR322, was obtained from Nancy Quintrell and J. Michael Bishop (13).

**Construction of a chimeric v-*erbB*/*src* gene.** The N-terminal extracellular and transmembrane domains of the v-*erbB* coding region were joined to the kinase and C-terminal domains of the v-*src* coding region by an *Hpa*I/*Pvu*II blunt-end ligation, as follows (see Fig. 1).

**(i) Steps 1 and 2: preparation of the v-*src* molecular clone.** The pBR322 subclone of the v-*src* *Eco*RI B fragment contains U3 sequences from the RSV long terminal repeat which would interfere with subsequent generation of infectious chimeric virus genomes (13). We therefore reversed the orientation of the *Eco*RI B v-*src* fragment in the pBR322 vector by *Eco*RI cleavage and religation; this reversed clone was subsequently cleaved by *Nru*I and religated in the presence of an excess of *Eco*RI oligonucleotide linkers (Pharmacia, Inc.) to delete the undesired U3 sequences (see Fig. 1). The plasmid DNA was then cleaved with *Pvu*II restriction endonuclease, and the 870-base-pair fragment (representing the kinase domain and all but the C-terminal-most 11 amino acid codons of the v-*src* coding region; 12, 46) was purified by preparative agarose gel electrophoresis.

**Step 3: preparation of the v-*erbB* molecular clone.** The creation and properties of the Alu321 mutant of the v-*erbB* gene, representing a fully transformation-competent, in-frame insertion of an *Hpa*I linker at an *Alu*I site within the v-*erbB* coding region, have been previously described (36). The v-*erbB* gene carrying this *Hpa*I oligonucleotide linker insertion was subcloned as a *Sal*I-*Pvu*II DNA fragment into the plasmid vector pBR329, cleaved with *Hpa*I and *Pvu*II, and treated with bacterial alkaline phosphatase to prevent self-ligation.

**Step 4: creation of a chimeric v-*erbB*/*src* oncogene.** The gel-purified *Pvu*II-to-*Pvu*II fragment of v-*src* isolated in step 1 was ligated to the phosphatase-treated v-*erbB* pBR329 vector prepared in step 3, and the ligation products were transformed into HB101 host cells. A molecular clone representing the desired construction was identified by restriction endonuclease mapping; the correct nature of the clone and preservation of the reading frame through the *Hpa*I/*Pvu*II junction were confirmed by subsequent DNA sequence analysis. The resulting construct (pErbB/src7) represents an in-frame linkage of the first 119 N-terminal amino acid codons of the v-*erbB* coding region to amino acid codons 226 to 515 of v-*src* (12, 46, 51).

**Step 5: completion of the construction.** Use of the 870-base-pair *Pvu*II-to-*Pvu*II v-*src* fragment in the ligation in step 3 of our construction resulted in a molecular clone, pErbB/src7, which lacked 11 amino acid codons that are present on the very C terminus of the wild-type v-*src* coding sequence. The pErbB/src7 plasmid DNA was therefore subsequently cleaved with PvuII and *Eco*RI, and the missing v-*src* sequences were introduced in the form of a 135-base-pair *Pvu*II-*Eco*RI DNA fragment obtained from the v-*src* plasmid. The final plasmid clone, pErbB/src7-36, contains an intact coding domain beginning at the N terminus of the parental v-*erbB* coding sequence and terminating at the very C terminus of the v-*src* sequence.

**Construction of an infectious molecular clone and transfection of avian fibroblasts.** The v-*erbB*/*src* chimeric oncogene was subsequently reconstructed into an infectious form by replacing the *Sal*I-to-*Eco*RI sequences in pAEV-11-3R, an infectious molecular clone of the wild-type AEV genome, with the corresponding *Sal*I-to-*Eco*RI sequences from the pErbB/src7-36 plasmid (47). The resulting vector, referred to here as pChimera, therefore encodes a variant of the AEV DNA genome in which the original v-*erbB* sequences have been replaced by the chimeric v-*erbB*/*src* gene. The pChimera vector possesses an intact v-*erbA* locus and is capable of generating infectious virus when transfected into avian fibroblasts in the presence of a suitable helper virus (pAEVchimera, like the original AEV genome, is replication defective) (36, 47).

Chicken embryo secondary cells were transfected with a molecular clone (pRAV-10R) of the Rous-associated virus type 1 (RAV-1) genome alone, the RAV-1 helper plus the *erbB*/*src* chimera plasmid, or the RAV-1 helper plus wild-type AEV (AEV-11-3R) by a calcium phosphate coprecipitation technique (47). Approximately 100 μg of pRAV-10R DNA plus 500 μg each of either wild-type or chimera AEV plasmid DNA was used for each 60-mm (diameter) plate. The transfected avian cells were passaged 1:5 every 2 days in DME 8+1 at 39°C. Culture supernatants from the transfected fibroblasts were subsequently used to infect fresh fibroblasts or bone marrow cells.

**Assays for oncogenic transformation of cells.** The ability of infected fibroblasts to grow in soft agar (demonstrate substrate-independent growth) was tested as previously described, with fibroblast-conditioned medium in place of a feeder cell monolayer (36). Hexose uptake was determined by measurement of [1-$^3$H]deoxyglucose transport (39, 44). Actin cables were visualized by fluorescent staining of permeabilized fibroblasts with rhodamine-conjugated phalloidin (2). Plasminogen activator protease secretion was determined by use of a casein-agarose overlay plaque assay (20). The ability of the mutant to transform erythroid cell progenitors was assayed by a methylcellulose colony method with bone marrow cells derived from 1- to 2-week-old SPAFAS chickens (21).

**Immunoprecipitation analysis and in vitro kinase assay.** Infected fibroblasts were radiolabeled for 2 h at 39°C in RPMI medium containing 250 μCi of [$^{35}$S]methionine per ml (800 Ci/mmol; 500 μCi/$10^6$ cells). The cells were then lysed, and the lysates were immunoprecipitated as previously described, with either tumor-bearing-rabbit (TBR) serum or serum directed against purified RSV virions (35, 37, 41). The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis and visualized by fluorography.

The in vitro kinase assay was performed as previously described for the viral *src* protein (7, 35), with the TBR serum used in the protein analysis described above.

**Subcellular fractionation and tunicamycin treatment.** Approximately $10^7$ fibroblasts infected by the *erbB*/*src* chimera were radiolabeled with [$^{35}$S]methionine for 2 h at 39°C as described above. The cells were then swelled on ice in hypotonic buffer (5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.1], 10 mM *N*-ethylmaleimide, 0.5% aprotinin), scrapped off the culture dish with a rubber policeman, and broken open by 30 strokes of a loose-fitting plunger in a Dounce homogenizer. The different subcellular fractions were subsequently isolated by differentiation centrifugation and isopycnic banding as previously described for the parental v-*erbB* and v-*src* proteins (10, 40). Samples of the different subcellular fractions were adjusted to 0.5 M NaCl–1 mg of bovine serum

albumin per ml–1% (wt/vol) Nonidet P-40 and subjected to immunoprecipitation with *src*-directed serum, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis.

One plate of two duplicate cultures of infected fibroblasts (approximately $10^6$ cells per plate) was treated with 1 μg of tunicamycin for 4 h at 39°C; the other plate was not exposed to the inhibitor. Both cultures were then washed and incubated for 2 h more in RPMI medium containing 500 μCi of [$^{35}$S]methionine per plate, retaining 1 μg of tunicamycin per ml in the labeling medium in the treated culture. The cells were subsequently washed with phosphate-buffered saline and lysed, and the lysates were analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis.

