JOURNAL OF VIROLOGY, May 1987, p. 1665–1669
0022-538X/87/051665-05$02.00/0

Vol. 61, No. 5

# Cell Transformation by a Virus Containing a Molecularly Constructed *gag-erbB* Fused Gene

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Received 22 September 1986/Accepted 30 January 1987

**A provirus DNA that contains a *gag-erbB* fused gene as the sole and transforming gene was molecularly constructed from plasmid pSRA2 containing the entire genome of Rous sarcoma virus and pAE7.7 containing the entire genome of an avian erythroblastosis virus (AEV), AEV-H. A virus containing the *gag-erbB* fused gene (GEV) was recovered from chicken embryo fibroblasts transfected with the proviral DNA and a helper virus DNA. GEV could transform chicken embryo fibroblasts as efficiently as could AEV-H. Anti-*erbB* and anti-*gag* sera immunoprecipitated a protein with a molecular weight of about 110,000 from GEV-transformed cells. The *erbB* and *gag-erbB* fused-gene products in AEV-H- and GEV-transformed cells were analyzed.**

In avian erythroblastosis viruses (AEVs), AEV-R, ES4, and AEV-H strains which induce erythroblastosis and sarcomas in chickens, the v-*erbB* gene is the transforming gene (6, 18). The v-*erbB* gene has been shown to be a truncated version of the cellular epidermal growth factor (EGF) receptor gene (4, 17, 19). The EGF receptor molecule consists of three domains, an extracellular EGF-binding domain, a transmembrane domain, and a cytoplasmic domain with tyrosine-specific protein kinase activity that is regulated by EGF binding to the extracellular domain (2, 17).

On the other hand, the v-*erbB* gene products of AEV-R, ES4, and AEV-H lack most of the extracellular domain and a short segment of the extreme carboxy terminus of the cytoplasmic domain of the EGF receptor (14, 19), although, like EGF receptor molecules, a mature form of the products is localized on the plasma membrane of cells (1, 7, 15). Because there is no EGF-binding domain, the tyrosine-specific protein kinase activity of the v-*erbB* gene product is considered to be deregulated and constitutively activated, and this unregulated expression of protein kinase activity is believed to be responsible for cell transformation (5, 13).

The v-*erbB* gene products in chicken erythroblasts transformed by the ES4 strain have been studied. The direct *erbB* gene product (molecular weight, 62,000) is known to be glycosylated and then phosphorylated, forming proteins with molecular weights of 66,000 and 68,000, respectively (8, 16). The glycosylated and phosphorylated protein is further processed to a mature form with a molecular weight of 74,000, part of which is extruded from the cell surface (1, 7, 15).

We characterized the AEV-H strain which has only the v-*erbB* gene as the transforming gene, unlike the AEV-R and ES4 strains, which have the *erbA* gene as an additional gene of cellular origin (18). Nucleotide sequence analysis revealed that the AEV-H v-*erbB* gene differs from those of the AEV-R and ES4 strains in the structure of its 3′ terminus (14). The AEV-H *erbB* gene product (molecular weight, 67,000), which is 42 amino acids longer than those of the AEV-R and ES4 strains as shown by comparison of the nucleotide sequences of the v-*erbB* genes of the three strains, is also glycosylated (14).

The *erbB* gene product can be identified with anti-*erbB* sera obtained from rats inoculated with AEV-transformed rat cells (8) and rabbit antisera against the *erbB* polypeptide synthesized in bacteria (16). However, since the *erbB* gene product is a truncated EGF receptor molecule, anti-*erbB* serum cross-reacts with cellular EGF receptors (unpublished data). Furthermore, antibodies against a synthetic peptide from the C terminus of human the EGF receptor were also shown to react with the v-*erbB* gene product (13). In studies on the mechanism of cell transformation by the *erbB* gene, this cross-reactivity between the v-*erbB* gene product and the normal EGF receptor (c-*erbB* gene product) may cause trouble. Although one way to avoid the problem would be to develop v-*erbB*-specific antibodies, it may not be easy because these gene products are so closely related. On one hand, most viral oncogenes other than *erbB*, *src*, and a few others are *gag*-fused genes and their products can be identified with anti-*gag* sera. In this study, therefore, we constructed a *gag*-fused *erbB* gene and examined whether the product of this *erbB* gene modified at its N terminus could still transform cells.

