Mammalian Cell Transformation by a Murine Retrovirus Vector Containing the Avian Erythroblastsis Virus \textit{erbB} Gene

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A recombinant murine retrovirus vector containing the \textit{v-erbB} gene of avian erythroblastosis virus was constructed to investigate \textit{v-erbB} as a transforming gene for mammalian cells. A restriction fragment containing the \textit{v-erbB} sequences from a molecular clone of avian erythroblastosis virus were inserted into a Moloney murine leukemia virus vector. The construct, designated MuLV/\textit{erbB}, transformed NIH 3T3 cells at a high efficiency in the DNA transfection assay. Individual MuLV/\textit{erbB} transfectants grew in soft agar and were tumorigenic. The transfectants contained \textit{v-erbB} DNA sequences, expressed \textit{v-erbB}-specific transcripts, and synthesized \textit{v-erbB}-related glycoproteins. The majority of transfectants produced two major \textit{v-erbB} gene products of 58 and 66 kilodaltons. However, some transfectants produced much smaller \textit{v-erbB}-specific proteins. Tunicamycin experiments revealed that the size heterogeneity observed between different transfectants was not due to variations in glycoprotein processing, implying that, in some cases, alterations in the MuLV/\textit{erbB} genome occurred during the transfection process. These findings indicate that expression of the complete \textit{v-erbB} gene product is not required for transformation of NIH 3T3 cells. A transmissible murine \textit{v-erbB} (M-\textit{erbB}) virus was generated by infection of nonproducer transfectants with amphotropic murine leukemia virus. Transmission of the rescued M-\textit{erbB} virus was confirmed by DNA, RNA, and protein analyses. The introduction of a transforming \textit{v-erbB} gene into mammalian cells by virus infection provides a means of analyzing the mechanism by which this epidermal growth factor receptor-related gene alters the growth and differentiation of cells from various lineages.

Avian erythroblastosis virus (AEV) is a replication-defective retrovirus which induces erythroblastosis, sarcomas, and carcinomas in chickens (16, 19, 20) and transforms immature erythroid cells and fibroblasts in vitro (for a review, see reference 19). The genome of AEV contains two oncogenes, \textit{v-erbA} and \textit{v-erbB}, which code for a hybrid protein, p75\textit{ga\textit{a-erbA}}, and for an integral membrane glycoprotein, gp74\textit{ga\textit{a-erbB}}, respectively (9, 23, 30, 34, 35). Studies with deletion mutants in \textit{v-erbA} or \textit{v-erbB} have indicated that \textit{v-erbB} is the principal transforming gene of AEV (17, 40). Moreover, a new virus isolate, AEV-H, which contains only the \textit{v-erbB} gene, retains the capacity to transform avian fibroblasts and induces weak erythroblast transformation in vitro (27, 49). Although \textit{v-erbB} alone is sufficient to induce fibroblast transformation, \textit{v-erbA} is thought to cooperate with \textit{v-erbB} to enhance transformation and block differentiation of avian erythroid cells (27).

The gene products of the \textit{src} family of retroviral oncogenes and the receptors for several growth factors exhibit tyrosine-specific kinase activity (for a review, see reference 25). The \textit{v-erbB} gene is a member of the \textit{src} gene family, and its protein product shares amino acid homology with other members of this group, particularly within the domain responsible for tyrosine-specific protein kinase activity (36, 50). Recently, \textit{v-erbB} has been found to be the predicted \textit{v-erbB} gene sequence possesses extensive homology with that of the human epidermal growth factor (EGF) receptor (14). The \textit{v-erbB} glycoprotein represents a truncated form of the mammalian EGF receptor which has retained transmembrane and cytoplasmic domains but lacks the EGF-binding domain. The \textit{v-erbB} gene product is also slightly truncated at its cytoplasmic terminus but still retains the tyrosine kinase activity in this region of the molecule (18, 29).

Analysis of the range of mammalian target cells susceptible to transformation by \textit{v-erbB} has been impaired by difficulties in introducing this avian virus into mammalian cells. Transformation of mammalian cells with AEV, by virus infection or by transfection, occurs only at very low frequencies (11, 37). In addition, transformation of mammalian fibroblasts by these approaches does not lead to productive infection or the ability to rescue infectious virus containing \textit{v-erbB}. With the advent of recombinant DNA technology, it has become possible to construct mammalian retroviruses containing a variety of foreign genes. In the present report, we describe the construction and characterization of a \textit{v-erbB}-containing murine retrovirus which allows for more extensive investigation of the function of this EGF receptor-related gene as a transforming or growth-promoting gene in well-defined mammalian systems.