## RESULTS

**A chimeric v-*erbB*/*src* oncogene was created by recombinant DNA methodology.** The N-terminal extracellular, glycosylated, and transmembrane domains of the v-*erbB* coding region were fused to the C-terminal kinase domain of the v-*src* coding region by an *Hpa*I-*Pvu*II ligation, using molecular clones of each of these two oncogenes and standard recombinant DNA techniques (Fig. 1A). This construction resulted in in-frame linkage of the first 119 codons of the v-*erbB* sequence to the C-terminal 301 codons of v-*src*. A single extra codon was introduced at the site of fusion because of the method of construction; a virtually identical in-frame insertion at the same site in the v-*erbB* protein sequence has been previously shown to have no detectable effect on oncogenic or biochemical properties (36). The resulting construction places the v-*src* kinase domain in a similar position, relative to the v-*erbB* transmembrane domain, as the v-*erbB* kinase domain it replaces (Fig. 1B). The very C-terminal region of the v-*erbB* polypeptide, previously implicated in erythroid transformation, possesses no obvious cognate in v-*src* and is absent from the chimeric construct (51).

**The v-*erbB*/*src* chimeric oncogene encodes a stable polypeptide of the expected molecular weight.** An infectious form of the chimeric oncogene was created by completely replacing the v-*erbB* sequences of pAEV-11-3R, a molecular clone of the AEV genome, with the v-*erbB*/*src* chimeric gene. The final infectious construct (referred to as pChimera) was subsequently transfected into avian fibroblasts in the presence of an RAV-1 genome. A RAV-1 helper virus is required by AEV and AEV-based vectors for replication (22, 26). Parallel cell cultures were transfected by the RAV-1 genome alone or the RAV-1 genome and an unmodified AEV genomic clone to serve as negative and positive controls, respectively. Virus stocks recovered from the transfected cells were used to infect fresh fibroblast cells, and the infected cells were propagated for at least five passages before being assayed for their biological and biochemical properties.

The polypeptides encoded by the chimeric virus were analyzed by immunoprecipitation of extracts of infected fibroblasts metabolically labeled with [$^{35}$S]methionine (Fig. 2). A polypeptide doublet of heterogeneous molecular weight (49,000 and 53,000 apparent molecular weight [49K and 53K polypeptides, respectively]) could be detected by v-*src*-directed antiserum, (TBR serum) in fibroblasts infected by the chimera (lane 2), which was not present in cells infected by the RAV-1 helper alone or by the AEV parent (lanes 1 and 3). The TBR serum was obtained from a rabbit bearing an RSV-induced tumor and therefore cross-reacts with a number of helper viral structural proteins (7, 35, 37, 48) (Fig. 2). The pattern of reactivity of the TBR serum can be compared to the pattern of structural and p74$^{gag\text{-}erbA}$ proteins detected in these same cells by a serum directed against purified virion proteins (Fig. 2, lanes 4 to 6). The 49K-53K protein was immunoprecipitated by neither the virion-specific antiserum (Fig. 2, lanes 4 to 6) nor normal rabbit serum (data not shown). Partial proteolysis mapping (not shown) confirmed the identity of the 49K-53K protein as a v-*erbB*/*src* chimeric polypeptide.

**The chimeric v-*erbB*/*src* polypeptide is glycosylated.** The heterogeneous size of the chimeric oncogene protein synthesized in chimera-infected cells closely resembled the pattern displayed by the parental AEV *erbB* protein (24, 41), although the chimera pattern migrated at a position some 15,000 daltons smaller than that of the wild-type v-*erbB* protein. This is the pattern predicted if the v-*erbB*-derived N terminus is glycosylated in the chimera as it is in the parental v-*erbB* protein (the v-*erbB*/*src* chimera protein should be 132 amino acid codons smaller than the v-*erbB* parent).

This hypothesis was confirmed by the use of tunicamycin, a specific inhibitor of N-linked protein glycosylation (Fig. 3). Fibroblasts infected by the chimera and radiolabeled with [$^{35}$S]methionine in the absence of tunicamycin synthesize the 49K-53K protein doublet as described above (lane 4; labeled gp49 and gp53). A duplicate culture of chimera-infected fibroblasts, treated with tunicamycin, synthesized a single, smaller polypeptide of homogenous size (about 46,000 in apparent molecular weight, labeled p46 in lane 3). The polypeptide synthesized by the chimera in the presence of tunicamycin was virtually identical in size to the primary translation (unglycosylated) product predicted from the nucleic acid sequence of the v-*erbB*/*src* chimeric oncogene. No such protein was detected in fibroblasts infected by RAV-1 helper virus alone (lanes 1 and 2). Serving as an internal control, the effect of tunicamycin on the proteins encoded by the helper virus can also be seen in Fig. 3. Tunicamycin treatment resulted in synthesis of a smaller form of the normally glycosylated RAV-1 envelope precursor protein (gPr92) but had no visible effect on the unglycosylated *gag*-related polypeptides of the helper virus (p27, for example).

**The v-*erbB*/*src* chimeric protein follows the biosynthetic pathway of the v-*erbB* parent.** The subcellular locations of the v-*erbB* and v-*src* proteins are distinct from one another. The v-*src* protein is synthesized on free polysomes and associates with the plasma membrane posttranslationally (33, 42; reviewed in reference 32). The v-*erbB* protein is synthesized on rough endoplasmic reticulum and subsequently translocated to lighter membrane fractions (4, 23, 40). The glycosylation observed for the v-*erbB*/*src* chimera protein strongly suggested that the N-terminal v-*erbB* sequences were capable of directing the chimeric polypeptide to the rough endoplasmic reticulum system despite the present of the v-*src* catalytic domain. This was confirmed by subcellular fractionation of chimera-infected fibroblasts (Fig. 4).

Fibroblasts infected by the chimera were radiolabeled for 2 h with [$^{35}$S]methionine and lysed, and the various subcellular fractions were separated by differential-velocity and isopycnic centrifugations. Most of the v-*erbB*/*src* chimeric protein was found in the P-154 membrane and nuclear wash fractions by these methods (Fig. 4A, lanes 3 and 5). When the membranes in the P-154 pellet were further fractionated by density, the majority of the v-*erbB*/*src* protein was found at the 40%/50% sucrose interface (lane 7). Both the nuclear wash and the 40%/50% sucrose interface