Plasmid pGEB-2, which contains most of the *erbB* gene, part of the *gag* gene, and two long terminal repeats as inserts, was constructed as shown in Fig. 1. pSRA2 DNA (3) containing the entire genome of Rous sarcoma virus was digested with *Sma*I and a fragment containing the 3′ part of the *src* gene and the 5′ part of the *gag* gene in addition to viral sequences (the c region, two long terminal repeats, and a leader sequence) required for viral replication was purified by gel electrophoresis, circularized by self-ligation, linearized by *Xho*I digestion, and inserted into the *Xho*I site of pACYC177. The resultant plasmid, pSBC-X, was double digested with *Bgl*II and *Mlu*I enzymes to remove the remaining *src* sequence. This treatment also removed a short stretch of nucleotides from the 3′ side of the remaining *gag* sequence. On one hand, the *erbB* sequence was recovered in two fragments, *Stu*I-*Eco*RI and *Eco*RI-*Fnu*DII fragments, from plasmid pAE7.7 (19). The *erbB* sequence recovered in this way contained most of the *erbB* coding region but not 43 nucleotides at the 5′ terminus including the initiation site, because the *Stu*I restriction site is located near the 5′ end but within the *erbB* coding region. The *Fnu*DII site is located in the c region following the *erbB* gene. These two fragments and a *Bgl*II-*Mlu*I fragment of pSBC-X treated with Klenow

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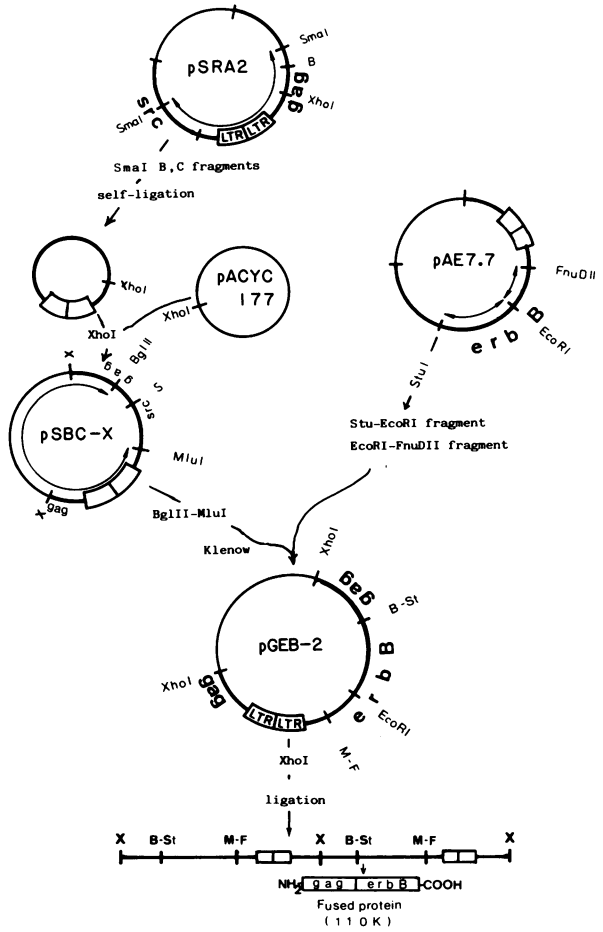


FIG. 1. Molecular construction of GEV DNA containing the *gag-erbB* fused gene.

fragment were ligated. pGEB-2, which contains these fragments in the right configuration for expression, was screened.

Before transfection, the viral sequence was cut out from pGEB-2 by digestion with *Xho*I and ligated to obtain the full viral sequence unit in the right configuration for expression. The expected gene product is a *gag*-fused *erbB* product consisting of a *gag* protein (p19-p10-p27) of 45 kilodaltons and an *erbB* protein of 65 kilodaltons (Fig. 2).

