Molecular Characterization of Three *erbB* Transducing Viruses Generated during Avian Leukosis Virus-Induced Erythroleukemia: Extensive Internal Deletion Near the Kinase Domain Activates the Fibrosarcoma- and Hemangioma-Inducing Potentials of *erbB*

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Three new *erbB* transducing viruses generated during avian leukosis virus-induced erythroblastosis have been cloned and sequenced, and their transforming abilities have been analyzed. Provirus 9134 E1 expresses an amino-terminally truncated *erbB* product that is analogous to the proviral insertionally activated *c-erbB gag-erbB* fusion product. This virus efficiently induces erythroblastosis, but does not transform fibroblasts in vitro or induce sarcomas in vivo. In contrast, virus 9134 S3 expresses an *erbB* product identical to the *erbB* product of 9134 E1, with the exception of a large internal deletion located between the kinase domain and the putative autophosphorylation site, P1. Interestingly, this virus is no longer capable of inducing erythroblastosis, but can induce both fibrosarcomas and hemangiomas in vivo. Provirus 9134 F3 has sustained an approximately 23-amino-acid carboxy-terminal truncation and is capable of inducing both erythroblastosis and sarcomagenesis. This virus expresses an *erbB* product with the shortest carboxy-terminal truncation sufficient to reveal the sarcomagenic potential of this protein. The distinct transforming properties of these viruses indicate that different structural domains of the *erbB* product confer distinct disease specificities.

Avian leukosis virus (ALV) can induce erythroleukemia by proviral insertional mutagenesis of the proto-oncogene *c-erbB* (10, 20, 26). The avian *c-erbB* gene encodes a growth factor receptor, and proviral integration results in separation of the ligand-binding domain of this receptor from its transmembrane and cytoplasmic regulatory domains (10, 20, 22, 26). Two alternate transcripts are expressed from the proviral insertionally activated *c-erbB* locus (IA *c-erbB*) and are translated to protein products that are differentially processed (N. J. Maihle, M. Raines, T. Flickinger, and H.-J. Kung, submitted for publication). The IA *c-erbB* products appear to be nearly identical in their sequences to the viral *erbB* products of the avian erythroblastosis viruses (AEVs) AEV-ES4 and AEV-H (7, 35). However, the viral *erbB* products of both these viruses have sustained multiple point mutations, carboxy-terminal deletions, and truncations relative to the native and insertionally activated *c-erbB* products (7, 34). Since proviral insertional activation of *c-erbB* results in erythroblastosis but not sarcomagenesis (10), these changes in the coding regions of the viral *erbB* products presumably confer the fibroblast transformation potential associated with their expression (30, 33, 34).

In addition, a number of novel, acutely transforming *erbB* viruses have recently been described (11, 20, 27, 32); some of these viruses can transform only hematopoietic cells (4, 20), whereas others, which carry C-terminally altered *c-erbB* products, exhibit distinct disease potentials (11, 32). The precise nature of the alterations in these *erbB* coding sequences, however, has not yet been defined. Furthermore,

these earlier studies were performed with viruses which were uncloned or only partially purified. In this report we describe the generation and characterization of three new *erbB* transducing viruses released from a single ALV-induced erythroblastosis sample. We have applied both biological and molecular approaches to obtain purified viral stocks, in order to unambiguously demonstrate the disease specificity associated with these newly isolated viruses, and have used these cloned viruses to precisely define the structural alterations associated with changes in transformation potential. Our structural analysis reveals that at least two of these viruses have not sustained point mutations in the *erbB* coding region, probably because of their limited passage history. Two of these viruses were selected for on the basis of their ability to transform fibroblasts in vitro and to induce sarcomagenesis in vivo. By studying these viruses, we have determined that specific alterations in the carboxy-terminal sequences of the *erbB* coding region are responsible for the alterations in the disease spectrum associated with infection by these viruses. This observation is particularly provocative since, by analogy to the human epidermal growth factor receptor, three potential tyrosine autophosphorylation sites, P3, P2, and P1, exist in *c-erbB* within 100 amino acids of the carboxy terminus (9). Although no evidence regarding the nature of the phosphate acceptor sites in the avian *c-erbB* product is currently available, our data suggest that perturbation of these putative regulatory sequences may influence disease specificity.