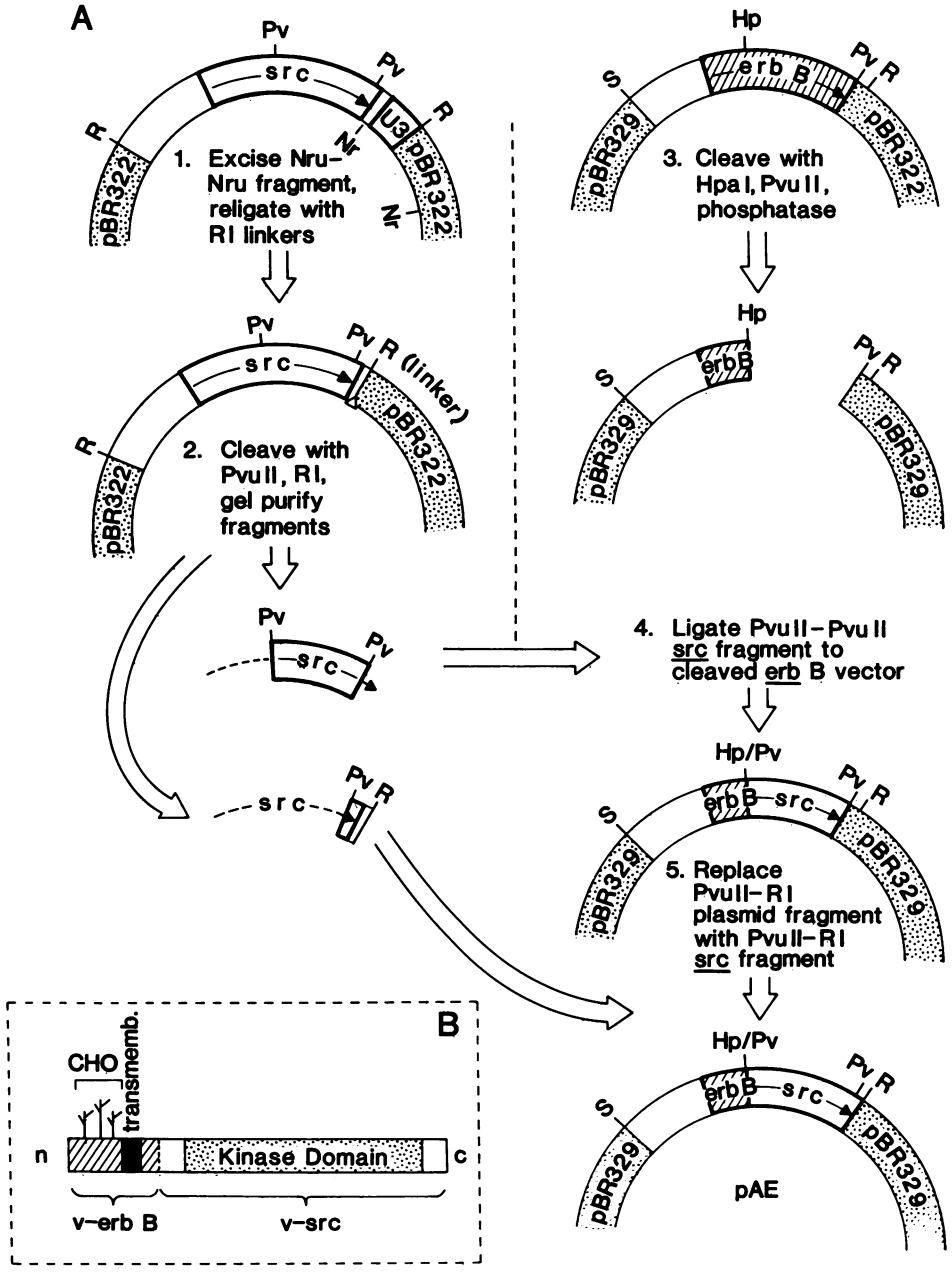


FIG. 1. Construction of a chimeric v-*erbB*/*src* oncogene. (A) Flow chart of the construction scheme used. The details of and rationale behind the steps used in the construction are explained in Materials and Methods. Briefly, a *Pvu*II-*Pvu*II fragment from the RSV DNA genome was excised by restriction endonuclease cleavage and used to replace the *Hpa*I-*Pvu*II DNA fragment originally in the v-*erbB* pAE-SAL-RI-Alu321 molecular clone (steps 1 to 4). A *Pvu*II-*Eco*RI fragment from the RSV clone (containing the C-terminal 11 amino acid codons missing from the *Pvu*II-*Pvu*II fragment described above) was next excised and used to replace the *Pvu*II-*Eco*RI fragment contributed by the pBR329 vector (step 5). The resulting chimeric oncogene contains the N-terminal coding sequences of v-*erbB* linked at an *Hpa*I-*Pvu*II site to the C-terminal coding sequences of v-*src*. Abbreviations: R = *Eco*RI, S = *Sal*I, Pv = *Pvu*II, Hp = *Hpa*I, and Nr = *Nru*I. Sequences contributed by v-*erbB* are represented as hatched boxes, and sequences contributed by v-*src* are represented by open boxes. Arrows indicate the orientation of the reading frames of both genes. (B) Schematic of the chimeric oncogene polypeptide. The expected structure of the chimeric polypeptide synthesized by the v-*erbB*/*src* fusion oncogene is shown schematically. v-*erbB*-related sequences are hatched; v-*src*-related sequences are shown as open boxes or stippled. CHO, Possible sites of N-linked protein glycosylation in v-*erbB*; transmemb., transmembrane domain; kinase domain, region of conserved amino acid sequence thought to define the active site of the tyrosine-directed protein kinases.

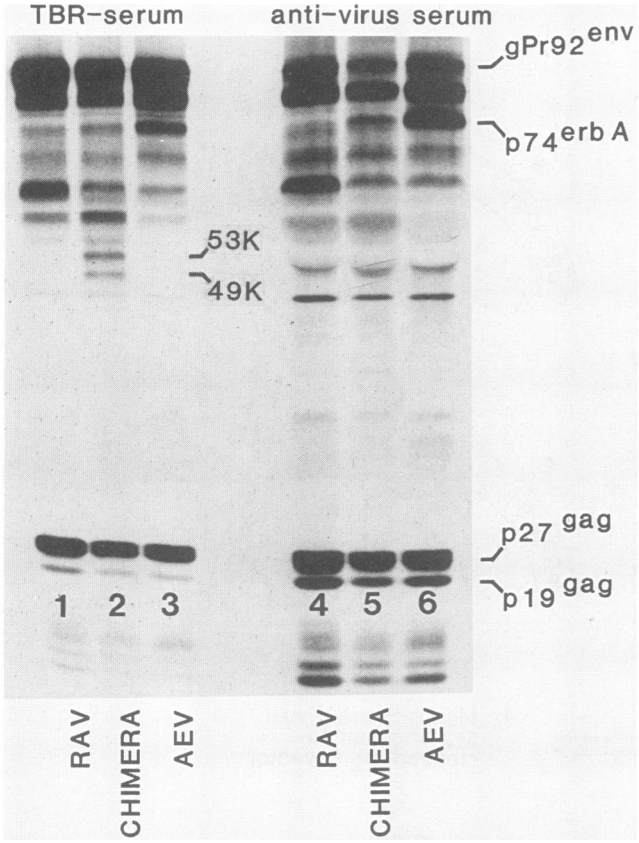


FIG. 2. Proteins synthesized by the chimeric oncogene in infected fibroblasts. Avian fibroblasts infected by the RAV-1 helper virus alone (lanes 1 and 4), by the RAV-1 helper and the v-*erbB*/*src* chimera (lanes 2 and 5), or by the RAV-1 helper and wild-type AEV (lanes 3 and 6) were metabolically radiolabeled with [$^{35}$S]methionine and lysed, and v-*src*-related proteins were immunoprecipitated with TBR serum (lanes 1 to 3) or anti-*gag* serum (lanes 4 to 6). 49K-53K indicates the 49,000-53,000-molecular-weight polypeptide doublet synthesized by the chimera. Helper virus-encoded polypeptides include the p27, and p19 *gag* proteins, as well as gPr92, an *env* gene product. Also visible in these immunoprecipitates is p74$^{gag\text{-}erbA}$, the product of the AEV v-*erbA* oncogene. Molecular weight standards, run in adjacent lanes, are not shown.

represent fractions highly enriched for endoplasmic reticulum (40). This pattern observed for the gp49/53 chimeric protein is virtually identical to the pattern seen for the parental v-*erbB* polypeptide (Fig. 4B), indicating that the N-terminal domain of the v-*erbB* parent directed the subcellular location of the chimeric protein (40; data not shown). In contrast, the subcellular distribution of the v-*src* parental protein was very different, with most of p60$^{v\text{-}src}$ in soluble and light-density (plasma membrane) fractions under the same conditions (10; data not shown).