Transfection of chicken embryo fibroblasts (CEF) was carried out by the Polybrene-dimethyl sulfoxide method (10). Briefly, $1.2 \times 10^6$ CEF were seeded in the presence of 10 μg of Polybrene, and on the next day 500 ng of plasmid DNA that had been digested with *Xho*I and then ligated as described above was added to the cells with 1.0 μg of pYAV-e DNA containing the entire viral genome of Y73-associated virus (YAV) as a helper virus DNA (14). Three hours later, the culture was treated with 28.5% dimethyl sulfoxide for 4 min and then fed with 5 ml of fresh medium. After 24 h, the culture was overlaid with soft agar-containing medium and incubated at 41°C.

Transformed-cell foci, which were indistinguishable from those induced by AEV-H infection, were observed 1 week after transfection (Fig. 3). The culture was transferred and again overlaid with soft-agar-containing medium so that transformed cells could grow selectively. When fully trans-

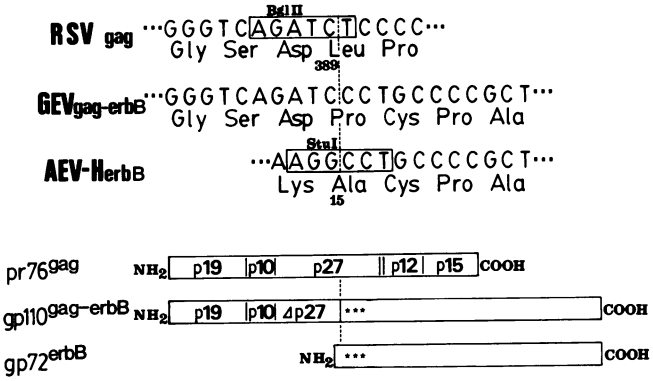


FIG. 2. Predicted in-frame fusion sequence around the junction of the GEV *gag-erbB* fused protein. Asterisks indicate possible glycosylation sites.

formed, the culture fluids were harvested and assayed for transforming virus (*gag-erbB*-containing virus [GEV]) and stored as a virus stock. The titer of the GEV stock (about $10^6$ focus-forming units/ml) was almost equivalent to that of AEV-H. When GEV-infected CEF were grown in soft-agar suspension cultures, transformed cell colonies morphologically similar to those induced by AEV-H were also formed. The results showed that the molecularly constructed *gag-erbB* gene can transform CEF, and the transcript of the fused gene was efficiently incorporated into a YAV virion.

We then characterized viral gene products produced in

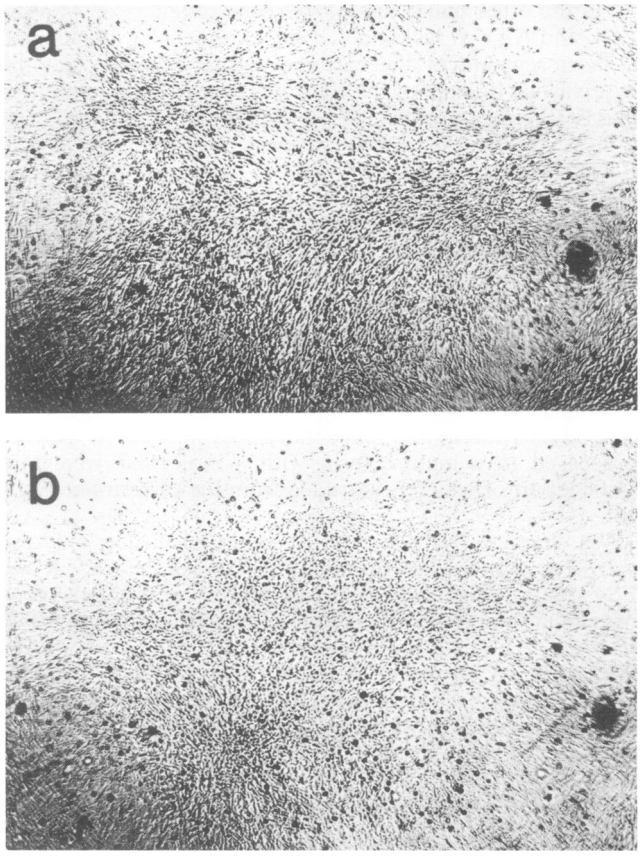


FIG. 3. Transformed-cell foci induced by infection with AEV-H (a) and GEV (b).