MATERIALS AND METHODS

Viruses, chickens, and induction of neoplasms. ALV-induced erythroblastosis was induced by injecting 10^2 to 10^3 infectious units of the RAV-1 strain of ALV into the peritoneums of 1-day-old line 15₁ chickens. Virus stocks derived

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FIG. 1. Purification scheme and passage history of 9134 *erbB* transducing viruses. The original viral extract, 9134 E1, was a liver homogenate derived from an ALV (RAV-1)-infected bird ($15_1 \times 151_4$). This viral extract was used both to molecularly clone provirus E1 (top) and to derive secondary leukemias (E2) which were used to obtain the viral extracts (liver homogenates) used in all subsequent experiments. Viruses capable of transforming fibroblasts were selected for in vivo (w.w.) by their ability to induce sarcomagenesis and were subsequently biologically (S3a, clones 1 to 8; S3b, clones 1 to 6) and molecularly (S3 provirus) cloned from soft-agar colonies. Fibroblast transformation was also selected for in vitro by infecting CEF and monitoring colony formation in soft agar. Selection of soft-agar colonies was used to biologically clone both the S3 and F3 viruses. Biologically cloned viruses (S3 and F3) or molecularly cloned and rescued E1 virus were used for testing disease potential and analysis of *erbB* expression products. Abbreviations: E, erythroblastosis samples; S, fibrosarcomas; F, infected CEF; i.v., intravenous; w.w., wing web.

from ALV-induced erythroleukemia samples or sarcomas were prepared by filtering tissue homogenates (10% [wt/vol] in 10% tryptose broth) or were plasma samples. The purification scheme used for isolation of the viruses described in this report is illustrated in Fig. 1. Briefly, we tested the viral extracts for their ability to induce erythroblastosis by injecting 0.1 ml intravenously into 13-day-old embryos (SPAFAS, Inc., Norwich, Conn.) The ability to induce sarcomas was determined by subcutaneous injections into the wing web. Clone-purified virus stocks were similarly tested by injection into 1-day-old line 15₁ × 15₄ or line 0 chickens. Birds were monitored for tumorigenesis, and sample collection was as previously described (10, 26). All neoplasms were diagnosed on the basis of gross morphology as well as histology.

Cells, cell culture, and chicken embryo fibroblast transformation. A stably transformed quail fibroblast line, QT6 (21), and line 0 chicken embryo fibroblasts (CEF) were obtained from the Regional Poultry Research Laboratory, East Lansing, Mich., and maintained in Dulbecco modified Eagle medium-high glucose (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 5% fetal bovine serum, 1% chicken serum, 10 μg of penicillin per ml, 0.5 μg of streptomycin per ml, and 1 μg of amphotericin B per ml. Cells were infected with virus by seeding in 60-mm dishes; within 5 h after seeding the medium was aspirated off and replaced with 1.0 ml of virus-containing medium. Polybrene (Sigma Chemical Co., St. Louis, Mo.) was added to 2 $\mu\text{g}/\text{ml}$ to enhance infection. Cells were incubated for 2 to 3 h before fresh medium was added and were passaged for 2 weeks prior to virus collection. Virus stocks were collected 8 to 12 h after medium was changed and were stored at -70°C . The presence of *erbB*-containing viruses was monitored by extracting viral RNA, blotting it onto nitrocellulose, and hybridizing it with *erbB* and virus-specific probes (29).

Soft-agar colonies from virus-infected cells were selected by seeding dilutions of cells in growth medium containing 0.3% Bacto-Agar (soft agar; Difco Laboratories, Detroit, Mich.). A 2.0-ml suspension of cells was placed onto a 4.0-ml base containing growth medium supplemented with 0.65% Bacto-Agar. After 1 week the cultures were fed with

an additional 20 ml of soft agar, and 2.0-ml additions of soft agar were made at 3- to 4-day intervals. After 2 to 3 weeks, discrete colonies were removed with a drawn pipette and seeded into growth medium in 35-mm petri dishes. Uninfected CEF were added as cells began to senesce.

QT6 cells were transfected with 20 μ g of total plasmid DNA by using the calcium phosphate technique (12). Approximately 15 μ g of test DNA was cotransfected with 25 μ g of pSV2neo (molar ratio, 3:1) (31). Cells were split 1:4 18 h prior to transfection, and the medium was changed 4 to 8 h before addition of DNA. Medium containing 1 mg of G418 (GIBCO) per ml was added 36 h posttransfection. After 2 weeks in selective medium, the remaining cells were trypsinized and replated or harvested for further analysis.

Northern (RNA) transfer and S1 nuclease analysis. Total cellular RNA was extracted from frozen tissue or cultured fibroblasts by the guanidinium isothiocyanate-hot phenol method (19). Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (1), electrophoresed in a 1% formaldehyde-agarose gel (19), and electrotransferred to Gene-Screen (New England Nuclear Corp., Boston, Mass.) as described by Radinsky et al. (25). Hybridization probes were synthesized by nick translation of gel-purified fragments with ³²P-labeled nucleotides (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.).