**The v-*erbB*/*src* chimera protein possesses in vitro kinase activity similar to that of the v-*src* parent polypeptide.** The v-*src* protein possesses strong tyrosine kinase activity in vitro, phosphorylating both itself (autophosphorylation) and the heavy chain of many *src*-directed immunoglobulins (9, 35). In contrast, the kinase activity of the v-*erbB* protein is much more difficult to demonstrate in vitro. Relatively low levels of autophosphorylation and phosphorylation of certain target polypeptides have been reported for v-*erbB* preparations in vitro, but none of the known *erbB*-directed antisera appear to be recognized as substrates by the v-*erbB* kinase (19, 31). We were therefore interested in characterizing the in vitro kinase activity of our v-*erbB*/*src* chimeric protein.

The chimeric polypeptide was immunoprecipitated with TBR serum, the immunoprecipitates were washed and incubated with [$\gamma$-$^{32}$P]ATP, and the products of the reaction were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Intense kinase activity directed against immunoglobulin G (IgG) heavy chain could be seen in immunoprecipitates of the v-*erbB*/*src* chimeric protein (lane 4). No kinase activity was detected in control immunoprecipitates with normal rabbit serum (lanes 1, 3, and 5), in TBR immunoprecipitates of cells infected with RAV-1 only (lane 2), or in TBR immunoprecipitates of wild-type AEV-infected cells (lane 6). The TBR serum used in this analysis had no detectable activity against c-*src* polypeptide (lane 2; also, reference 37). Similar immunoprecipitates of the parental v-*erbB* protein with v-*erbB*-directed serum were essentially negative in these assays (data not shown). These results demonstrate that the v-*src* kinase domain in the chimeric polypeptide retains the structural and enzymatic

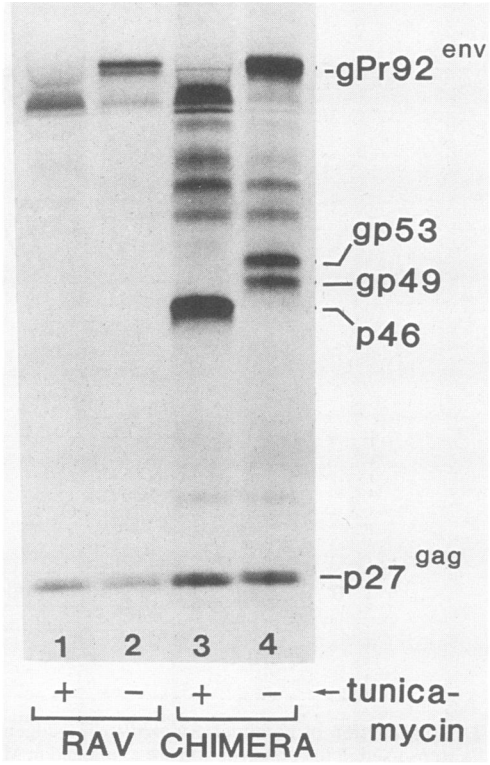


FIG. 3. Tunicamycin treatment of chimera-infected cells. Fibroblasts infected by the chimera plus the RAV-1 helper virus (lane 3 and 4) or by the helper virus alone (lanes 1 and 2) were radiolabeled with [$^{35}$S]methionine in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of tunicamycin. The cells were subsequently lysed, the lysates were immunoprecipitated with TBR serum, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Glycosylated (gp49 and gp53) and unglycosylated (p46) forms of the chimeric oncogene protein are indicated. Helper virus-encoded proteins also detected by TBR serum include the normally glycosylated gPr92$^{env}$ protein and the unglycosylated p27$^{gag}$ protein. Molecular weight standards, run in adjacent lanes, are not shown.

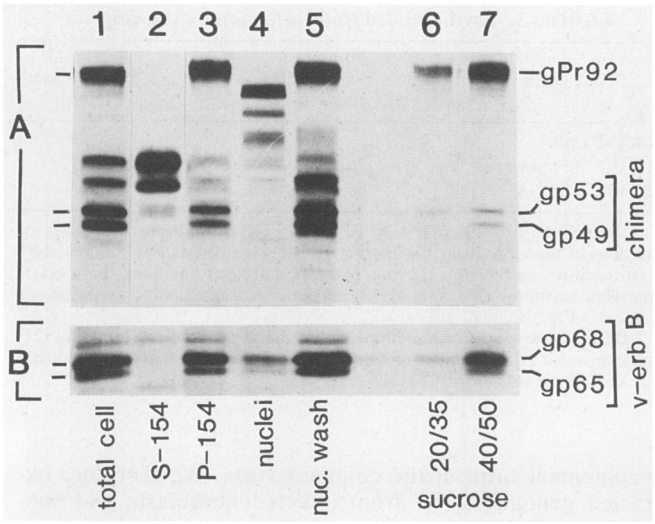


FIG. 4. Subcellular localization of the v-*erbB*/*src* chimeric protein. Fibroblasts infected by either the v-*erbB*/*src* chimera (panel A) or by wild-type AEV (panel B) were metabolically radiolabeled with [$^{35}$S]methionine for 2 h and then lysed, and the different subcellular fractions were isolated as described in Materials and Methods. Equal amounts of each fraction were immunoprecipitated with *src*-directed TBR serum (panel A) or anti-v-*erbB* serum (panel B), and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The different fractions were loaded on the electrophoretogram as follows (lanes): 1, total cell lysate sample before fractionation; 2, 154,000 × *g* supernatant; 3, 154,000 × *g* pellet; 4, purified nuclear fraction; 5, detergent wash of crude nuclei. Lanes 6 and 7 represent the 154,000 × *g* pellet further fractionated by density. Material at the 20%/35% (wt/vol) sucrose interface, lane 6; material at the 40%/50% sucrose interface, lane 7.

properties necessary for TBR immunoglobulin phosphorylation, properties that are not shared by the v-*erbB* parent kinase domain (virtually no activity against TBR serum by the wild-type AEV v-*erbB* protein was detected; Fig. 5, lane 6).

To better compare the kinase activity of our chimeric oncogene protein with that of the RSV parental v-*src* polypeptide, we assayed in parallel the abilities of these two polypeptides to function in the in vitro immunoglobulin kinase assay (Table 1). Immunoprecipitates from fibroblasts infected by the v-*erbB*/*src* chimera demonstrated slightly higher kinase activity per cell in this assay than did immunoprecipitates derived from cells expressing the v-*src* parent. However, there was also slightly more gp49/53$^{v\text{-}erbB/src}$ protein in chimera-infected cells (detected as [$^{35}$S]methionine radiolabel) than pp60$^{v\text{-}src}$ protein in RSV-infected cells (Table 1). Partial-proteolysis mapping (data not shown) indicated that both v-*src* and the chimeric protein phosphorylated the same site(s) within the IgG molecule. We conclude that there is little or no significant difference between the in vitro kinase activities of the v-*erbB*/*src* and v-*src* oncogene proteins.

**The chimeric v-*erbB*/*src* polypeptide is fully capable of transforming fibroblasts to an oncogenic state.** Fibroblasts infected by the chimera quickly developed the distinctive spindle-shaped, fusiform, criss-crossed morphology exhibited by cells transformed by the AEV parent (Fig. 6b and c; reference 44). This transformed morphology was different from the round, loosely adherent morphology exhibited by fibroblasts transformed by the RSV parent (panel d) and from the flat, nonrefractile, organized monolayers of untransformed fibroblasts infected by the RAV-1 helper alone (panel a).