MATERIALS AND METHODS

Cells and viruses. Continuous mouse NIH 3T3 (26), normal rat kidney (15), mink epithelial MvLu (CCL64) (24), and 501 normal human fibroblast (3) cell lines have been described. Clonal simian sarcoma virus-transformed nonproducer normal rat kidney cell line (2) and Snyder-McDonough feline sarcoma virus-transformed nonproducer lines (7) were used. Murine leukemia virus (MuLV) pseudotypes of replication-defective viruses were obtained by superinfection of appropriate nonproducer cells with a clonal strain of the mouse type C helper virus, amphotropic MuLV (22, 38). The titers of defective transforming viruses, expressed as focus-forming units (FFU) per milliliter, were determined on NIH 3T3 cells as previously described (1). The 6C2 line, a chicken
erythroid cell line transformed by AEV (8), was kindly provided by C. Moscovici.

**Molecular cloning.** The Ch4A-A9 clone of Moloney-MuLV (Mo-MuLV) was obtained from H. Fan, University of California, Irvine (5), and subcloned in pBR322 devoid of BamHI and PstI sites. The AEV-11 plasmid was the kind gift of J. M. Bishop, University of California, San Francisco (46). Construction of the MuLV/erbB vector was performed as described in Fig. 1. Briefly, the Mu-MuLV DNA clone was digested with BamHI (New England Biolabs, Beverly, Mass.) and after purification, the 12.4-kilobase-pair (kb) BamHI fragment was religated with T4 DNA ligase (New England Biolabs). This DNA was then digested with PstI, and the 12.2-kbp purified fragment was religated with T4 DNA ligase. The permuted AEV provirus was purified from pBR313 sequences by digestion with EcoRI and religation of the 5.3-kbp fragment containing AEV sequences. This circular DNA molecule was then digested with PstI, and the resulting 3.5-kbp PstI fragment was inserted into the Mol-MuLV vector by using bacterial alkaline phosphatase (P-L Biochemicals, Inc., Milwaukee, Wis.) and T4 DNA ligase. The ligated MuLV/erbB molecules were then used to transform *Escherichia coli* C600 by the method of Cohen et al. (10).

**Transfection assays.** DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique (21), as modified by Wigler et al. (48). For transfection of cloned DNA, 40 μg of normal calf thymus DNA was coprecipitated as a carrier. NIH 3T3 cells, seeded 24 h earlier at 10^5 cells per 10-cm dish in Dulbecco modified Eagle medium supplemented with 10% calf serum, were changed for 18 to 20 h to the DNA precipitate. After medium change, cultures were maintained with twice-weekly changes of Dulbecco modified Eagle medium with 5% calf serum. Monolayers were screened 14 to 21 days after transfection for focus formation.

**Soft agar cloning and analysis of tumor induction.** Individual transformed foci that arose upon transfection with the MuLV/erbB construct were selected by the cloning cylinder technique and grown up to mass cultures. Cell suspensions from mass cultures were plated at 10-fold serial dilutions in semisolid agarose medium containing Dulbecco modified Eagle medium supplemented with 10% calf serum, were changed for 18 to 20 h in the DNA precipitate. After medium change, cultures were maintained with twice-weekly changes of Dulbecco modified Eagle medium with 5% calf serum. Visible colonies comprising ≥100 cells were scored at 14 days. For tumorigenicity testing, 10 NFR nude mice were inoculated subcutaneously with 10^5 cells of each clonal line. Tumor formation was monitored twice weekly.

**DNA blotting analysis.** DNA samples were digested with appropriate restriction endonucleases (New England Biolabs), electrophoresed in horizontal agarose gels, and transferred to nitrocellulose as described by Southern (42). Filters were hybridized under stringent conditions (50% formamide, 0.75 M NaCl, 0.075 M sodium citrate; 42°C) with nick-translated ^32^P-labeled DNA probes (10^6 cpm/ml) (39) for 18 h.

