MOLECULAR AND CELLULAR BIOLOGY, May 1986, p. 1751–1759
0270-7306/86/051751-09$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 6, No. 5

A Single Amino Acid Substitution in v-*erbB* Confers a Thermolabile Phenotype to *ts*167 Avian Erythroblastosis Virus-Transformed Erythroid Cells

OK-RYUN CHOI,[1] CECELIA TRAINOR,[1] THOMAS GRAF,[2] HARTMUT BEUG,[2] AND JAMES DOUGLAS ENGEL[1]*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201,[1] and EMBL, Heidelberg, Federal Republic of Germany[2]

Received 4 November 1985/Accepted 13 February 1986

A library of recombinant bacteriophage was prepared from *ts*167 avian erythroblastosis virus-transformed erythroid precursor cells (HD6), and integrated proviruses from three distinct genomic loci were isolated. A subclone of one of these proviruses (pAEV1) was shown to confer temperature-sensitive release from transformation of erythroid precursor cells in vitro. The predicted amino acid sequence of the v-*erbB* polypeptide from the mutant had a single amino acid change when compared with the wild-type parental virus. When the wild-type amino acid was introduced into the temperature-sensitive avian erythroblastosis virus provirus in pAEV1, all erythroid clones produced in vitro were phenotypically wild type. The mutation is a change from a histidine to an aspartic acid in the temperature-sensitive v-*erbB* polypeptide. It is located in the center of the tyrosine-specific protein kinase domain and corresponds to amino acid position 826 of the human epidermal growth factor receptor sequence.

Avian erythroblastosis virus (AEV) is a replication-defective retrovirus which causes fibrosarcomas and (predominantly) rapid erythroblastosis in vivo in newborn chicks and is capable of transforming both fibroblasts and erythroid precursor cells in vitro (17). The ES4 strain of AEV is unusual, however, in that it has transduced parts of two unlinked cellular loci as integral components of the viral genome; these have been termed v-*erbA* and v-*erbB* (1, 35). v-*erbA* is a fusion polypeptide containing *gag* sequences and is synthesized from genomic RNA, whereas v-*erbB* is translated from a spliced subgenomic mRNA (21, 22, 29, 31).

Deletion studies have demonstrated that v-*erbA* is dispensable for transformation in vitro, whereas v-*erbB* expression is absolutely required (12, 33, 34). An adjunct to the argument that v-*erbB* is sufficient for in vitro transformation was provided by analysis of a new isolate of AEV (termed AEV-H), which has acquired only a transduced v-*erbB* gene and which is different (in several portions of the predicted polypeptide sequence) from the *erbB* protein in AEV-ES4 (44, 45). The v-*erbB* gene of AEV-ES4 encodes a 61-kilodalton polypeptide which is glycosylated and phosphorylated to generate a series of precursor glycoproteins, gp62.5, gp66, and gp68 (20). The intermediate gp68 is further glycosylated to form a mature gp74 whose localization to the plasma membrane is required for cell transformation (4).

The amino acid sequence of v-*erbB* as deduced from the DNA sequence has extensive homology with the receptor for human epidermal growth factor (EGF) (9) and is related to the *src* family of oncogenes (44) which contain tyrosine-specific protein kinase activity (30). Recently, tyrosine-specific kinase activity for the *erbB* protein has been demonstrated in vitro and in vivo (13, 23).

To date, five conditional mutants have been isolated which allow temperature-sensitive release from the AEV-induced self-renewal of erythroid precursors that leads to a partial block in differentiation (15, 27). The two of these mutants which have been studied in the greatest detail are designated *ts*34 and *ts*167, both originally isolated from mutagenized cells which were infected with AEV-ES4 (15, 27). Phenotypically, the two temperature-sensitive mutants differ slightly in that *ts*167 appears to allow a greater proportion of the thermally released CFU-E precursor erythroid cells to progress to maturity (27).

Our interest in erythroid cell development led us to undertake the present study, which was designed to reveal the nature of the lesion(s) which confers the temperature sensitivity to *ts*167 AEV-transformed erythrocyte precursor cells. Our aim was to identify the thermosensitive lesion(s) in the hope that analysis of such mutants might shed light on the mechanism by which the *erb* gene products are able to restrict the further development of erythroid cells. At the same time, we anticipated that an analysis of how this lesion affected the transforming potential of the transduced viral oncogene might also provide further insight into the question of why this truncated receptor for the EGF (9) induces the self-renewal of erythroid cells at a particular stage of development.

MATERIALS AND METHODS

Cells and cell lines. Chicken embryo fibroblasts (CEF) were prepared from 10- to 11-day-old embryos purchased from SPAFAS, Inc. (Norwich, Conn.) by the procedure of Vogt (43) and were propagated in growth medium consisting of Dulbecco modified Eagle medium, 10% tryptose phosphate broth, 5% calf serum, and 1% heat-inactivated chicken serum (GIBCO Laboratories, Grand Island, N.Y.). Normal bone marrow cells were prepared from 1- to 3-week-old SPAFAS chicks as described by Graf (14), and mature erythrocytes were removed with lymphocyte separation medium (Histopaque; density, 1.077; Sigma Chemical Co., St. Louis, Mo.) as previously described (5). A continuous cell line (HD6) transformed with *ts*167 AEV and erythroblast clones transformed with various molecular clones of AEV were

* Corresponding author.

grown in a nutrient medium (EBM+H) consisting of Dulbecco modified Eagle medium, 8% fetal bovine serum, 2% heat-inactivated chicken serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.3), and 5×10^{-5} M thioglycerol.

Genomic blotting. High-molecular-weight DNA from HD6 cells and normal chicken erythrocytes was prepared as previously described (10). After digestion with restriction enzymes, DNA (2.5 μg per lane) was subjected to electrophoresis on a 0.8% agarose gel and then blotted to nitrocellulose (10, 38).

Construction and screening of HD6/λEMBL3 library. The genomic DNA library of HD6 DNA was constructed according to published procedures (11, 24). High-molecular-weight DNA of HD6 cells was prepared and partially digested with *Mbo*I. Fragments of 20 to 22 kilobases (kb) were purified on 10 to 40% sucrose density gradients. Bacteriophage λEMBL3 (11) DNA was double digested with *Bam*HI and *Eco*RI after ligation of the cohesive ends. To reduce the background from internal fragment religation, oligonucleotides produced by digestion of the *Bam*HI-*Eco*RI linker sequence were removed by differential precipitation (11) from the DNA containing the essential genes for λ replication and the internal nonessential fragments to be replaced by inserted HD6 DNA. After dephosphorylation of the λEMBL3 *Bam*HI ends (by treatment with calf intestine alkaline phosphatase), 3.75 μg of 20- to 22-kilobase-pair (kbp) HD6 DNA was ligated to 7.5 μg of λEMBL3 arms. The ligation mixture was directly packaged in vitro with extracts prepared as previously described (7, 25) followed by a CsCl step gradient (1.7, 1.5, and 1.45 g of CsCl per ml in 0.1 M NaCl–0.05 M Tris hydrochloride [pH 7.5]–0.001 M $MgCl_2$) to remove unreacted components of the packaging extracts which are toxic to host-cell growth. The titer of the library was 5×10^6 PFU, and the unamplified library was used directly for screening on host strain *Escherichia coli* 803 *supF*. Approximately 5×10^5 PFU were screened by plaque hybridization (2), using a nick-translated 0.5-kbp *Bam*HI-*Eco*RI fragment of v-*erbB* and a 0.45-kbp *Pst*I fragment of v-*erbA* (42) as a mixed probe in the hybridization reaction. The positive plaques were picked and subjected to secondary and tertiary screening with separate probes for v-*erbA*, v-*erbB*, and pRAV10R (see below). Phage DNA was prepared and purified according to published procedures (24).