Infected fibroblasts were also tested for four other phenotypic manifestations of oncogenic transformation (Table 2; reference 44). Chimera-infected fibroblasts were capable of anchorage-independent growth, a relatively stringent test of oncogenic transformation, yielding soft-agar colonies indistinguishable in number and morphology from those generated by AEV-infected cells. Chimera-infected fibroblasts also demonstrated high levels of plasminogen activator protease secretion, another characteristic of oncogenic transformation (44), comparable to those demonstrated by AEV-infected cells although much lower than the protease levels seen in RSV-infected cells. Chimera-infected fibroblasts contained few intact actin cable bundles, similar to the disaggregation of actin cables seen in AEV- and RSV-induced transformation, whereas most fibroblasts infected by the helper alone retained actin cables. When tested for hexose uptake, a fifth criterion of fibroblast oncogenic transformation, fibroblasts infected by the chimera demonstrated

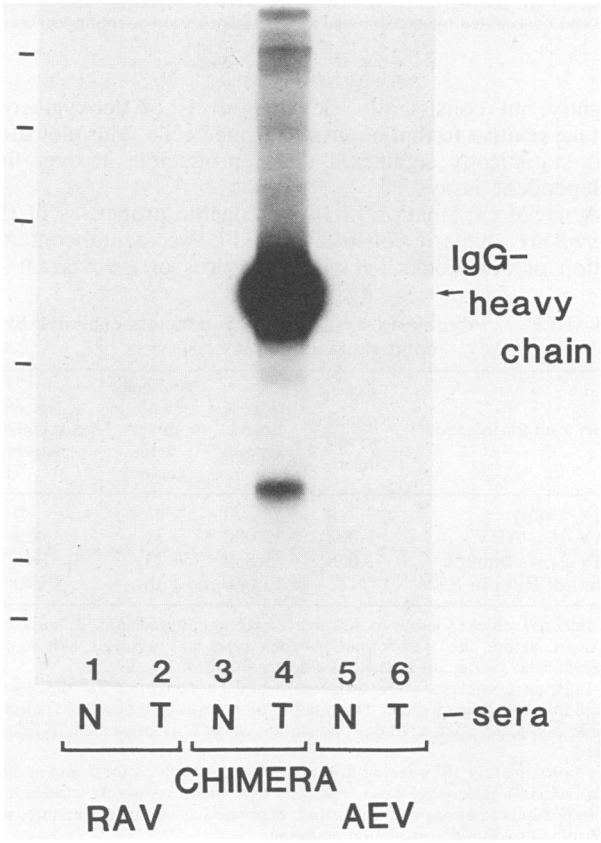


FIG. 5. In vitro kinase assay of v-*erbB*/*src* chimera protein. Fibroblasts infected by the RAV-1 helper virus alone (lanes 1 and 2), by the helper and the chimera (lanes 3 and 4), or by the helper and the parental AEV (lanes 5 and 6) were lysed, and the lysates were immunoprecipitated with normal rabbit (N) serum (lanes 1, 3, and 5) or RSV TBR (T) serum (lanes 2, 4, and 6). The immunoprecipitates were washed, incubated with [$\gamma$-$^{32}$P]ATP for 15 min at 23°C, washed again, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Molecular weight standards, run in adjacent lanes, are indicated on the left.

TABLE 1. Kinase activity of the v-*erbB*/*src* chimeric oncogene protein compared with that of the v-*src* parent

| Protein | Kinase activity (cpm)/$10^6$ infected cells[a] | [$^{35}$S]methionine-labeled oncogene protein (cpm)/$10^6$ infected cells[b] | Kinase/labeled protein ratio ($10^2$) |
|---|---|---|---|
| v-*erbB*/*src* chimera | 592,200 | 2,595 | 2.28 |
| v-*src* parent | 225,138 | 1,474 | 1.53 |

[a] Kinase activity was measured as described previously (9, 35). Briefly, cells infected by either the v-*erbB*/*src* chimera virus or wild-type Schmidt-Ruppin were lysed, and the extracts were immunoprecipitated with RSV TBR serum as described in the legend to Fig. 5. The immunoprecipitates were washed and incubated with 2 μCi each of [γ-$^{32}$P]ATP for 15 min at 23°C, and the radioisotope incorporated into IgG heavy chain was measured by SDS-polyacrylamide gel electrophoresis and a liquid scintillation counting technique. Kinase activity is represented as $^{32}$P counts per minute incorporated into IgG heavy chain during the 15-min incubation (1,000 cpm = 0.151 fmol of phosphate incorporated).

[b] Infected cultures of fibroblasts, prepared and maintained in parallel to those used in the kinase assay described above were radiolabeled for 2 h with [$^{35}$S]methionine as previously described (41). The cells were lysed, the extracts were immunoprecipitated with TBR serum, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The radiolabeled v-*src* and v-*erbB*/*src* protein bands were visualized by autoradiography and quantitated by excision and a liquid scintillation counting technique.

slightly but consistently elevated levels of deoxyglucose uptake relative to that of untransformed cells. This elevation was statistically significant and reproducible in over five independent assays.

A trivial explanation of the oncogenic properties of the v-*erbB*/*src* chimeric construct would be accidental contamination of our stocks by wild-type virus or a revertant or recombinant form of the chimeric virus. We therefore extracted genomic DNA from infected fibroblasts and subjected the DNA to restriction endonuclease-Southern blotting analysis by using restriction enzymes and hybridization probes that would distinguish the AEV genome from that of the chimera. The restriction digestion pattern obtained from the DNA from chimera-infected cells was identical to that of the original construction and ruled out large-scale rearrangements within the chimeric oncogene or possible contamination with wild-type AEV or RSV (data not shown).

TABLE 2. Fibroblast transformation parameters exhibited by chimera-infected fibroblasts

| Virus used for infection[a] | No. of colonies in soft agar[b] | Hexose uptake (cpm)[c] | % of cells with intact actin cables[d] | No. of caseinolytic plaques[e] |
|---|---|---|---|---|
| RAV-1 only | 0 | 700 | 85 | 0 |
| RAV-1 + AEV | 1,022 | 13,000 | 31 | 489 |
| RAV-1 + chimera | 1,058 | 1,839 | 23 | 345 |
| Schmidt-Ruppin RSV | NT | 3,066 | 16 | 3,740[f] |

[a] Infected chicken embryo fibroblasts were cultured for at least five passages before the transformation phenotype was assayed. All assays represent the average of at least two determinations.

[b] Infected fibroblasts were trypsinized and counted, and $10^5$ cells were plated into soft agar medium. The number of macroscopic fibroblast colonies visible per plate after a 10-day incubation at 39°C is presented. NT, Not tested.

[c] Approximately $10^5$ infected fibroblasts were incubated for 5 min at 39°C with 4 μCi of [$^3$H]deoxyglucose. The cells were then extensively washed, and the radiolabel remaining cell associated, expressed as counts per minute, was determined by liquid scintillation counting.

[d] Infected fibroblasts were plated on cover slips, washed, fixed, and permeabilized, and the actin cables were visualized with rhodamine-conjugated phalloidin. The number of cells exhibiting intact actin cables is presented as a percentage of the total number of cells counted (about 300 cells per assay).

[e] Infected (RAV, AEV, or Chimera) fibroblasts were trypsinized, and $5 \times 10^5$ cells were plated into 60-mm (diameter) petri plates. The cells were then washed and overlaid with casein agar overlay medium as previously described (20). The number of zones of caseinolysis (plaques) were counted after a 16-h incubation at 37°C.

[f] RSV-infected cells were plated at $5 \times 10^4$ cells per plate, and the number of plaques observed was multiplied by 10.