**RNA purification and analysis.** Total cellular RNA was purified by a modification of the guanidine hydrochloride extraction method (12) as described by Adams et al. (4). poly(A)-containing RNA was isolated by chromatography on oligot(dT)-cellulose columns. RNAs were fractionated in the presence of formaldehyde by agarose gel electrophoresis and transferred to nitrocellulose filters as described previously (32). Transcripts were analyzed by hybridization with ^32^P-labeled nick-translated v-erbB DNA (39) under the conditions described by Wahl et al. (47).

**Biopsynthetic labeling and immunoprecipitation.** The procedures for metabolic labeling and immunoprecipitation were as previously described (6). Subconfluent cultures containing about 5 × 10^6 cells per 10-cm petri dish were labeled for 3 h at 37°C with 4 ml of methionine-free Dulbecco modified Eagle medium containing 200 μCi of ^35^S)methionine (800 ci/nmol; New England Nuclear Corp., Boston, Mass.). To inhibit glycoprotein processing, cells were labeled in the presence of 1 μg of tunicamycin (Sigma Chemical Co., St. Louis, Mo.) per ml. Cell extracts were prepared by lysing with 1 ml of a buffer which contained 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 0.1 mM phenylmethylsulfonyl fluoride. Clarified extracts were divided into 200-μl portions and then incubated with 5 μl of rabbit anti-v-erbB serum (kindly provided by M. Hayman, Imperial Cancer Research Fund, London, England) or normal rabbit serum for 30 min at 4°C. Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.) and boiled for 3 min in sample buffer which contained 30% glycerol, 0.04% sodium dodecyl sulfate, 0.1 M Tris hydrochloride (pH 6.8), and 8% mercaptoethanol (reducing conditions). All samples were analyzed by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide.

**RESULTS**

**Construction and characterization of a murine retrovirus vector containing the v-erbB gene.** Our strategy for construction of a murine retrovirus vector containing v-erbB is summarized in Fig. 1. The MuLV vector was derived from a Mo-MuLV provirus subcloned in pBR322 (5) by the procedure shown in Fig. 1A. We deleted major portions encompassing *gag*, *pol*, and *env* gene sequences from the vector but retained the two Mo-MuLV long terminal repeats (LTRs) needed for initiation and polyadenylation of viral transcripts (28, 44). The deleted Mo-MuLV vector also retained the region containing packaging sequences, the *r*NA-binding site needed for efficient encapsidation of the viral genome, and sequences involved in the synthesis of plus-strand DNA (33).

The method used to obtain a colinear v-erbB gene fragment for insertion into the MuLV vector is shown in Fig. 1B. The vect-erbB gene used for insertion into the Mo-MuLV vector was present in a 3.5-kbp PstI fragment derived from a permutated molecular clone of AEV (46). Our strategy in making the construct was to attempt to express the v-erbB gene product by using the ATG codon present at the beginning of its open reading frame. This was facilitated by the fact that the PstI fragment containing the insert has no open reading frame upstream of the ATG codon located 6 codons into the v-erbB open reading frame (13). In addition, the *gag* gene sequence of the MuLV vector used was deleted of a small PstI-PstI fragment which contained the putative AUG for its initiation.

To establish whether the DNA construct, designated MuLV/erbB, was biologically active, we transfected NIH 3T3 cells with serial dilutions of the recombinant plasmid DNA. MuLV/erbB DNA had a transforming efficiency of 4 × 10^3 to 15 × 10^3 foci per μg of DNA insert (Table 1). Cells within the MuLV/erbB-transformed foci exhibited a dense, fusiform morphology (Fig. 2) similar to that of AEV-transformed chicken embryo fibroblasts. Cell lines established from individual MuLV/erbB transfectants were shown
TRANSFORMATION BY A MURINE erbB RETROVIRUS

A

B

FIG. 1. Construction of the MuLV/erbB plasmid. (A) Construction of the MuLV vector. Mo-MuLV integrated DNA subcloned in pBR322 was digested with BamHI enzyme. After purification of the 12.4-kbp BamHI fragment and religation, the DNA was digested with PstI, and the purified 12.2-kbp PstI fragment was religated. The resulting MuLV vector contained a unique PstI site. (B) Insertion of the v-erbB coding sequence into the MuLV vector. The permuted AEV provirus (44) was purified from pBR313 sequences by EcoRI digestion and religation of the 5.3-kbp EcoRI AEV fragment. The circular AEV provirus molecule was then digested with PstI, and the 3.5-kbp PstI fragment encompassing the colinear v-erbB region was purified and inserted into the PstI site of the MuLV vector DNA. Symbols: O, Mo-MuLV LTRs; □, avian LTRs; ■, the v-erbB gene.

to possess known properties of malignant cells, including the ability to grow in soft agar and to form tumors in vivo (Table 1).