DNA sequencing and construction of reciprocal mutants. The plasmid containing temperature-sensitive AEV provirus, pAEV1, was constructed by ligation of a 7.7-kb *Hin*dIII fragment (from λAEV6a) to pAT153 DNA which had been cleaved at the *Hin*dIII site (39). The plasmid pAEV11-3 (described by Sealy et al. [34]) was used as the molecular clone representative of wild-type AEV-ES4. All plasmids were propagated in *E. coli* MC1061 and prepared by alkaline lysis and polyethylene glycol precipitation methods described in published procedures (6, 24).

The DNA sequence of the v-*erbB* region of pAEV1 was determined by the chemical cleavage method of Maxam and Gilbert (26) as modified by Smith and Calvo (36) and compared with the sequence of same region of pAEV11-3 (J. M. Bishop, unpublished observations).

The reciprocal mutants between pAEV1 (temperature sensitive) and pAEV11-3 (wild type) were prepared by exchange of a 558-base-pair (bp) *Bam*HI fragment from the v-*erbB* region of the two viruses. To simplify the construction, a 2.2-kbp *Sal*I-*Eco*RI fragment containing parts of both *erbA* and *erbB* from pAEV1 and pAEV11-3 was individually subcloned into pBR322 digested with *Sal*I and *Eco*RI. After exchanging the 558-bp *Bam*HI fragments in each of the two intermediate recombinants, the temperature-sensitive 2.2-kbp *Sal*I-*Eco*RI fragment containing the wild-type *Bam*HI fragment was isolated and ligated into pAEV1 which had been digested to completion with *Sal*I and partially with *Eco*RI. The reciprocal mutant was prepared in the same manner. Both mutants were confirmed by restriction enzyme mapping and by DNA sequencing of the exchanged regions.

Transfection of CEF. Secondary CEF were plated 24 h before transfection, and a 50 to 70% confluent monolayer was refed with fresh medium 4 h before the addition of the DNA precipitate. Transfections were performed essentially by the method of Graham and van der Eb (19) as modified by Parker and Stark (28). A solution containing 10 parts of AEV plasmid DNA and 1 part of pRAV10R (a generous gift from H. J. Kung) Rous-associated virus-1 (RAV1) helper virus DNA (final total DNA concentration, 20 μg/ml) was adjusted to 250 mM $CaCl_2$ and added slowly to an equal volume 2× HBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.13) with constant agitation. The mixture was allowed to form a fine visible precipitate for 30 min at room temperature, and then 0.5 ml of this DNA-$CaPO_4$ precipitate was added directly to 5 ml of medium in a 60-mm culture plate.

After 4 h of incubation at 37°C, the medium containing the $CaPO_4$-DNA precipitate was aspirated, and cell monolayers were treated with 2 ml of 15% glycerol in HBS for 3 min at room temperature, washed twice with medium (without serum), and then overlaid with complete growth medium. Cells were passaged three times (1:4 split) before assay for reverse transcriptase or AEV-specific RNA by dot hybridization. For focus assays (see below), second and third passage-transfected cells were plated under soft agar.

Dot hybridization. For viral RNA isolation, virions in culture supernatant were pelleted by centrifugation at 40 krpm in an SW50.1 rotor at 4°C for 1 h after removal of cell debris by low-speed centrifugation (7,000 × *g*) for 10 min. Virus pellets were suspended in 300 μl of 20 mM Tris hydrochloride (pH 7.4)–0.1 M NaCl–10 mM EDTA containing 0.5 mg of freshly prepared proteinase K per ml and 0.5% sodium dodecyl sulfate, and 5 μg of carrier RNA was added. The mixture was incubated at 37°C for 1 h, and RNA was extracted twice with neutral phenol-$CHCl_3$-isoamyl alcohol (25:24:1) and once with chloroform, followed by ethanol precipitation. The RNA pellet was dissolved in 200 μl of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–7.4% formaldehyde and then denatured at 63°C for 5 min; the solution was then filtered through a dot-blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.) onto a nitrocellulose filter which had been presoaked in 20× SSC. Each sample well was then rinsed with 400 μl of 6× SSC. The nitrocellulose filter was then baked in a vacuum oven at 80°C for 2 h, followed by prehybridization, hybridization, and washing under previously described conditions (6). The nick-translated v-*erbB* probe containing the 0.5-kbp *Bam*HI-*Eco*RI fragment (described above) was used to detect AEV viral sequences. RAV1 RNA was also prepared and used as a negative control, while HD6 culture supernatant served as the positive control.

Clonal cell propagation. Focus assays were performed as described previously (16). CEF foci were picked into 24-well dishes and grown in either EBM+H or growth medium.

Bone marrow cells were infected with virus from culture supernatants of transformed CEF and seeded in semisolid medium containing methylcellulose as previously described (18). Erythroid colonies were picked from methylcellulose into 24-well dishes after 8 to 10 days of incubation at 37°C.

These cells were grown in EBM+H for 5 to 10 days at 37°C and then tested for inducibility of differentiation by temperature shift to 42°C as described previously (3). Hemoglobin production was monitored as the assay for terminal erythroid differentiation by acid and neutral benzidine staining (3, 27).

Miscellaneous. Restriction enzyme maps of phage were determined by gel electrophoresis of single and double digests of phage DNA and by hybridization of double blots to v-*erbA* and v-*erbB* probes (7, 24). For detailed maps of the subclone pAEV1, the technique of Smith and Birnsteil (37) was employed. ^{32}P-labeled probes were prepared by nick translation of fragments excised from plasmids: a 0.45-kbp *Pst*I fragment for the v-*erbA* probe and a 0.5-kbp *Bam*HI-*Eco*RI fragment for the v-*erbB* probe (described above). Reverse transcriptase activities in virions were measured with a synthetic template by the method of Sealy et al. (34).