TABLE 3. Erythroid cell transformation by the chimera

| Virus stock | Fibroblast-transforming titer ($10^4$)[a] | Erythroid cell-transforming titer[b] |
|---|---|---|
| RAV-1 only | 0 | 0 |
| RAV-1 + chimera | 4.7 | 0 |
| RAV-1 + AEV | 4.3 | 107 |

[a] Determined by exposing $5 \times 10^5$ uninfected fibroblasts to a series of dilutions of the virus stock, incubating the cells for 12 h at 39°C, followed by trypsinization and plating of the cells in soft agar medium. Fibroblast-transforming titer is expressed as the number of soft agar colonies induced per milliliter of the original virus stock.

[b] Determined by use of a methylcellulose-bone marrow colony assay (21) and expressed as the number of macroscopic erythroid cell colonies induced per 4 ml of the original virus stock.

**The chimeric v-*erbB*/*src* gene does not transform erythroid cells in an unsupplemented bone marrow colony assay.** The wild-type v-*erbB* protein is capable of oncogenic transformation of immature erythroid cells as well as fibroblasts (5, 14, 18, 22). This erythroid cell-directed activity has been localized, in part, to the very C-terminal domain of the v-*erbB* protein (51; unpublished data). However, actual determination of target cell specificity in the tyrosine kinase family of oncogenes appears to be a complex phenomenon; a number of other tyrosine kinase oncogenes, including v-*src*, appears to be capable of at least limited erythroid cell transformation activity (28, 29, 38). It was therefore of interest to test the ability of our chimera to transform avian erythroid cells in an in vitro bone marrow colony assay.

Stocks of the v-*erbB*/*src* chimera showed no erythroid cell-transforming activity in our assay, in contrast to the hundreds of erythroid cell colonies induced by the AEV parent (Table 3). Both the AEV parental virus stock and the chimera stock possessed approximately equal fibroblast-transforming titers (Table 3). We conclude that the erythroid cell-transforming potential of the chimera is at least 2 orders of magnitude lower than that of the v-*erbB* parent oncogene despite the presence of an intact, expressed copy of the v-*erbA* gene in the chimeric construct.

## DISCUSSION

**Nature of the v-*erbB*/*src* chimeric oncogene.** The chimeric construction we have generated should encode a 46,000-molecular-weight primary translation product bearing the extracellular, glycosylated, and transmembrane domains of the AEV v-*erbB* protein linked to the kinase catalytic domain of the RSV v-*src* polypeptide. The amino acid sequence Val-Ala-Ile-Lys (VAIK), a highly conserved domain in all of the known tyrosine kinases (25), is thought to represent a portion of the ATP-binding site (30) and is situated 74 amino acid codons C terminal to the end of the transmembrane domain in the parental v-*erbB* protein. The

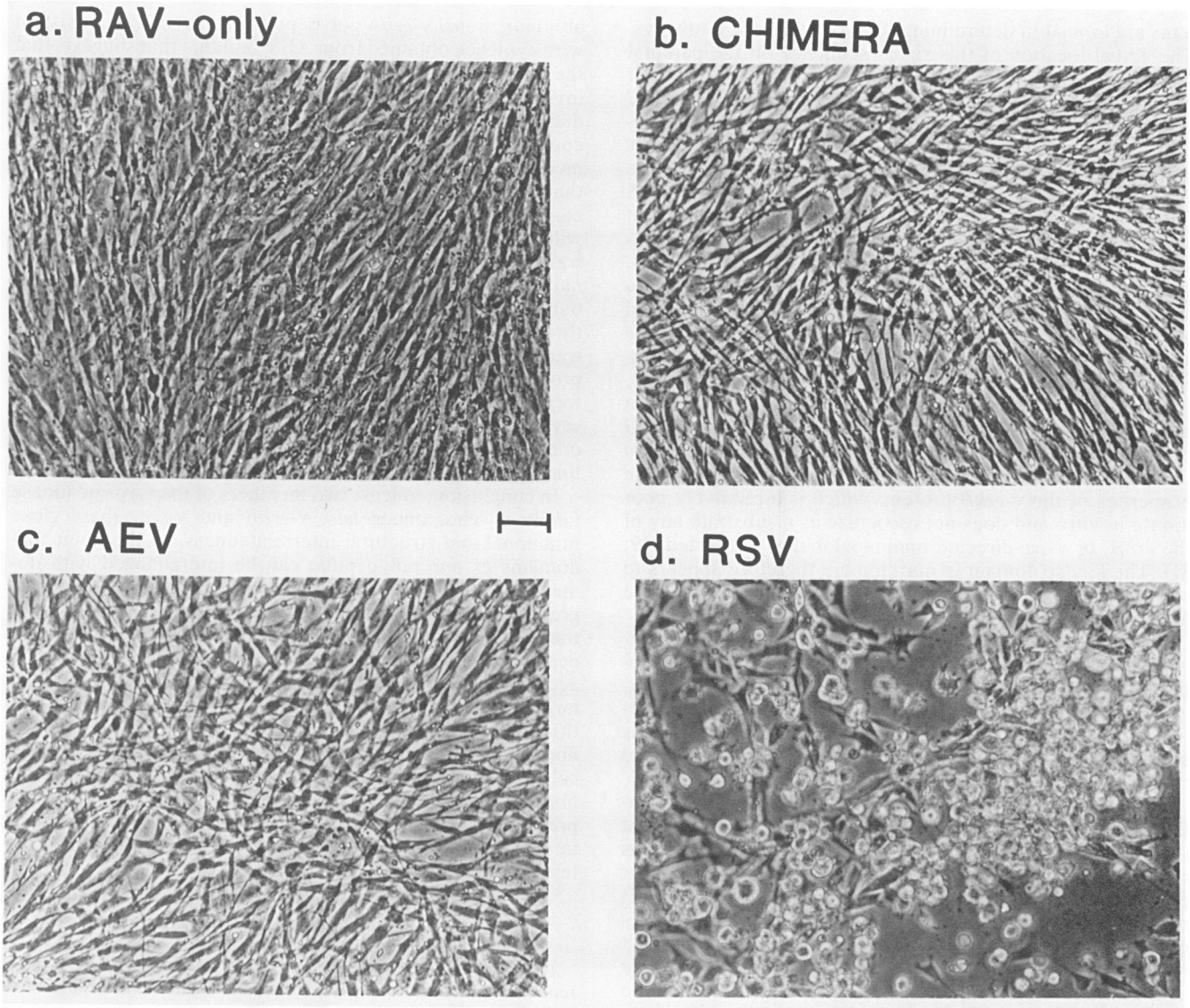


FIG. 6. Morphology of fibroblasts infected by the chimera. Representative microscope fields of fibroblast monolayers infected by the RAV-1 helper virus alone (a), the helper plus the chimera (b), the helper plus the AEV parent (c), or the RSV parent (d) are shown. Bar, about 20 μm.

chimeric construction places the same VAIK sequence in the v-*src* kinase domain 86 amino acids from the end of the transmembrane domain contributed by the v-*erbB* gene.

The ability of our chimeric construct to transform fibroblasts suggests that the exact spacing between the catalytic and membrane-association domains of the tyrosine kinases is not crucial for biological activity. This result supports evidence previously obtained from site-directed mutagenesis experiments on v-*erbB* (36). Furthermore, the ability to excise the kinase domain of v-*src* and introduce it into an unrelated protein sequence background (v-*erbB*-encoded sequences) in an active form strongly suggests that the kinase region is itself a functionally and conformationally discrete domain. This is in agreement with data obtained from site-directed mutagenesis and partial-proteolysis mapping (11, 17, 34).