To confirm that the v-erbB gene was responsible for induction of the transformed phenotype, DNAs from individual transfectants were analyzed for the presence of v-erbB sequences by Southern blot analysis. As shown in the restriction map of the MuLV/erbB construct in Fig. 3C, the v-erbB gene possessed two internal BamHI restriction sites and one EcoRI site. Digestion with BamHI generated a 0.55-kbp v-erbB-specific fragment spanning the middle portion of v-erbB, and double digestion with EcoRI and BamHI
produced an additional 0.55-kbp BamHI-EcoRI fragment encompassing the 3' region of v-erbB. High-molecular-weight DNAs from three individual transfectants, designated 422.14.1, 422.12.3, and 422.13.1, were digested with BamHI and hybridized with a v-erbB-specific probe composed of the two fragments described. The predicted 0.55-kbp fragment was observed in each case (Fig. 3A, lanes 2 through 4), while control NIH 3T3 DNA lacked this fragment (Fig. 3A, lane 1). These results confirmed that exogenously transferred v-erbB DNA was integrated in all MuLV/erbB transfectant DNAs analyzed. The detection of various larger-sized v-erbB-specific BamHI fragments downstream from the 0.55-kbp BamHI fragment in all three transfectant DNAs (Fig. 3A, lanes 2 through 4) suggested that multiple MuLV/erbB-specific gene copies had integrated in each.

To further analyze the molecular structure of the integrated MuLV/erbB DNA, transfectant DNAs were restricted with XbaI, which uniquely cleaves the MuLV/erbB construct within the Mo-MuLV LTRs (Fig. 3C), and hybridized with the 0.55-kbp v-erbB BamHI probe. Among the
products of 58 and 66 kDa (Fig. 4, lane 1A). Several other individual MuLV/erbB-induced transfectants produced v-erbB-specific products of identical sizes (data not shown). However, some transfectants synthesized v-erbB-related proteins of smaller sizes. The 422.12.3 and 422.13.1 transfectants synthesized three v-erbB-related gene products with molecular masses of 48, 50, and 52 kDa (Fig. 4, lanes 3A and 5A).

The size heterogeneity of v-erbB products observed among the different transfectants analyzed might be accounted for by differences in glycoprotein processing or could result from alterations which may have occurred in the genomic structure of the MuLV/erbB construct during the transfection process. To determine whether differences in glycosylation of the v-erbB gene products were the cause of the size variations, transfectants were exposed to tunicamycin, a specific inhibitor of glycosylation (43, 45), during the radiolabeling period. Tunicamycin treatment altered the mobility of v-erbB-related proteins in all of the transfectants analyzed (Fig. 4). The sizes of the major v-erbB translational products synthesized by the 422.14.1 transfectant were shifted from 58 and 66 kDa (Fig. 4, lane 1A) to 48, 62, and 64 kDa in the presence of tunicamycin (Fig. 4, lane 2A). These sizes were similar to those of v-erbB-specific proteins observed in avian cells transformed by the wild-type AEV genome in the presence of tunicamycin (Fig. 4, lane 8A).

The mobility of v-erbB gene products in the 422.12.3 and 422.13.1 transfectants, which expressed 48-, 50-, and 52-kDa v-erbB-related proteins, was reduced to 46 kDa in the presence of the inhibitor (Fig. 4, lanes 4A and 6A, respectively). These results substantiate the theory that the smaller v-erbB gene products in some MuLV/erbB transfectants resulted from deletions in the structure of the integrated MuLV/erbB DNA. Since transfectants that exhibited smaller v-erbB-related proteins still possessed malignant properties, these results further indicate that expression of the complete v-erbB gene product is not required for transformation of NIH 3T3 cells.