RESULTS

Analysis of integrated proviruses in HD6 cells. An initial decision was made to employ HD6 cells as the source of DNA for cloning integrated *ts*167 AEV provirus. HD6 is a transformed erythroid precursor cell line which has been established from bone marrow cells initially infected with *ts*167 AEV and RAV2 as a helper virus (5). HD6 cells shed active *ts*167 AEV virus particles, and this supernatant virus has been shown to confer conditional erythroblast-transforming activity on freshly infected bone marrow cells in vitro (see below).

To determine the number of provirus copies which integrated into the host chromosome in HD6 cells, genomic DNA was prepared from cultured HD6 cells and from normal chicken erythrocytes by standard procedures (10) and analyzed by Southern blotting (38) with a nick-translated v-*erbB* fragment as a probe. The restriction enzyme fragments hybridizing to the v-*erbB* probe in normal chicken erythrocyte DNA were those expected from hybridization to the c-*erbB* locus (41) (Fig. 1). Additional bands in lanes containing digested HD6 DNA imply that there exist three different cellular loci for provirus integration, although it is not clear whether the provirus integrated into three separate loci in a homogeneous cell population or whether proviruses integrated into one or more loci in a heterogeneous population of cells (see Discussion).

Molecular cloning of integrated copies of *ts*167 AEV provirus. Since it has been shown that virus recovered from HD6 cells confer temperature sensitivity to erythroid cell transformation (5; and therefore that at least one of these loci must harbor a temperature-sensitive provirus) we prepared a genomic recombinant DNA library of HD6 DNA which had been partially digested with *Mbo*I in the vector λEMBL3 (11) and screened the bacteriophage library (without amplification) with nick-translated v-*erbA* and v-*erbB* probes (2, 7). A total of 37 recombinants reacted with this mixed probe on primary screening; on further analysis with separate v-*erbA* and v-*erbB* probes, 11 of the 37 recombinants which showed positive hybridization response to both probes were identified as integrated AEV provirus clones. The rest of the recombinants were subsequently identified as either c-*erbA* or c-*erbB* coding regions by comparison of these recombinants to those already isolated and described (41; O. Choi and J. D. Engel, unpublished observations).

Abbreviated restriction enzyme maps of 10 of the integrated provirus recombinants are shown in Fig. 2. As expected from the genomic blotting analysis (Fig. 1), the

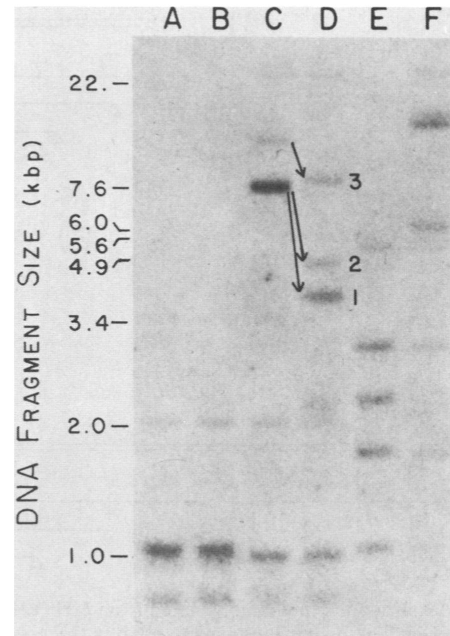

FIG. 1. *erbB* homologous sequences in normal chick erythrocytes and in *ts*167 AEV-transformed erythroid cell line HD6. Genomic erythrocyte and HD6 DNAs were isolated as previously described (10). A 2.5-μg portion of each DNA sample was digested with *Hin*dIII (lanes A and C), *Hin*dIII and *Sal*I (lanes B and D), or *Eco*RI (lanes E and F), electrophoresed on a neutral 0.8% agarose gel, and blotted to nitrocellulose as described previously (10, 38). A subclone containing a 0.5-kbp *Bam*HI-*Eco*RI fragment of the v-*erbB* gene from wild-type AEV-ES4 was nick translated and hybridized to a nitrocellulose filter which was then exposed for autoradiography. The bands visualized in the chick erythrocyte DNA (lanes A, B, and E) correspond to those from c-*erbB* coding sequences (41). The additional bands visualized in the lanes containing HD6 DNA (lanes C, D, and F) are due to hybridization to homologous AEV v-*erbB* sequences contained within the proviruses integrated into the HD6 cell line (see Fig. 2). Numbers in the gel define loci.

recombinants containing both v-*erbA* and v-*erbB* complementary sequences can be divided into three different groupings based on the restriction enzyme patterns of flanking cellular sequences. Each of these three separate loci contained either all or part of a proviral copy of AEV, and the restriction fragments of cellular flanking sequences of individual groups did not cross-hybridize with one another (data not shown). The loci were arbitrarily numbered; the groups numbered 1, 2, and 3 in Fig. 2 were shown to be derived from the loci labeled 1, 2, and 3 in genomic HD6 DNA (Fig. 1) by comparison of restriction enzyme digest patterns (data not shown).

The provirus locus 1 (in Fig. 1 and 2) was chosen as the first possible locus for harboring active temperature-sensitive AEV because of the relatively greater intensity of the genomic DNA band which hybridized to the v-*erbB* probe (Fig. 1). The 7.7-kb *Hin*dIII fragment containing an entire provirus copy of AEV from recombinant λAEV6a was subcloned into the plasmid pAT153 at the *Hin*dIII site and is subsequently referred to as pAEV1. A detailed restriction enzyme map of this plasmid is shown in Fig. 3.

Phenotype of cells transformed by provirus in pAEV1. To determine whether the virus from the cloned DNA in pAEV1 is temperature sensitive for erythroblast transformation, DNA transfection was employed to recover active virus