For S1 nuclease analysis, gel-purified fragments with appropriate restriction enzyme sites (see Fig. 3) were radio-labeled at their 5' ends by dephosphorylation with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and treatment with polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as outlined by Maniatis et al. (19). Fragments labeled at their 3' ends were synthesized by treatment with Klenow fragment in the presence of ^{32}P -deoxynucleoside triphosphates. Probe and poly(A)⁺ RNA were used at 0.02 to 1.0 pmol and 1 mg per hybridization reaction, respectively. Optimal hybridization temperatures were determined empirically, and all hybridizations took place overnight in 10 mM piperazine-*N-N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA–

80% formamide. Unhybridized probe was digested with 200 U of S1 nuclease (Sigma), and the resistant fragments were precipitated and analyzed by polyacrylamide gel electrophoresis (5% polyacrylamide, 7 M urea). End-labeled *Hinf*I-*Hae*III-digested ϕ X174 DNA samples were used as molecular weight standards.

Molecular cloning and nucleotide sequence analysis. Transducing proviruses were cloned from total cellular DNA derived from leukemic liver samples or infected fibroblasts, as described in the accompanying report (27). Appropriate fragments were subcloned into M13mp18 or M13mp19, and their nucleotide sequences were determined by using the dideoxy chain termination method of Sanger et al. (28). DNA sequences were confirmed by generating overlapping clones and by determining the sequence of both strands.

In vivo labeling, immunoprecipitation, and SDS-PAGE analysis. Approximately 0.5×10^6 to 1×10^7 uninfected or virally infected CEF or stably transformed QT6 cells were preincubated for 30 min at 37°C in Dulbecco modified Eagle medium (methionine-free) (GIBCO), and then 100 μ Ci of [35 S]methionine (800 Ci/mmol; Amersham) was added. The cells were incubated with the radiolabel for 90 min and then washed twice with phosphate-buffered saline at 4°C. Immunoprecipitation was as described previously (Maihle et al., submitted). Briefly, cells were scraped from the plate and lysed in 2.5% sodium dodecyl sulfate–0.1% Nonidet P-40–1% deoxycholate for 3 min at 100°C. Lysates were diluted 10-fold (50 mM Tris hydrochloride [pH 7.4], 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100), and 1 μ l of anti-*erbB* (Maihle et al., submitted) was added per sample. Protein A-Sepharose (30- μ l suspension; Pharmacia, Inc., Piscataway, N.J.) was added to each sample prior to incubation at 4°C for 12 h. Immunoprecipitates were harvested by centrifugation and sequential washing prior to analysis by 0.1% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide). Gels were fluorographed with En 3 Hance (New England Nuclear) prior to autoradiography at –70°C.

RESULTS

Identification of *erbB* transducing viruses from 9134-infected leukemic tissue samples. In this report, we describe studies on acutely transforming viruses released from birds infected with the nonacute virus ALV (RAV-1 strain). These new isolates are particularly interesting since with time some of them have acquired sarcomagenic potential—a feature that is not characteristic of most newly released *c-erbB* transducing viruses. In the ensuing sections, we describe in detail the derivation of these isolates and their passage history. This information is summarized in diagrammatic form in Fig. 1.

Plasma and liver homogenates from the original 9134 leukemic tissue samples (E1, induced by RAV-1) were shown by Southern analysis to contain transducing proviruses (31) and were injected into chicken embryos. Four of nine injected birds developed erythroblastosis rapidly, with latencies between 7 and 15 days, confirming the presence of acute viruses. RNA from two secondary leukemic tissue samples (Fig. 2; E2a and E2b) was compared with RNA isolated from birds infected with the original virus (E1). Two major *erbB*-related RNAs, 4.0 and 4.5 kilobases (kb) in size, persist in all three samples. The 4.0-kb species is consistently expressed at a higher level than the 4.5-kb species. We presume that in each case the larger viral component represents genomic RNA and the smaller one represents the subgenomic mRNA. The provirus responsible for generation

FIG. 2. Northern analysis of *erbB*-related RNAs in sarcomas induced with 9134 viral extracts. Poly(A) $^+$ RNA was isolated from the original 9134 erythroleukemic sample (E1), secondary erythroleukemias (E2 and E2b), fibrosarcomas (S3a and S3b), or transformed fibroblasts (F3, F3Sa, and F3Sb) and fractionated on 1% formaldehyde-agarose gels. Following transfer to filters, these samples were hybridized to an *erbB*-specific probe (1.7-kb *Apal*-to-*SacI* fragment derived from 1A *c-erbB* cDNA clone [22]). The positions of the genomic (4.5-kb) and subgenomic (4.0-kb) 9134 E1 viral transcripts are indicated.

of the 4.5-kb genomic and 4.0-kb subgenomic mRNAs has been cloned (27), and its characteristics are discussed below. These studies identify the 4.5-kb RNA species as the leukemogenic viral component.