AEV-H- and GEV-infected cells. First, extracts of AEV-H-transformed non-virus-producing cells that had been labeled with [$^{35}$S]methionine or $^{32}P_i$ in the presence or absence of tunicamycin (1 μg/ml) were immunoprecipitated with rat anti-*erbB* serum obtained from a tumor-bearing rat (8) and subjected to polyacrylamide gel electrophoresis. Three major viral gene products, p67, gp72, and a heavily phosphorylated material, were identified as *erbB* gene products (Fig. 4). pr76, the *gag* gene product of AEV-H, was also detected by rat anti-*erbB* serum because it contains anti-*gag* antibody as well as anti-*erbB* antibody (anti-*erbB* serum preabsorbed with Rous-associated virus-2 (RAV-2) virion proteins could not detect pr76 protein). The results (Fig. 4) indicated that gp72 protein is a glycosylated form of the p67 *erbB* gene product. As a phosphorylated form of the *erbB* gene product, in addition to a faint band with slightly lower mobility than p67 (Fig. 4, lanes 5 and 6, arrowhead), a very heavily phosphorylated material with a molecular mass of 78 to 80 kilodaltons (indicated by arrows) was detected (gp72 is probably phosphorylated but phosphorylation of gp72 is not clearly shown in Fig. 4 because of the high background due to the heavily phosphorylated material). This material was easily detectable when labeled with $^{32}P_i$ but was usually not seen as a clear band when labeled with [$^{35}$S]methionine. This material may correspond to gp74, a mature and possibly functional form of the ES4 *erbB* gene product present at the cell surface of erythroblasts transformed by the ES4 strain (1, 7). There is no report of whether gp74 of ES4 is heavily phosphorylated in vivo (1, 7, 8). Using an antibody against a synthetic peptide from the C terminus of the human EGF receptor, Kris et al. demonstrated that 68- and 75-kilodalton proteins of the ES4 *erbB* gene were autophosphorylated in the in vitro kinase reaction of the immunoprecipitates (13). Furthermore, they showed that 78- and 85-kilodalton products of the AEV-H *erbB* gene were phosphorylated in the in vitro kinase reaction by using the same antibody, although we could not demonstrate phosphorylation of these proteins in our immunoprecipitates with rat anti-*erbB* serum. The phosphoproteins of 78 and 85 kilodaltons may correspond to gp72 and the heavily phosphorylated protein described above, respectively, or the heavily phosphorylated material with an apparent molecular mass of 78 to 80 kilodaltons may in fact consist of two components of 78 and 85 kilodaltons. Although further analysis of this heavily phosphorylated form appears to be interesting and important, we did not examine this material further in this study.