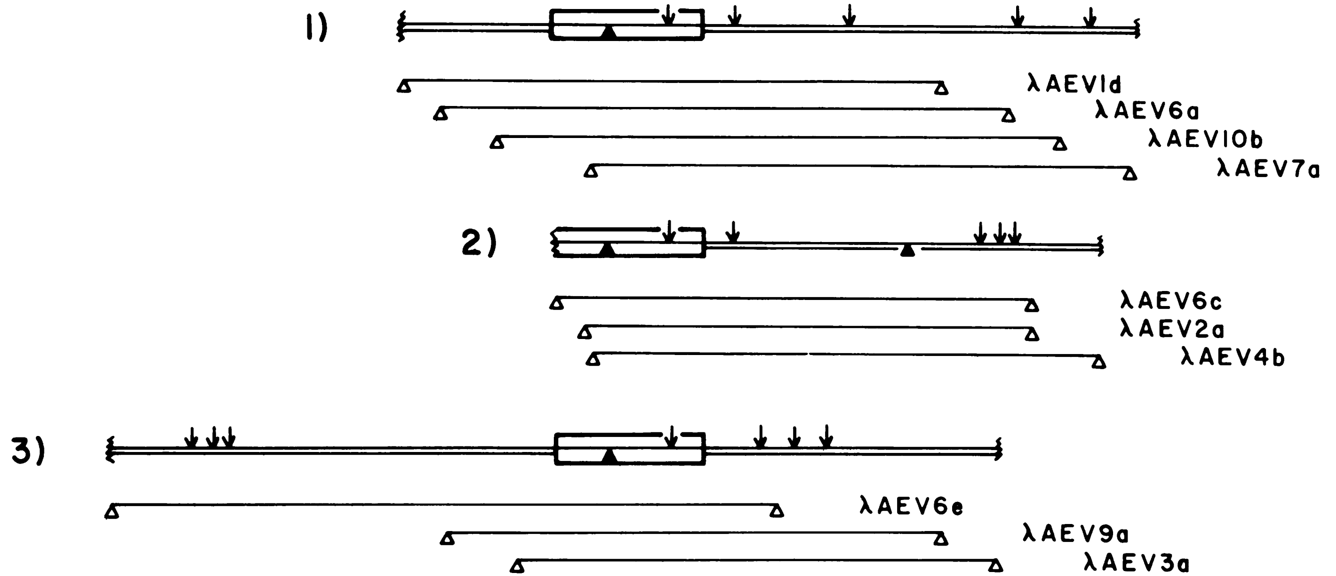

FIG. 2. Provirus recombinants isolated from HD6 cells. Recombinants hybridizing to both v-*erbA* and v-*erbB* probes were mapped with *Sal*I and *Eco*RI. The restriction maps overlap in three separate groups, thus identifying three separate loci of viral integration in HD6 cells. Symbols: △, *Sal*I linker in λEMBL3; ▲, *Sal*I; ↓, *Eco*RI. Recombinants were mapped as previously described (7). The position of the provirus is displayed as a box containing both a single *Sal*I and *Eco*RI site.

particles, followed by analysis of the thermal inducibility and differentiation capacity of erythroid precursor cells transformed with the recovered virus.

To obtain infectious replication-defective AEV, CEF were transfected with DNA employing a cotransfection procedure adding cloned RAV1 DNA as a helper virus. This helper (pRAV10R) has been used successfully in previous studies to recover replication-defective AEV (42). We transfected secondary CEF with 10 parts of pAEV1 DNA to 1 part of pRAV10R DNA (containing a single copy of the RAV1 helper virus) by modification of the $CaPO_4$ coprecipitation method (19, 28). Virus production was monitored by assay for reverse transcriptase activity within gently lysed virions (34). The abundance of AEV-specific genomic sequences in supernatants from the transfected CEF was assayed by dot-blot hybridization with radiolabeled v-*erbB* as probe. The levels of reverse transcriptase activity recovered from CEF supernatants were the same when comparing pAEV1-

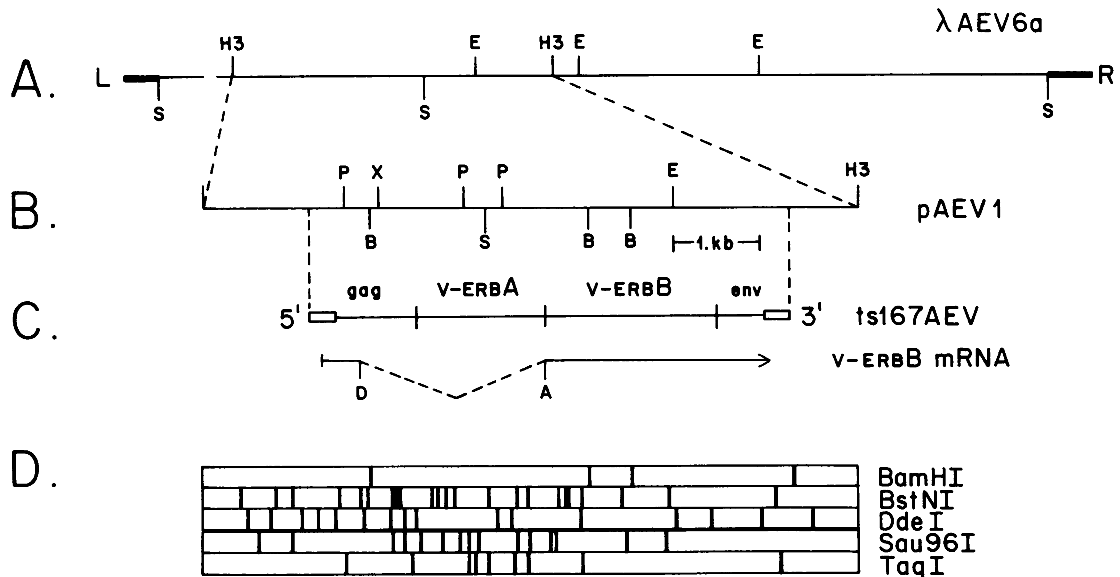

FIG. 3. Restriction enzyme map of pAEV1. (A) Recombinant λAEV6a was digested with *Hin*dIII; a 7.7-kb fragment containing the entire provirus and cellular flanking sequences was subcloned into the *Hin*dIII site of pAT153 (B; 39). The restriction maps shown in panels B and D were resolved by conventional double-digest (24) and end-labeled partial digest (37) restriction endonuclease methods. Letters in lines A and B correspond to restriction endonuclease sites for *Bam*HI (B), *Pst*I (P), *Xho*I (X), *Sal*I (S), *Eco*RI (E), and *Hin*dIII (H3). D and A in line C correspond to the 5′ and 3′ splice sites used for generation of the v-*erbB* subgenomic mRNA, respectively (1).

pRAV10R cotransfected cultures to pRAV10R transfection supernatants alone. Virion RNA hybridizing to *erbB* probe was obtained only from the supernatants of cultures transfected with both pAEV1 and pRAV10R (data not shown).

As a biological assay for the transforming activity of the virus and as a procedure for the cloning of transformed cells, we employed a standard focus assay (16). Transfected cells were passaged two or three times and then overlaid with soft agar; after 10 to 14 days of incubation, typical (diffuse) AEV foci were scored and picked into 24-well dishes containing 0.5 ml of EBM+H (Materials and Methods). An average of two to three foci were obtained per 60-mm dish. Individual foci were expanded to confluency in 100-mm dishes, and the culture supernatant of these cloned foci was examined for the presence of high-titer AEV by dot hybridization analysis. The hybridization signal intensity from the culture supernatant of individual expanded foci was usually about 100-fold stronger than the signal intensity from mass culture supernatants of transfected cells.

To determine whether the recovered virus was temperature sensitive for erythroid cell transformation, bone marrow cells were infected in vitro with supernatant virus from expanded foci of CEF and then seeded in methylcellulose at a density of 1×10^6 to 2×10^6 bone marrow cells per ml (5). Resulting erythroid colonies were picked 8 to 10 days later, and individual colonies were then grown in 0.5 ml of EMB+H after seeding into a 24-well dish. Individual erythroid precursor cell clones were then tested for the induction to maturity by determining the proportion of cells expressing hemoglobin as analyzed by acid benzidine staining (3, 27). By this assay, the virus contained in pAEV1 was shown to be temperature sensitive for erythroid transformation (Fig. 4). Therefore, we assume that pAEV1 harbors an active, integrated proviral form of *ts*167 AEV.