**Biochemical properties of the chimera-encoded polypeptide.** The chimeric v-*erbB*/*src* oncogene encoded a 46,000-molecular-weight primary translation product that was consistent with the peptide predicted from the construction. This chimeric protein was glycosylated in infected cells to multiple species of higher apparent molecular weight, indicating that the N-terminal glycosylation sites contributed by the v-*erbB* sequences could be fully recognized by the host cell glycosyl transferases despite the linkage of C-terminal sequences from the normally unglycosylated v-*src* protein.

Glycosylation of the v-*erbB*/*src* protein also implied the presence in the chimera of an appropriate signal sequence capable of directing the association of the chimeric protein with microsomal fractions. The subcellular distribution of the v-*erbB*/*src* protein bore out this prediction; the chimeric protein is found in the same subcellular fractions as the v-*erbB* protein, principally fractions enriched for rough endoplasmic reticulum (40). This distribution of the chimera is distinct from that of the v-*src* polypeptide (10), confirming that the N-terminal sequences of these two oncogene pro-

teins are crucial in determining their biosynthetic pathways. The actual location of this signal sequence on the parental v-*erbB* protein remains unclear. We feel that the strongest hypothesis is that the transmembrane sequence of the v-*erbB* protein is itself the signal sequence for membrane association. We base this hypothesis on the absence of an obvious consensus signal sequence at the extreme N terminus of the AEV-ES-4 v-*erbB* coding region (unpublished data) and on the properties of a mutant of v-*erbB* we have isolated that lacks the transmembrane domain (Δ-transmemb.; manuscript in preparation). If the extreme N terminus of v-*erbB*, which is retained in our Δ-transmemb. mutant, encoded a signal sequence, the mutant polypeptide would be expected to be sequestered into microsomal fractions and perhaps secreted. Instead, the Δ-transmemb. v-*erbB* protein is synthesized as a soluble cytoplasmic protein (unpublished data).

The chimeric polypeptide is fully capable of acting as a protein kinase in vitro, phosphorylating *src*-directed IgG heavy chain at levels comparable to those exhibited by v-*src* protein itself. This result for the chimera contrasts to the properties of the v-*erbB* protein, which is a relatively poor kinase in vitro and does not recognize as a substrate any of the *erbB*- or v-*src*-directed immunoglobulins yet tested (19, 31). The kinase domain in our chimera therefore appears to retain the in vitro enzymatic properties demonstrated by the RSV parent.

**Biological properties of the v-*erbB*/*src* chimera.** The v-*erbB*/*src* chimera was fully capable of transforming fibroblasts to an oncogenic state, as judged by morphology, growth in soft agar, plasminogen activator protease secretion, and loss of actin cables, although hexose uptake was only slightly elevated compared with levels in untransformed cells.

The morphology of v-*erbB*/*src*-transformed cells was identical to that of fibroblasts transformed by the v-*erbB* parent (fusiform cells forming criss-crossed monolayers) and was readily distinguished from that of the round, refractile, poorly adherent cells transformed by the RSV parent (44). It therefore appears that replacement of N-terminal v-*src* sequences with v-*erbB* sequences can alter at least one aspect of the transformed phenotype. Intriguingly, a number of different mutations within the v-*src* gene are also known to yield a fusiform morphology (1, 15, 43). These fusiform mutations, as a group, tend to map to the N-terminal portion of the v-*src* sequence (15, 16, 27). The morphological properties of cells infected by our chimera may therefore be due to the absence of *src* sequences that are necessary for full manifestation of the transformed state, perhaps resulting in alteration of the substrate specificity of the chimera tyrosine kinase. An alternative hypothesis is that the distinct subcellular localizations of the v-*src* and v-*erbB*/*src* chimera proteins results in different accessibility of the kinases to host cell protein substrates that are important in determination of morphology. This latter hypothesis is supported by a study which revealed that several fusiform mutants of RSV synthesized a v-*src* polypeptide with altered subcellular distribution (43). A shared theme in both of these hypotheses is that the v-*src* kinase domain itself is not the sole determinant of the transformed phenotype and that the differences seen in the phenotype of v-*src*- and v-*erbB*-transformed cells cannot be solely attributed to the divergence in amino acid sequence within the kinase domains of these two oncogene polypeptides.

Our chimeric oncogene failed to transform erythroid progenitor cells detectably in our in vitro bone marrow assay despite the ability of the construct to synthesize a (presumably) functional v-*erbA* polypeptide. This result is consistent with evidence obtained from AEV mutants that suggests that the C-terminal domain of the v-*erbB* protein is intimately involved in erythroid target cell specificity (51; unpublished data). This C-terminal domain is missing from our chimeric construct. On the other hand, our results appear to be inconsistent with those of Kahn et al., who demonstrated that the parental v-*src* gene, in association with v-*erbA*, could induce erythroid cell transformation similar to that of wild-type AEV (29). It has been reported that v-*src*-transformed erythroid cells propagate only under a very narrow range of pH and temperature (29). It is possible that our relatively simple bone marrow culture conditions, although capable of supporting growth of wild-type AEV-transformed erythroid cells, are not capable of permitting propagation of perhaps more fastidious v-*erbB*/*src*-transformed erythroid precursors or that a very low level of erythroid cell-transforming ability exists for our chimeric oncogene but could not be detected within the statistical limitations of our assay.

In conclusion, at least two members of the tyrosine kinase family of viral oncogenes, v-*erbB* and v-*src*, share close functional and structural interrelatedness, to the point that domains of one polypeptide can be interchanged with domains of the other and yield a fully functional polypeptide product. The properties of the resulting chimera suggest that the N-terminal v-*erbB* sequences play an important role in determining fibroblast morphology, perhaps by affecting the accessibility or affinity of the chimeric protein for certain target polypeptides. It has been previously suggested that the ratio of pp36 to pp42 phosphorylation or differences in fibronectin attachment in v-*src*- versus v-*erbB*-transformed cells may account for the differences in morphology of fibroblasts infected by AEV versus RSV (19, 27, 43). We are presently analyzing the phosphorylation pattern of known target substrate polypeptides in our chimera-infected cells to test this hypothesis.

## ACKNOWLEDGMENTS

The expert and enthusiastic technical assistance of Cathy Judelson is gratefully acknowledged. I also thank Blake Millikan for helpful discussions.

This work was supported by Public Health Service grant CA38823 from the National Cancer Institute.

## LITERATURE CITED

1. **Anderson, D. D., R. P. Beckmann, E. H. Harms, K. Nakamura, and M. J. Weber.** 1981. Biological properties of "partial" transformation mutants of Rous sarcoma virus and characterization of their pp60$^{src}$ kinase. J. Virol. **37**:445–458.
2. **Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. Webb.** 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3 diazole phallacidin. Proc. Natl. Acad. Sci. USA **77**:980–984.
3. **Barker, W. C., and M. O. Dayhoff.** 1982. Viral *src* gene products are related to the catalytic chain of mammalian cAMP-dependent protein kinases. Proc. Natl. Acad. Sci. USA **79**: 2836–2840.
4. **Beug, H., and M. J. Hayman.** 1984. Temperature-sensitive mutants of avian erythroblastosis virus: surface expression of the *erb* B product correlates with transformation. Cell **36**: 963–972.
5. **Beug, H., A. von Kirchbach, G. Doderlein, J. F. Conscience, and T. Graf.** 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell **18**:375–390.