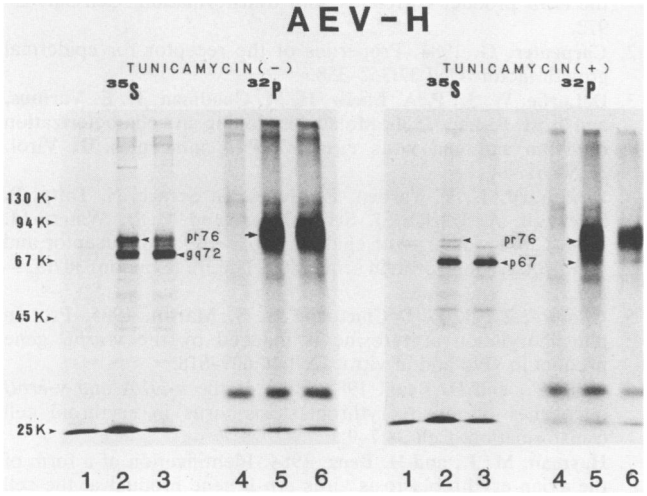


FIG. 4. Immunoprecipitation of the *erbB* gene product from extracts of cells transformed by AEV-H. Non-virus-producing CEF transformed by AEV-H were prepared as described previously (12), and separate cultures containing about $1.5 \times 10^6$ cells were starved for 1 h with 2 ml of methionine- or phosphate-free medium supplemented with 5% dialyzed calf serum in the presence (+) or absence (−) of tunicamycin (1 μg/ml) and labeled with [$^{35}$S]methionine (50 μCi/ml) or $^{32}P_i$ (750 μC/ml) for 6 h in the same medium. Since labeling of cultures for longer periods (up to 18 h) produced essentially the same results, we routinely labeled cultures for 6 h. Extracts of transformed cells were immunoprecipitated with normal rat serum (lanes 1 and 4), rat anti-*erbB* serum (lanes 2 and 5), and rat anti-*erbB* serum preabsorbed with disrupted RAV-2 virion proteins (lanes 3 and 6). The resulting immunoprecipitates were analyzed by 10% (wt/vol) polyacrylamide gel electrophoresis. The conditions for immunoprecipitation and gel electrophoresis were essentially as described previously (11).

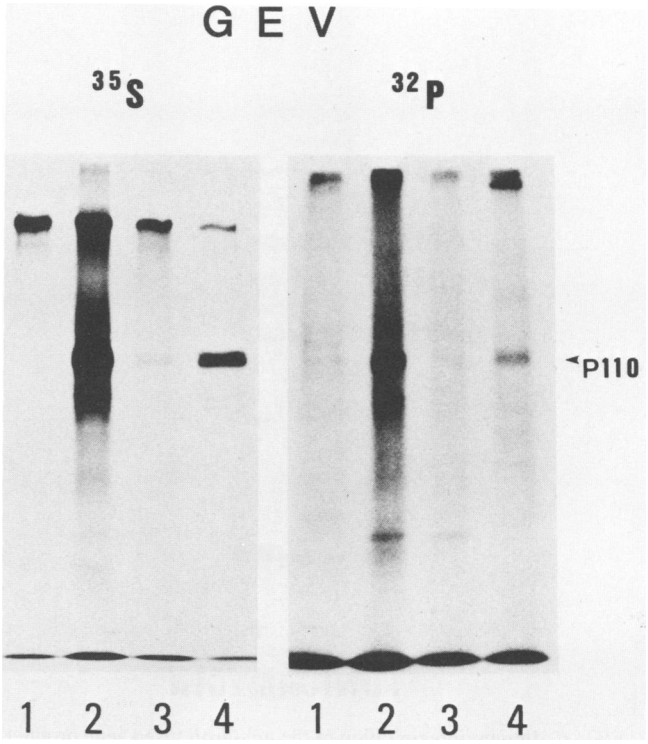


FIG. 5. Immunoprecipitation of the *gag-erbB* fused gene product. Non-virus-producing transformed CEF cultures induced by GEV were prepared and labeled with [$^{35}$S]methionine and $^{32}P_i$ as described in the legend to Fig. 4. Cell extracts were immunoprecipitated with normal rabbit serum (lane 1), anti-RAV-2 virion protein serum (lane 2), anti-RAV-2 virion protein serum preincubated with purified RAV-2 virion proteins (lane 3), or anti-*erbB* serum (lane 4) and subjected to 8.5% polyacrylamide gel electrophoresis.

Next, a non-virus-producing cell clone transformed by GEV was isolated as described previously (12), and two separate cultures of the same non-virus-producing clone were labeled with [$^{35}$S]methionine and $^{32}P_i$. Gene products were immunoprecipitated from extracts with rabbit antiserum against viral structural proteins of RAV-2 and rat anti-*erbB* serum. As expected from the structure of the *gag-erbB* gene, a protein with a molecular weight of about 110,000 was detected with either antiserum as a [$^{35}$S]methionine- and $^{32}$P-labeled protein (Fig. 5). This result showed that anti-virion serum was useful for immunoprecipitating the *gag-erbB* product (p110). When virus-producing cells transformed by GEV (YAV) were labeled with [$^{35}$S]methi-