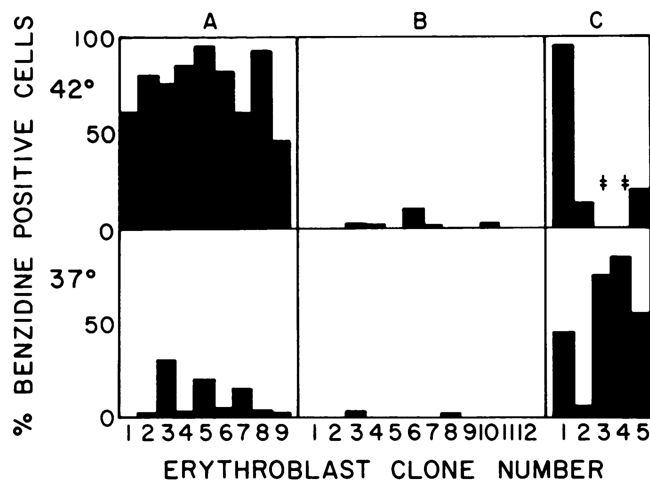

FIG. 4. Histographic representation of benzidine-stained erythroblasts at 37 and 42°C. Individual erythroblast clones obtained after infection of bone marrow cells with AEV supernatants from transfected CEF were incubated at permissive (37°C) and restrictive (42°C) temperatures for 3 days as described in the text and then stained for hemoglobin with acid benzidine (3, 27). (A) The data for erythroblast clones infected with virus recovered from pAEV1 transfection, the provirus recombinant which is representative of locus 1 (Fig. 1 to 3); (B) the data for cells infected with pAEV1 virus containing the 558-bp *Bam*HI fragment in the v-*erbB* gene substituted from pAEV11-3; (C) the data for pAEV11-3 virus-transfected erythroblasts in which the provirus has now been modified by replacement of the 558-bp *Bam*HI fragment in v-*erbB* with the homologous fragment from pAEV1. ‡ indicates clones which died after incubation at 42°C for 3 days.

Nature of the temperature-sensitive lesion in *ts*167 AEV. The product of the v-*erbB* gene has been identified as the transforming protein of AEV by analysis of v-*erbA* and v-*erbB* deletion mutants (12, 33, 34). Furthermore, in studies with *ts*34 and *ts*167 AEV, it has been observed that cell surface expression of the glycoprotein encoded by the v-*erbB* gene is required for transformation of cells (4, 20) but that correct carbohydrate processing of this glycoprotein is not required for subcellular localization or transformation of cells (32). These results taken together suggested that the lesion conferring the conditional transforming activity of v-*erbB* might be exhibited as a thermally inducible conformational change which affects the transport of v-*erbB* to the cell surface, an enzyme activity, or another property of the v-*erbB* protein (or some combination of these factors). To determine the mutation(s) responsible for the temperature sensitive lesion, we sequenced the v-*erbB* gene of the cloned temperature-sensitive AEV locus in pAEV1.

The nucleotide sequence of temperature-sensitive v-*erbB* shows five nucleotide changes but only a single predicted amino acid sequence change in the entire peptide (see the legend to Fig. 5); this mutation occurs in the tyrosine-specific protein kinase region (30) when compared with the sequence of wild-type AEV-ES4 (Fig. 5). This nonconservative amino acid change in v-*erbB* is a histidine in the wild-type *erbB* gene product which has been changed to an aspartic acid residue in pAEV1 at amino acid 826 (numbered relative to the predicted human EGF receptor protein sequence; 40).

Temperature sensitivity of erythroid cells infected with reciprocal virus mutants. To determine whether the identified lesion of v-*erbB* protein was both necessary and sufficient for the conditional transforming activity of *ts*167 AEV, we constructed two reciprocal substitution mutants between temperature-sensitive AEV (pAEV1) and wild-type AEV-ES4 (pAEV11-3) (Fig. 6). Transfection, infection, and assay of transformed erythroblast clones for conditional differentiation were performed as described above.

Figure 4B shows that the erythroblasts transformed with an in vitro reconstructed temperature-sensitive provirus with the wild-type 558-bp *Bam*HI fragment (containing the histidine) display a phenotype indistinguishable from wild-type AEV-transformed erythroblasts. In contrast, the wild-type virus containing a substituted *Bam*HI fragment from pAEV1 (with an aspartate at position 826) displays an unusual temperature-sensitive phenotype. The yield of virus from transfection experiments was consistently lower than that of the temperature-sensitive AEV provirus containing the wild-type *Bam*HI fragment recombinant virus. Thus, infection of bone marrow cells with wild-type AEV containing the temperature-sensitive *Bam*HI fragment construction gave rise to a total of only five transformed erythroblast colonies, whereas 24 random colonies from several hundred were picked for testing from the temperature-sensitive AEV containing the wild-type *Bam*HI fragment virus. These former clones (wild-type AEV containing the temperature-sensitive *Bam*HI fragment transformants) underwent spontaneous differentiation, and about 50% of the cells from these erythroblast clones showed hemoglobin expression without thermal induction (Fig. 4C). Only one clone of the five showed a substantive increase in the number of benzidine-positive cells after temperature shift to 42°C (Fig. 4C; two clones died during the incubation).