Generation of a transmissible murine retrovirus containing the avian v-erbB gene. Transfectant foci induced by the MuLV/erbB vector were nonproductive due to the defective nature of the viral construct. In an effort to generate infectious transforming virus, transformants were infected with amphotrophic MuLV, a replication-competent helper virus. The presence of rescued murine erbB (M-erbB) virus was assayed after 2 weeks by measuring the ability of culture supernatants to transmit the transformed phenotype to uninfected NIH 3T3 cells. Foci of transformed cells with a morphology similar to those obtained by transfection with MuLV/erbB DNA were induced by supernatant fluids from 422.14.1 and 422.15.1 transfectants infected with amphotropic MuLV. The titer of virus released in each case was very low and ranged from 5 to 20 FFU/ml. However, when individual foci induced by the transmitted virus were grown up to mass culture, they were found to release focus-forming virus at high titers, ranging from 10^5 to 10^7 FFU/ml, on NIH 3T3 cells. These findings suggested that infections, rescuable virus was generated as a rare event but was subsequently selected for upon further virus passage. The two transfectants which exhibited deletions in the structure of the integrated MuLV/erbB DNA construct (Fig. 3B, lanes 3 and 4) did not produce any transforming virus despite numerous attempts to detect virus release.

To examine the structure of the rescued virus genome, high-molecular-weight DNAs from three mass cultures independently transformed by M-erbB virus were digested with...
We also compared v-erbB-containing RNA transcripts present in the parental 422.14.1 transfectant with those present in one of the mass cultures of M-erbB virus-infected NIH 3T3 cells. The transfectant contained two major v-erbB-containing mRNA species of around 4.3 and 3.3 kilobases (kb), as well as some minor high-molecular-weight species (Fig. 7, lane 1). The sizes of the high-molecular-weight species were consistent with a complete transcript of the 8.0-kbp genomic MolLV/erbB DNA construct, while the smaller species were consistent with unspliced or spliced subgenomic transcripts (or both) that prematurely terminated at the internal AEV LTR polyadenylation site (Fig. 3C). Although precise comparisons between sizes of RNAs are difficult with gel analysis, the two major v-erbB RNAs in the M-erbB virus-infected cells were consistent with genomic transcripts of the two genomic M-erbB viral DNAs shown to be present in the same cells (Fig. 5, lane 2). It should be noted that neither of these transcripts corresponded to any of the v-erbB RNA species in the parental 422.14.1 transfectant. We conclude from all of these results that the internal avian LTR sequences in the original construct led to inefficient virus rescuability due to premature termination of the genomic transcript and that efficiently rescuable M-erbB virus was associated with viral genomic deletions.

M-erbB virus-infected NIH 3T3 cells were next analyzed for the expression of v-erbB-specific products by immunoprecipitation with anti-v-erbB serum. Two separately infected mass cultures were each shown to produce two v-erbB-specific protein products of 58 and 66 kDa (Fig. 8,

**FIG. 4.** Immunoprecipitation and electrophoretic analysis of v-erbB-specific translational products in MolLV/erbB-induced transfectants in the absence and presence of tunicamycin. Cell extracts are from [35S]methionine-labeled MolLV/erbB-induced transfectant 422.14.1 (lane 1), tunicamycin-treated 422.14.1 (lane 2), 422.12.3 (lane 3), tunicamycin-treated 422.12.3 (lane 4), 422.13.1 (lane 5), tunicamycin-treated 422.13.1 (lane 6), AEEV-transformed avian erythroblasts, 6C2 (lane 7), and tunicamycin-treated 6C2 (lane 8). Extracts were treated with rabbit anti-v-erbB serum (A lanes) or normal rabbit serum (B lanes) and subjected to immunoprecipitation analysis, as described in Materials and Methods. Positions of molecular weight markers (shown in thousands) are indicated to the left of the gel.

XbaI and hybridized with the 0.55-kbp v-erbB-specific BamHI fragment (Fig. 5, lanes 2 through 4). Two major v-erbB-specific XbaI fragments of 4.2 and 5.4 kbp were detected in all three samples. Smaller fragments were also observed in one culture at a lower intensity (Fig. 5, lane 2). Thus, all three virus-infected lines exhibited MolLV/erbB genomic deletions when compared to the original construct or the 422.14.1 transfectant, where XbaI cleavage generated 8-kbp v-erbB-specific genomic fragments (Fig. 3B, lane 2).