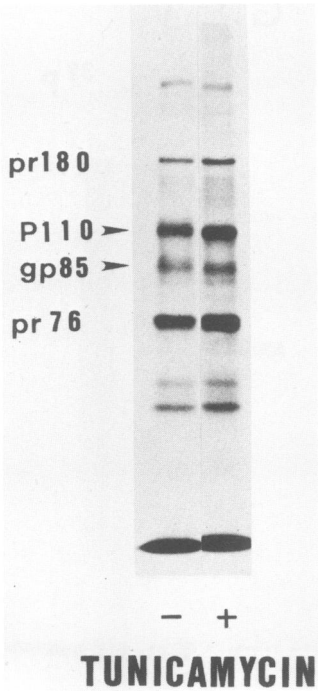


FIG. 6. Immunoprecipitation of the *gag-erbB* fused gene product produced in the presence or absence of tunicamycin. Cells infected with GEV which was associated with a leukosis virus, YAV, were labeled with [$^{35}$S]methionine in the presence (+) or absence (−) of tunicamycin (1 μg/ml) for 6 h. The viral gene products immunoprecipitated with rabbit anti-RAV-2 virion protein serum were analyzed by 8.5% polyacrylamide gel electrophoresis. pr76 and pr180 are a product of the *gag* gene and a readthrough product of the *gag* and *pol* genes of YAV, respectively.

onine in the presence of tunicamycin, the *gag-erbB* fused gene product, as well as gp85 of the helper virus, the protein beneath p110, moved slightly faster, suggesting that the p110 protein was glycosylated (Fig. 6). This result is consistent with the expected structure of the *gag-erbB* gene product in which all possible glycosylation sites of the *erbB* gene product of AEV-H should be retained. Only p110 was detected as a phosphorylated form. In contrast to the AEV-H *erbB* gene product, no heavily phosphorylated material was formed. Therefore, at least in this case, further modification of the *erbB* gene product leading to formation of heavily phosphorylated material does not appear to be required for cell transformation. It is not known whether the absence of further modification is due to removal of the extreme N-terminal 15 amino acids of the *erbB* gene product or addition of *gag* protein to the *erbB* gene product.

For determination of whether the p110 protein is autophosphorylated in a kinase reaction in vitro, the material immunoprecipitated from extracts of GEV (YAV)-transformed cells by anti-virion serum was incubated with [γ-$^{32}$P]ATP and the reaction products were separated on polyacrylamide gel. The p110 protein was phosphorylated (data not shown), but incorporation of $^{32}$P into p110 was very low, and it could not be determined in this study whether p110 was autophosphorylated or phosphorylated by a cellular protein kinase contaminating the immunoprecipitate.

The v-*erbB* gene is known to be a truncated version of the EGF receptor gene. The EGF receptor has a large extracellular EGF-binding domain that is believed to control the activity of tyrosine-specific protein kinase of the cytoplasmic domain of the EGF receptor through a conformational change brought about by EGF binding to this domain. Therefore, study of the effects of modification of the extracellular region of the v-*erbB* gene product on protein kinase activity may shed light on the mechanism of cell transformation by the *erbB* gene. In this sense, it is noteworthy that addition of the large *gag* polypeptide to the *erbB* gene product does not affect its transforming activity. Furthermore, since AEV-transformed erythroblasts are known to differentiate to some extent (6, 9), it may be interesting to examine the effect of the *gag* modification of the *erbB* gene on transformation and differentiation of erythroblasts.

Finally, in a study of the mechanism of cell transformation by the *erbB* gene, GEV appeared to be more suitable than other AEV strains because anti-*gag* sera that can immunoprecipitate the transforming gene product easily and specifically are available in this system, whereas only anti-*erbB* serum is available for immunoprecipitation of the gene product in other AEV systems, and coimmunoprecipitation of cellular EGF receptors might mask effects of the *erbB* gene product.

We are grateful to M. Hayman, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., for a gift of rat anti-*erbB* serum and H. Shinno-Kohno for excellent technical assistance.

This work was supported in part by a grant-in-aid for cancer research from the Japanese Ministry of Education, Science, and Culture.

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