6. **Bishop, J. M., and H. E. Varmus.** 1984. Functions and origins of retroviral transforming genes, p. 999–1108. *In* R. Weiss, N. Teich, H. Varmus, and J. M. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Brugge, J., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
8. **Buss, J. E., M. P. Kamps, and B. M. Sefton.** 1984. Myristic acid is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. **4**:2697–2704.
9. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. USA **75**:2021–2024.
10. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{protosrc}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. USA **77**:3783–3787.
11. **Cross, F. R., E. A. Garber, and H. Hanafusa.** 1985. N-terminal deletions in Rous sarcoma virus $p60^{src}$: effects on tyrosine kinase and biological activities and on recombination in tissue culture with the cellular *src* gene. Mol. Cell. Biol. **5**:2789–2795.
12. **Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischer, and H. M. Goodman.** 1983. Nucleotide sequence of an avian sarcoma virus oncogene (*src*) and proposed amino acid sequence for the gene product. Nature (London) **301**:736–738.
13. **DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop.** 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. **36**:50–61.
14. **Engelbreth-Holme, J., and A. Rothe Meyer.** 1935. On the connection between erythroblastosis (haemocytoblastosis), myelosis and sarcoma in chicken. Acta Pathol. Microbiol. Scand. **12**:352–377.
15. **Feuerman, M. H., B. R. Davis, P. K. Pattengale, and H. Fan.** 1985. Generation of a recombinant Moloney murine leukemia virus carrying the v-*src* gene of avian sarcoma virus: transformation in vitro and pathogenesis in vivo. J. Virol. **54**:804–816.
16. **Fujita, D. J., J. Bechberger, and I. Nedic.** 1981. Four Rous sarcoma virus mutants which affect transformed cell morphology exhibit altered *src* gene products. Virology **114**:256–260.
17. **Garber, E. A., F. R. Cross, and H. Hanafusa.** 1985. Processing of $p60^{v\text{-}src}$ to its myristylated membrane-bound form. Mol. Cell. Biol. **5**:2781–2788.
18. **Gazzolo, L., J. Samarut, M. Bouabdelli, and J. P. Blanchet.** 1980. Early precursors in the erythroid lineage are the specific target cells of avian erythroblastosis virus *in vitro*. Cell **22**:683–691.
19. **Gilmore, T., J. E. DeClue, and G. S. Martin.** 1985. Protein phosphorylation at tyrosine is induced by the v-*erb* B gene product in vivo and in vitro. Cell **40**:609–618.
20. **Goldberg, A. R.** 1974. Increased protease levels in transformed cells: a casein overlay assay for the detection of plasminogen activator production. Cell **2**:95–102.
21. **Graf, T.** 1975. *In vitro* transformation of chicken bone marrow cells with avian erythroblastosis virus. Z. Naturforsch. Sect. C Biosci. **30**:847–849.
22. **Graf, T., B. Royer-Pokora, G. E. Schubert, and H. Beug.** 1976. Evidence for the multiple oncogenic potential of cloned leukemia virus: in vitro and in vivo studies with avian erythroblastosis virus. Virology **71**:423–433.
23. **Hayman, M. J., and H. Beug.** 1984. Identification of a form of the avian erythroblastosis virus *erb* B gene product at the cell surface. Nature (London) **309**:460–462.
24. **Hayman, M. J., G. Ramsey, K. Savin, G. Kitchener, T. Graf, and H. Beug.** 1983. Identification and characterization of the avian erythroblastosis virus *erb* B gene product as a membrane glycoprotein. Cell **32**:579–588.
25. **Hunter, T., and J. A. Cooper.** 1985. Protein tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
26. **Ishizaki, R., and T. Schimizu.** 1979. Heterogeneity of strain R avian (erythroblastosis) virus. Cancer Res. **30**:2827–2831.
27. **Iwashita, S., N. Kitamura, and M. Yoshida.** 1983. Molecular events leading to fusiform morphological transformation by partial *src* deletion mutant of Rous sarcoma virus. Virology **125**:419–431.
28. **Kahn, P., B. Adkins, H. Beug, and T. Graf.** 1984. *src* and *fps* containing avian sarcoma viruses transform chicken erythroid cells. Proc. Natl. Acad. Sci. USA **81**:7122–7126.
29. **Kahn, P., L. Frykberg, C. Brady, I. Stanley, H. Beug, B. Vennstrom, and T. Graf.** 1986. V-*erb* A cooperates with sarcoma oncogenes in leukemic cell transformation. Cell **45**:349–356.
30. **Kamps, M. P., S. S. Taylor, and B. M. Sefton.** 1984. Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. Nature (London) **310**:589–592.
31. **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-*erb* B protein. Cell **40**:619–625.
32. **Krueger, J. G., E. A. Garber, and A. R. Goldberg.** 1983. Subcellular localization of $pp60^{src}$ in RSV-transformed cells. Curr. Top. Microbiol. Immunol. **107**:51–124.
33. **Lee, J. S., H. E. Varmus, and J. M. Bishop.** 1979. Virus-specific messenger RNAs in permissive cells infected by avian sarcoma virus. J. Biol. Chem. **254**:8015–8022.
34. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$). Proc. Natl. Acad. Sci. USA **78**:1624–1628.
35. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
36. **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.
37. **Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (*src*). Proc. Natl. Acad. Sci. USA **76**:1804–1808.
38. **Palmieri, S.** 1985. Transformation of erythroid cells by Rous sarcoma virus (RSV). Virology **140**:269–280.
39. **Palmieri, S., H. Beug, and T. Graf.** 1982. Isolation and characterization of four new temperature-sensitive mutants of avian erythroblastosis virus (AEV). Virology **123**:296–311.
40. **Privalsky, M. L., and J. M. Bishop.** 1984. Subcellular localization of the v-*erb* B protein, the product of a transforming gene of avian erythroblastosis virus. Virology **135**:356–368.
41. **Privalsky, M. L., L. Sealy, J. M. Bishop, J. P. McGrath, and A. D. Levinson.** 1983. The product of the avian erythroblastosis virus *erb* B locus is a glycoprotein. Cell **32**:1257–1267.
42. **Purchio, A. F., S. Jovanovich, and R. L. Erikson.** 1980. Sites of synthesis of viral proteins in avian sarcoma virus-infected chicken cells. J. Virol. **35**:629–636.
43. **Rohrschneider, L., and S. Reynolds.** 1985. Regulation of cellular morphology by the Rous sarcoma virus *src* gene: analysis of fusiform mutants. Mol. Cell. Biol. **5**:3097–3107.
44. **Royer-Pokora, B., H. Beug, M. Claviez, H. J. Winkhardt, R. R. Friis, and T. Graf.** 1978. Transformation parameters in chicken fibroblasts transformed by AEV and MC29 avian leukemia viruses. Cell **13**:751–760.
45. **Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa.** 1985. Amino terminal myristylation of the protein kinase $p60^{src}$, a retroviral transforming protein. Science **227**:427–429.
46. **Schwartz, D. C., R. Tizzard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–861.

47. **Sealy, L., M. L. Privalsky, G. Moscovici, C. Moscovici, and J. M. Bishop.** 1983. Site-specific mutagenesis of avian erythroblastosis virus: v-*erb* B is required for oncogenicity. Virology **130:** 155–177.
48. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28:**957–971.
49. **Teich, N.** 1984. Taxonomy of retroviruses, p. 25–208. *In* R. Weiss, N. Teich, H. Varmus, and J. M. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Vennström, B., L. Fanshier, C. Moscovici, and J. M. Bishop.** 1980. Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic viruses by transfection of chicken cells. J. Virol. **36:**575–585.
51. **Yamamoto, T., Y. Nishida, N. Miyajimi, S. Kawai, T. Ooi, and K. Toyoshima.** 1983. The *erb* B gene of avian erythroblastosis virus is a member of the *src* gene family. Cell **35:**71–78.