By double digestion with XbaI and EcoRI, which cleaves the original construct uniquely at the 3′ end of v-erbB (Fig. 3C), we showed that the proviral deletions in each case affected the 3′ end of the v-erbB coding region (Fig. 5, lanes 6 through 8).

To investigate the extent to which deletions could occur within the erbB/MolLV provirus, we analyzed a number of clonally infected cell lines. The majority showed integrated proviruses of either 5.4 or 4.2 kbp as observed in the infected mass cultures. However, cells infected with one clonal virus, designated M-erbB virus clone 1, contained a single integrated provirus of 2.8 kbp as determined by XbaI digestion (Fig. 6A, lane 3). Restriction analysis with different enzymes revealed the preservation of the 5′ PstI site, indicating the absence of any detectable deletion at the 5′ terminus of the v-erbB coding sequence. However, a major deletion beginning distal to the second BamHI site in the v-erbB open reading frame and encompassing essentially the entire 3′ region of the viral construct up to the 3′ MolLV LTR (Fig. 6A, lanes 6, 9, and 12) was detected by restriction analysis. This deletion not only removed avian LTRs from the original construct but also may have deleted as many as 500 nucleotides of v-erbB coding sequence from its 3′ terminus (Fig. 6B).

**FIG. 5.** Detection of v-erbB DNA sequences in M-erbB virus-infected NIH 3T3 cells. High-molecular-weight cell DNAs were digested with XbaI (lanes 1 through 4) and XbaI and EcoRI (lanes 5 through 8) and hybridized with a 32P-labeled, nick-translated v-erbB-specific probe. DNAs are from NIH 3T3 cells (lanes 1 and 5), M-erbB virus-infected culture 1 (lanes 2 and 6), M-erbB virus-infected culture 2 (lanes 3 and 7), and M-erbB virus-infected culture 3 (lanes 4 and 8).
FIG. 6. Restriction analysis of NIH 3T3 cells clonally infected with M-erbB virus clone 1. (A) DNAs from NIH 3T3 (lanes 1, 4, 7, and 10), MuLV/v-erbB-induced transfectant 422.14.1 (lanes 2, 5, 8, and 11) and NIH 3T3 cells clonally infected with M-erbB virus clone 1 (lanes 3, 7, 9, and 12) were cleaved with XbaI (lanes 1 through 3), BamHI (lanes 4 through 6), BamHI and EcoRI (lanes 7 through 9), or BamHI and PstI (lanes 10 through 12) and hybridized with the 0.55-kb BamHI-EcoRI fragment of v-erbB as a probe. (B) Restriction map of M-erbB virus clone 1 (bottom), shown in comparison to that of the MuLV/v-erbB construct (top). Symbols: \( \square \), Mo-MuLV LTRs; \( \Box \), avian LTRs; \( \blacksquare \) the v-erbB gene. Dashed lines indicate the extent of the deletion in M-erbB virus clone 1.

lanes 1A and 3A). The molecular sizes of these proteins shifted to 48, 62, and 64 kDa after tunicamycin treatment (Fig. 8, lanes 2A and 4A). The sizes of v-erbB-related translational products synthesized by M-erbB virus-infected NIH 3T3 cells closely resembled those observed in AEV-transformed avian erythroblasts (Fig. 8, lanes 5A and 6A) and the parental 422.14.1 transfectant (Fig. 4, lanes 1A and 2A). The cells infected with the smallest provirus, M-erbB clone 1, demonstrated a 56-kb v-erbB product, which migrated as a 48-kDa protein in tunicamycin-treated cells (data not shown). Thus, genomic deletions mapped to distal v-erbB coding sequences, in most cases, did not significantly alter the sizes of v-erbB products expressed by M-erbB virus-infected cells, although we identified one virus whose v-erbB transforming product was significantly decreased in size.