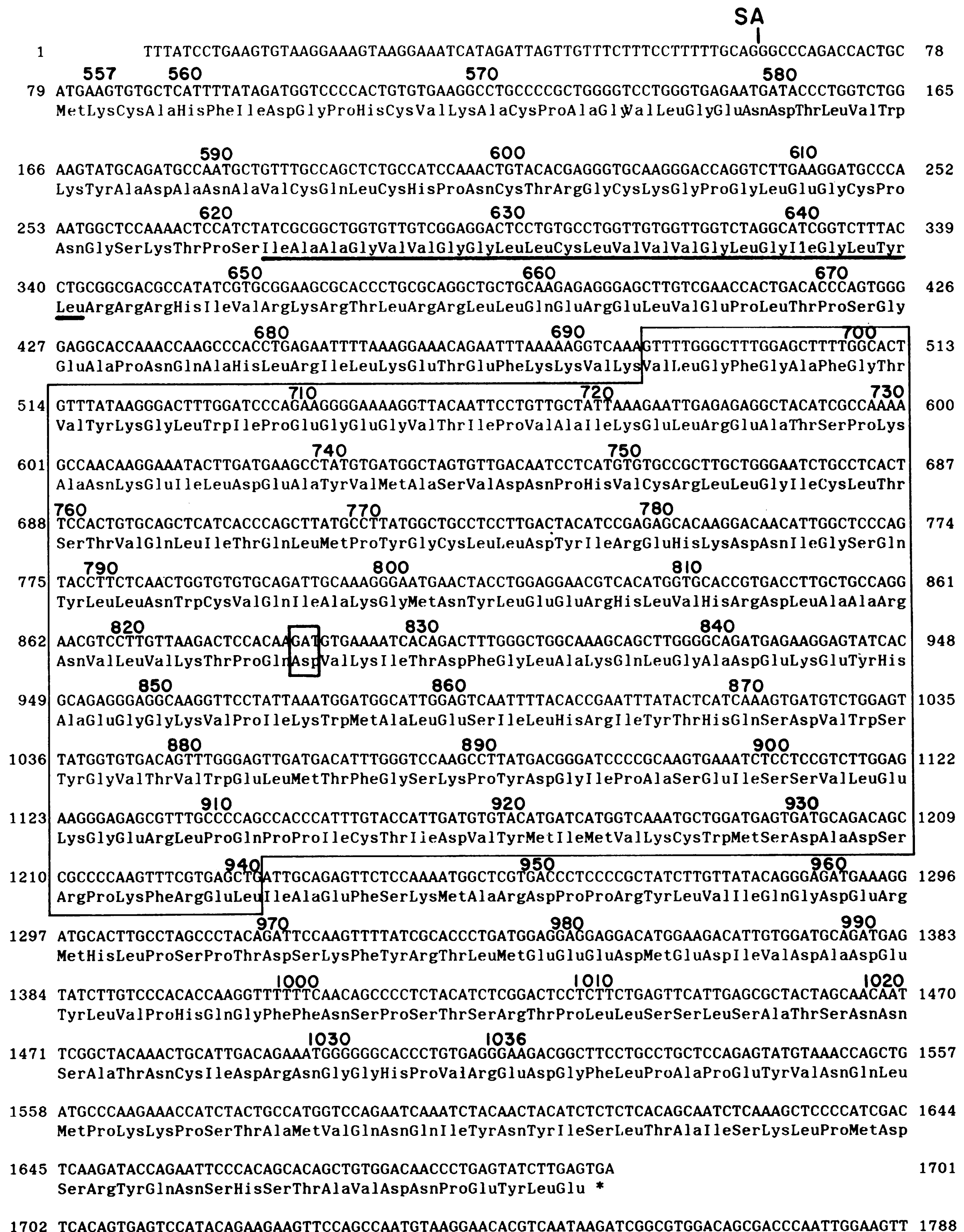

FIG. 5. DNA sequence and predicted amino acid sequence of the v-*erbB* gene in pAEV1. The DNA sequence of the v-*erbB* gene in pAEV1 was determined by modifications (36) of the standard chemical reactions (26). Over 95% of the DNA sequence was confirmed by both 3′ and 5′ end labeling of DNA fragments and by overlapping various sequences from multiple labeling sites. The predicted amino acid sequence is aligned and numbered with the amino acid sequence prediction from the human EGF receptor (40) from amino acid 557 to 1037. The correspondence between the two is excellent, and therefore we chose that amino acid numbering for comparison. The homology between the two predicted proteins indicates that there exist a missing Thr in *erbB* at amino acid position 614, a small deletion after amino acid position

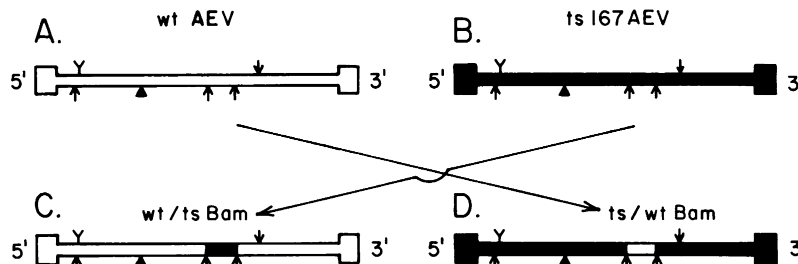

FIG. 6. Strategy for reciprocal replacement of amino acid substitutions between wild-type (wt) and *ts*167 AEV. Recombinants containing wild-type AEV (from pAEV11-3; blank virus) (A), *ts*167 AEV (from pAEV1; black virus) (B), wild-type AEV substituted with the (black) *ts*167 AEV *Bam*HI fragment (containing the aspartate) (C), and *ts*167 AEV substituted with the (blank) wild-type AEV *Bam*HI fragment (containing the histidine) (D) were transfected into CEF; foci were expanded, and the supernatants were used to infect bone marrow cells (Materials and Methods). Resulting erythroid colonies were subjected to differentiation conditions (5), and the thermally induced cultures were scored for erythroid maturation by acid benzidine staining (3, 27) (Fig. 4). Symbols used in the abbreviated restriction enzyme maps shown for the viruses are as follows: ↑, *Bam*HI; Y, *Xho*I; ▲, *Sal*I; ↓, *Eco*RI. The *Bam*HI fragment exchanged is from nucleotide 530 to nucleotide 1088 of Fig. 5 (corresponding to amino acid numbers 707 through 709 to 893 through 895). ts, Temperature sensitive.

DISCUSSION

The salient features of the current study are the following. First, an integrated provirus recombinant isolated from a cell line which produces conditional mutant (*ts*167) AEV can itself produce active transforming virus which is indistinguishable in transforming properties of erythroid precursor cells from *ts*167 AEV; second, within this virus, a single amino acid change (in comparison with wild-type AEV) occurred within the sequence encoding v-*erbB*, the primary transforming oncogene of AEV-ES4; third, the aspartate residue in *ts*167 AEV is a histidine that is conserved in *erbB* proteins from wild-type AEV-ES4, AEV-H, and in the cellular gene counterpart of *erbB*, the EGF receptor (40, 45); and fourth, when a molecular clone of *ts*167 AEV is altered to include the wild-type histidine at position 826 of v-*erbB*, pAEV1 becomes phenotypically indistinguishable from wild-type AEV. Thus, as illustrated in the diagram of Fig. 7, this single amino acid substitution is necessary for conferring a temperature-sensitive phenotype to AEV.

Unexpectedly, the clones of erythroid precursor cells infected with wild-type AEV containing the temperature-sensitive amino acid change in *erbB* displayed a different phenotype from those infected with temperature-sensitive AEV from pAEV1 in that they grew very poorly and differentiated at the permissive temperature and in that they did not exhibit a clear-cut temperature-sensitive phenotype. A possible explanation for this observation is that the phenotype displayed by *ts*167 AEV does not result from a single amino acid change; rather there exists another alteration which is undefined by the present analysis. This alteration either may have been present in the original mutant or may have arisen as a result of the cloning procedure.

Although the transformation activity of recovered virus (from pAEV1-transformed CEF foci) in erythroid cells is indistinguishable from that of *ts*167 AEV, we did not test all transformation parameters of CEF infected with the virus. What is the likelihood, then, that pAEV1 does not contain the genuine *ts*167 AEV, but is the result of reintegration of a slightly mutated form of *ts*167 AEV into what was (originally) a clonal population of erythroid precursor cells containing a single integrated temperature-sensitive virus? We chose a representative recombinant from locus 1 (Fig. 1 and 2) in that eventuality; on the original Southern blot (Fig. 1), the band containing locus 1 was the most intense. We reasoned that if the other integrated provirus loci arose in the population at a later time, they would be less represented in the total genomic DNA of HD6 cells, since these cells would now be a mixed population containing either one, two, or three integrated proviruses, all having originally arisen from a cell line harboring the virus only at locus 1. The less likely alternative is that either locus 2 or 3 was the original integration site and that reinfecting defective virus integrated at a new site (locus 1) which conferred a growth advantage to the population of cells containing that integrant. We are presently analyzing which of these possibilities is true by recloning HD6 from single cells (and subsequent analysis of genomic DNA) and by comparing the erythroblast-transforming phenotypes of the recombinant viruses encoded by locus 2 and locus 3 (Fig. 2).

Erythroid precursor cells transformed with *ts*167 AEV or *ts*34 AEV display a unique phenotype with respect to the *erbB* gene product when propagated at 42°C under conditions which allow differentiation (5). The mature $gp74^{erbB}$ plasma membrane protein is absent from the surface of these transformed erythroid cells when they are grown at the nonpermissive temperature in differentiation medium. The glycosylated and phosphorylated precursor polypeptides $gp66^{erbB}$ and $gp68^{erbB}$ are synthesized and found in intracellular membranes under these conditions but are not correctly processed or transported to the plasma membrane (4). However, inhibitors of glycosylation do not interfere with the transport of the *erbB* product to the cell surface, nor do these

1037, and subsequent substantial 3′ sequence discrepancies. The predicted amino acid sequence shown differs in three places from the published prediction (30) for the wild-type v-*erbB* protein in AEV-ES4. We resequenced parts of pAEV11-3 (containing wild-type AEV-ES4), and showed that amino acid 809 (amino acid 136 in reference 30) should be a leucine instead of a methionine as originally reported; further, M. Privalsky (personal communication) has determined that amino acid 930 (amino acid 260 in reference 30) is actually an aspartate, rather than a glycine as originally reported. Thus, the only amino acid sequence change predicted by the DNA sequence is found at position 826 (boxed). The transmembrane domain is heavily underlined, while the tyrosine-specific kinase domain is lightly outlined. SA, position of the 3′ splice junction used in formation of the v-*erbB* mRNA; *, termination codon used for v-*erbB*.

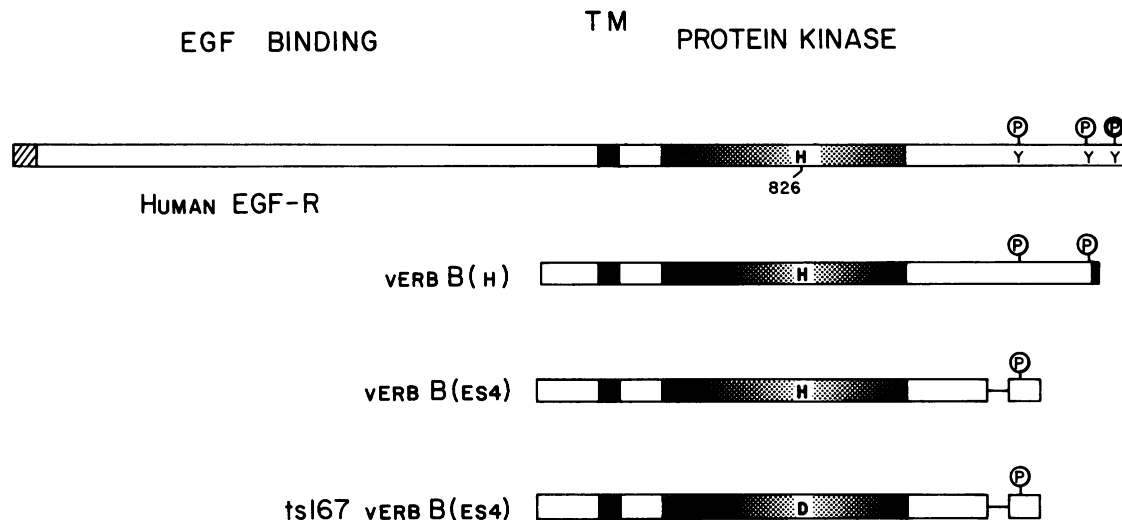

FIG. 7. Comparison of the human EGF receptor to cloned v-*erbB* polypeptides. Amino acid comparisons are diagramatically represented showing the best correlation of overlap of three sequenced v-*erbB* genes to the human receptor for EGF (9, 30, 44). The sequences are aligned to demonstrate the alteration in the histidine (corresponding to amino acid 826 of the human EGF receptor sequence) to aspartate in *ts*167 AEV as compared with wild-type AEV-ES4 and AEV-H. The sequences have been separated into the EGF-binding (extracellular) domains, the transmembrane region (TM; black box), the protein kinase region (stippled area), and sites for tyrosine phosphorylation in the human EGF receptor (P) (8).

inhibitors alter the transformed state of these cells (32). The data indicate that cell surface expression of *erbB* is required for transformation and that the *erbB* transport process is thermolabile in erythroid precursor cells infected with temperature-sensitive AEV. For this reason it seemed likely that the lesion(s) conferring the temperature-sensitive phenotype to these viruses would be located in the *erbB* gene and might be in the region which is involved in the transport process.

Perhaps surprisingly, the mutation in the *erbB* polypeptide in pAEV1 was not found to be in the extracellular domain (in which the glycosylation sites reside) nor in the transmembrane region but in the middle of cytoplasmic domain. This single mutation (substitution of a basic amino acid [histidine] for an acidic amino acid [aspartate]) might simply be responsible for the folding of one domain of the v-*erbB* protein. By this passive model, then, at the restrictive temperature, the altered conformation of the protein might not be recognized by factors which facilitate glycoprotein transport from intracellular membranes to the plasma membrane or, alternatively, does not allow a stable conformation of the temperature-sensitive v-*erbB* gene product when it is in the plasma membrane. It is not known whether *ts*167 AEV also displays temperature-sensitive tyrosine kinase activity in addition to temperature-sensitive transforming activity, but surprisingly, the dramatic change from a basic amino acid (histidine) to an acidic amino acid (aspartic acid) in the middle of the tyrosine-specific kinase region (Fig. 7) did not abolish transforming activity, but only caused the rather mild phenotypic change from a nonconditional (wild type) to a temperature-sensitive conditional (*ts*167) mutation for transformation. While this amino acid substitution does not occur at a position which is homologous to other amino acids within the conserved sequence for tyrosine kinase activity of related oncogene proteins (30) nor within the ATP-binding domain, it nonetheless may or may not be critical for formation of an active site for tyrosine kinase activity. Which of these alternatives is the correct one awaits in vitro analysis of the wild-type and mutant v-*erbB* polypeptides.