M-erbB virus transforming activity for different cell types. The mass population of M-erbB virus was able to induce foci on two continuous rodent fibroblast cell lines, NIH 3T3 and normal rat kidney, as well as on diploid, noncontinuous human fibroblasts (Table 2). In addition, this virus induced foci on the MvLu mink cell line, which is of epithelial origin. The M-erbB clone 1 virus showed similar relative titers on the same cell types. Thus, truncation of its carboxy terminus did not appreciably impair transforming ability for the cell lines analyzed. The focus-forming activity of M-erbB viruses was similar to that observed with an fms-containing retrovirus, Snyder-McDonough feline sarcoma virus, pseudotyped
with the same amphotropic helper virus (Table 2). The oncogene of Snyder-McDonough feline sarcoma virus like v-erbB, has been reported to possess homology with a growth factor receptor. The normal counterpart of v-fms is thought to be the macrophage colony-stimulating factor (M-CSF) receptor (41). In contrast, simian sarcoma virus whose sis oncogene encodes a PDGF-2-like growth factor polypeptide (33), effectively transformed each of the fibroblast-derived cell lines but induced no detectable alterations of MvLu epithelial cells. These findings support the concept that the range of target cells whose growth can be altered by oncogenes encoding growth factors is limited to those possessing receptors for such factors (31). While fibroblasts and epithelial cells analyzed possess EGF receptors, v-erbB can alter the growth of avian erythroblasts, which presumably lack this receptor (16, 19, 20). Similarly, v-fms transformed fibroblasts and epithelial cells, which do not appear to express detectable levels of the CSF-1 receptor (41). Thus, oncogene products derived from growth factor receptors appear to have a much wider range of target cells.

**DISCUSSION**

In the present studies, we describe the construction of a murine retrovirus which contains the v-erbB gene of AEV. By deletion of sequences required for replication by the replication-competent Mo-MuLV provirus and by insertion of a restriction fragment containing a linearized v-erbB gene, we generated an efficiently transforming DNA. Characterization of transfectants induced by the MuLV/erbB construct revealed the presence of v-erbB-specific DNA from the construct, as well as the expression of v-erbB-specific mRNAs and proteins. The v-erbB-specific proteins were glycosylated in mammalian cells, as is known to be the case for avian cells infected with AEV (23, 36). We did observe variant MuLV/erbB DNA transfectants whose v-erbB-related proteins were significantly smaller in both their glycosylated and nonglycosylated forms than those synthesized by the wild-type AEV genome. While we have not precisely mapped the deletions, these findings imply that

**TABLE 2. M-erbB virus transforming activity for different cell lines**

<table>
<thead>
<tr>
<th>Cell line (source)</th>
<th>Transforming activity (FFU/ml)</th>
<th>M-erbB</th>
<th>M-erbB clone 1</th>
<th>SM-FeSV</th>
<th>SSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3 (mouse fibroblast)</td>
<td>1 x 10^6</td>
<td>1 x 10^5</td>
<td>1 x 10^5</td>
<td>1 x 10^5</td>
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<tr>
<td>NRK (rat fibroblast)</td>
<td>2 x 10^4</td>
<td>1 x 10^4</td>
<td>3 x 10^4</td>
<td>5 x 10^4</td>
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<tr>
<td>S01 (human fibroblast)</td>
<td>2 x 10^5</td>
<td>5 x 10^5</td>
<td>NT^a</td>
<td>1 x 10^5</td>
<td>1 x 10^5</td>
</tr>
<tr>
<td>MvLu (mink epithelial cell)</td>
<td>2 x 10^2</td>
<td>4 x 10^2</td>
<td>5 x 10^2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

^a The titer of FFU per milliliter for virus stock was determined in the NIH 3T3 focus assay and adjusted to 10^6 FFU/ml. These standardized virus stocks were then pseudotyped with amphotropic MuLV for determining FFU per milliliter on each of the other cell lines.

^b SM-FeSV, Snyder-McDonough feline sarcoma virus.

^c SSV, Simian sarcoma virus.

^d NT, Not tested.
v-erbB gene products substantially smaller than wild type can be transforming, at least for target cells of fibroblastic origin.