ACKNOWLEDGMENTS

We thank J. M. Bishop and H. Varmus for providing the initial v-*erb* probes used in these studies, access to unpublished observations, and useful exchange of information, H.-J. Kung for encouragement and pRAV10R, M. Privalsky for access to unpublished data, B. Vennstrom for many helpful discussions, M. Jansson for help with transfection experiments, and G. Doederlein, S. Grieser, and S. J. Stamler for excellent technical assistance.

This work was supported by Public Health Service grants HL24415 and GM28896 from the National Institutes of Health to J.D.E. and by an NSRA Training grant award to C.T. (GM08061). The training experience provided by T. Graf and H. Beug in virology and erythroid cell culture to O.-R.C. and J.D.E. was partially supported by a Fogarty Senior International Fellowship. J.D.E. is an American Cancer Society Faculty Research Awardee.

LITERATURE CITED

1. **Anderson, S. M., W. S. Hayward, B. G. Neel, and H. Hanafusa.** 1980. Avian erythroblastosis virus produces two mRNAs. J. Virol. **36:**676–683.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196:**180–182.
3. **Beug, H., G. Doederlein, C. Freudenstein, and T. Graf.** 1982. Erythroblast cell lines transformed by a temperature-sensitive mutant of avian erythroblastosis virus: a model system to study erythroid differentiation *in vitro*. J. Cell. Physiol. **1:** (Suppl.)195–207.
4. **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.
5. **Beug, H., S. Palmieri, C. Freudenstein, H. Zentgraf, and T. Graf.** 1982. Hormone-dependent terminal differentiation *in vitro* of chicken erythroleukemia cells transformed by ts mutants of avian erythroblastosis virus. Cell **28:**907–919.
6. **Brush, D., J. B. Dodgson, O.-R. Choi, P. Wilkins Stevens, and J. D. Engel.** 1985. Replacement variant histone genes contain intervening sequences. Mol. Cell. Biol. **5:**1307–1317.
7. **Dodgson, J. B., J. Strommer, and J. D. Engel.** 1979. Isolation of the chicken β-globin gene and a linked embryonic β-like globin

gene from a chicken DNA recombinant library. Cell **17**:879–887.
8. **Downward, J., P. Parker, and M. D. Waterfield.** 1984. Autophosphorylation sites on the epidermal growth factor receptor. Nature (London) **311**:483–485.
9. **Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield.** 1984. Close similarity of epidermal growth factor receptor and *v-erbB* oncogene protein sequences. Nature (London) **307**:521–527.
10. **Engel, J. D., and J. Dodgson.** 1978. Analysis of the adult and embryonic chicken globin genes in chromosomal DNA. J. Biol. Chem. **253**:8239–8246.
11. **Frischauf, A.-M., H. Lehrach, A. Poustka, and M. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
12. **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.
13. **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.
14. **Graf, T.** 1973. Two types of target cells for transformation with avian myelocytomatosis virus. Virology **54**:398–413.
15. **Graf, T., N. Ade, and H. Beug.** 1978. Temperature sensitive mutant of avian erythroblastosis virus suggests a block in differentiation as a mechanism of leukaemogenesis. Nature (London) **275**:496–501.
16. **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.
17. **Graf, T., and D. Stehelin.** 1982. Avian leukemia viruses. Oncogenes and genome structure. Biochim. Biophys. Acta **651**: 245–271.
18. **Graf, T., A. von Kirchbach, and H. Beug.** 1981. Characterization of the hematopoietic target cells of AEV, MC29 and AMV avian leukemia viruses. Exp. Cell Res. **131**:331–343.
19. **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.
20. **Hayman, M. J., and H. Beug.** 1984. Identification of a form of the avian erythroblastosis virus *erbB* gene product at the cell surface. Nature (London) **309**:460–462.
21. **Hayman, M. J., G. M. Ramsay, K. Savin, G. Kitchener, T. Graf, and H. Beug.** 1983. Identification and characterization of the avian erythroblastosis virus *erbB* gene product as a membrane glycoprotein. Cell **32**:579–588.
22. **Hayman, M. J., B. Royer-Pokora, and T. Graf.** 1979. Defectiveness of avian erythroblastosis virus: synthesis of a 75K *gag*-related protein. Virology **92**:31–45.
23. **Kris, R. M., I. Lax, W. Gullick, M. D. Waterfield, A. Ullrich, M. Friedkin, 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.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from library of eukaryotic DNA. Cell **15**:687–701.
26. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
27. **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.
28. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
29. **Privalsky, M., and J. M. Bishop.** 1982. Proteins specified by avian erythroblastosis virus: coding region localization and identification of a previously undetected *erbB* polypeptide. Proc. Natl. Acad. Sci. USA **79**:3958–3962.
30. **Privalsky, M., R. Ralston, and J. M. Bishop.** 1984. The membrane glycoprotein encoded by the retroviral oncogene *v-erbB* is structurally related to tyrosine-specific protein kinase. Proc. Natl. Acad. Sci. USA **81**:704–707.
31. **Privalsky, M., 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.
32. **Schmidt, J. A., H. Beug, and M. J. Hayman.** 1985. Effects of inhibitors of glycoprotein processing on the synthesis and biological activity of the *erbB* oncogene. EMBO J. **4**:105–112.
33. **Sealy, L., G. Moscovici, C. Moscovici, and J. M. Bishop.** 1983. Site-specific mutagenesis of avian erythroblastosis virus: *v-erbA* is not required for transformation of fibroblasts. Virology **130**:179–194.
34. **Sealy, L., M. L. Privalsky, G. Moscovici, C. Moscovici, and J. M. Bishop.** 1983. Site-specific mutagenesis of avian erythroblastosis virus: *erbB* is required for oncogenicity. Virology **130**:155–178.
35. **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.
36. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2273.
37. **Smith, H. O., and M. L. Birnsteil.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3**:2387–2399.
38. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
39. **Twigg, A. J., and D. Sherratt.** 1980. *Trans*-complementable copy-number mutants of plasmid ColE1. Nature (London) **283**:216–218.
40. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. 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.
41. **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.
42. **Vennstrom, B., L. Fanshier, C. Moscovici, and J. M. Bishop.** 1980. Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic virus by transfection of chicken cells. J. Virol. **36**:575–585.
43. **Vogt, P. K.** 1969. Focus assay of Rous sarcoma virus. p. 198–211. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press, Inc., New York.
44. **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**:225–234.
45. **Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Ooi, and K. Toyoshima.** 1983. The *erbB* gene of avian erythroblastosis virus is a member of the *src* gene family. Cell **35**:71–78.