It was possible to generate a transmissible murine retrovirus by helper virus infection of certain MuLV/\(v\)-erbB transfectants. The ability to rescue transforming MuLV/\(v\)-erbB was restricted to those transfectants shown to contain full-length integrated MuLV/\(v\)-erbB DNA. Thus, DNA rearrangements or deletions in the MuLV/\(v\)-erbB constructs present in the variant transfectants presumably account for their lack of virus rescuability. Although \(M\)-erbB viruses were generated from transfectants containing the entire MuLV/\(v\)-erbB construct, such virus was initially rescued only at a very low efficiency. However, after a single cycle of selection, progeny \(M\)-erbB virus demonstrated a high titer associated with MuLV/\(v\)-erbB genomic deletions. The two major proviruses were about 5.0 and 6.1 kbp, taking into account the 0.7-kbp size of an Mo-MuLV LTR and the 4.3- and 5.4-kbp sizes of the integrated genomes after restriction with an enzyme that cuts within each LTR once.

Several lines of evidence argue that selection for efficiently rescued \(M\)-erbB virus required deletion of internal avian LTR sequences present in the original construct. Infectious \(M\)-erbB viruses preserved the \(v\)-erbB coding sequences required for transformation and expressed \(v\)-erbB-specific protein products whose molecular sizes closely resembled those of proteins encoded by AEV. Thus, the 2.0- and 3.0-kbp deletions in the two major viral genomic species generated must map to the 3' half of the original MuLV/\(v\)-erbB construct which contained the avian LTR sequences. In one clonal virus, we directly mapped the 3' deletion of \(v\)-erbB to encompass sequences distal to the second BamHI site in the \(v\)-erbB open reading frame as well as the entire avian LTR sequence and contiguous MuLV sequences up to the 3' LTR.

It has been reported that polyadenylation signals introduced within a retroviral DNA construct may lead to premature termination of a complete viral transcript and dramatically impair its rescuability as an infectious virus (44). Since avian LTRs contain such polyadenylation signals, it is likely that the presence of internal LTRs led to a MuLV/\(v\)-erbB DNA transcript containing the \(U_3\) portion of the Mo-MuLV LTR and terminating within the \(U_2\) portion of the avian LTR. The observed major 4.3- and 3.3-kb \(v\)-erbB-related mRNA species in the parental MuLV/\(v\)-erbB DNA transfectants are consistent with such subgenomic transcripts. Reverse transcription of these subgenomic transcripts conceivably could generate a provirus with hybrid LTRs containing avian \(U_2\) and Mo-MuLV \(U_3\) sequences. However, the \(v\)-erbB transcripts detected in \(M\)-erbB virus-infected cultures had different sizes from those detected in the parental transfectant. These findings imply that the major 4.3- and 3.3-kb RNA species in the transfectant did not give rise to new viral genomic RNAs of those sizes. We also detected two major species of genomic viral RNAs in mass culture of \(M\)-erbB virus-infected cells. Since \(Xh\)al cuts once in the \(U_2\) region of the Mo-MuLV LTR and does not cut within the avian LTR, these results imply that the genomic viral DNA detected in mass cultures of virus-infected cells must contain the \(U_2\) region of Mo-MuLV LTR, as well as its \(U_3\) region. All of these findings argue that viruses with hybrid LTRs may be impaired in provirus formation, circularization, or specific cleavage at the LTR-LTR junction necessary for provirus integration into the host cell genome.

Analysis of the host range of \(M\)-erbB viruses for various cell lines in culture revealed them to be capable of altering growth properties of epithelial as well as fibroblastic cell types. One \(M\)-erbB virus deletion mutant with a significant truncation at the carboxy terminus of the \(v\)-erbB product was shown to be capable of transforming these same cell types with similar efficiency. In a previous report, a naturally occurring AEV mutant, \(td\), was shown to exhibit premature termination in a region similar to that observed for our \(M\)-erbB virus clone 1 (49). This AEV mutant was unable to induce avian erythroleukosis, although it induced sarcomas. It will be of interest to analyze target cells for in vivo transformation by the \(M\)-erbB virus clone 1 in a mammalian system.

The \(sis\) transforming gene encodes a PDGF-like growth factor with a stringent specificity for target cells which possess PDGF receptors and, therefore, does not transform epithelial cells (31). The generation of \(v\)-erbB-containing retroviruses capable of infecting a wide variety of mammalian target cells should be useful in characterizing the mechanisms by which a gene coding for the activated form of a growth factor receptor may alter the growth and differentiation of such cells. This will be of particular interest with respect to cell types that normally lack EGF receptors. If cell types not responsive to this activated oncogene can be detected, it is possible that such cells may be deficient in pathways of growth factor-mediated proliferation distal to receptor activation.

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