We then asked whether these newly released *erbB* viruses possess the sarcomagenic potential exhibited by the other *erbB* transducing viruses such as AEV-ES4 and AEV-H (30, 33, 34). Leukemic E2a extracts were either injected into the wing webs of chickens or applied to CEF in vitro. Wing web injection resulted in the development of sarcomas in 6 of the 14 birds inoculated (Fig. 1). Likewise, transformed foci (F3) were readily identified in CEF infected with the 9134 E2 extracts (Fig. 1). Culture medium from F3 foci also induced acute fibrosarcomas when subsequently injected into birds. These studies clearly reveal the presence of sarcomagenic viruses in the original 9134 E2 extracts. However, it was not clear whether sarcomagenesis and fibroblast transformation were caused by the same viral component responsible for the leukemia. We therefore analyzed the RNA patterns of the sarcoma samples and transformed foci (Fig. 2). Interestingly, the predominant viral components in these samples were distinct from those in the leukemic tissue samples. The sibling S3a and S3b tumors shared viral components of a common size when probed with *erbB*, whereas the F3 series expressed yet another size class of *erbB*-related transcripts. The most straightforward interpretation of these data is that the original 9134 extracts contain a mixture of viruses, some of which exhibit leukemogenic potential, while others express sarcomagenic potential. The critical question addressed below is whether the *erbB* structures in these viruses are different.

Structure of *erbB* in the newly released transducing viruses. To probe the structure of the *c-erbB* sequences present in these viruses, we used S1 nuclease protection analysis. We have focused on the C-terminal domain of the *erbB* gene, since this region has previously been implicated in the

FIG. 3. S1 nuclease protection analysis of *erbB* sequences in sarcomas and transformed fibroblasts arising from infection with 9134 viral extracts. RNA was isolated from erythroleukemic samples (E1), fibrosarcomas (s), or infected fibroblasts (F). Poly(A)⁺ RNA (1 µg) or total RNA (30 µg) samples were hybridized to (A) a 1,874-nucleotide *NcoI*-to-*BglII* fragment labeled at its 3' end (probe A), (B) a 726-nucleotide *BamHI* fragment labeled at its 5' end (probe B), and (C and D) a 726-nucleotide *BamHI* fragment labeled at its 3' end (probe C). Samples were treated with S1 nuclease, precipitated, and run on a 6% denaturing polyacrylamide gel. Sizes of protected fragments were determined relative to the mobility of ³²P-labeled *HinfI*-*HaeIII*-digested ϕ X174 DNA. Undigested probe is also shown (lanes probe). RNA from AEV-R-infected (leukemic) chickens was used as control (lanes AEV-R). (E) Diagram of proviral insertionally activated *c-erbB* cDNA clone, indicating restriction sites and origin of the probes used to detect deletions in *erbB* coding sequences. Symbols: *, locations of the radioactive labels; ▨, *gag* and 5' viral sequences; ■, transmembrane domain; ▤, protein kinase domain (PK); ▤, untranslated sequence (3UT); pA1, pA2, first and second polyadenylation signals, respectively; P3, P2, P1, tyrosine residues homologous to the autophosphorylation sites present in the human epidermal growth factor receptor (6). Restriction enzyme sites: B, *BamHI*; N, *NcoI*.

control of disease potential (11, 32). We have purified the S3 and F3 viral components by soft-agar colony selection as discussed below.

Three S1 probes were developed (Fig. 3E). Probe A was labeled at the *NcoI* site present in the 3' coding region of *c-erbB* and extends to the *BglII* site in the 3' untranslated region. This probe was designed to examine the intactness of the *c-erbB* carboxy-terminal sequences. There are two adjacent *NcoI* sites 57 nucleotides apart. Owing to incomplete digestion by *NcoI*, both fragments were labeled. As a result, for a given RNA species a pair of bands differing by 57 nucleotides is predicted. The *NcoI* sites are situated 335 and 278 base pairs 5' of the *c-erbB* termination codon and therefore indicated the minimum size that should be protected by a viral RNA encoding a C-terminally intact *c-erbB* product. A fragment larger than this is protected in the

leukemic samples E1 and E2 and the sarcoma samples S3a and S3b, but not in the soft-agar colony F3 cl.2 or the sarcoma nodules (F3Sa and F3Sb) derived following F3 infection (Fig. 3). The F3-infected samples protected fragments about 70 nucleotides shorter than expected for an *erbB* gene expressing a C-terminally intact *erbB* product. This suggests that the F3 viral species has sustained a carboxy-terminal truncation of approximately 23 amino acids, including the putative autophosphorylation site P1.

To probe the integrity of the internal structure of the *c-erbB* sequences, we used a 726-base-pair *BamHI* fragment labeled at the 5' end of the antisense strand (Fig. 3, probe B). Only fully protected fragments are present in samples E1, E2, F3 cl.2, and F3Sa, indicating the intactness of this domain in these viral RNAs (Fig. 3B). Samples S3a and S3b, on the other hand, appeared to carry a deletion (or rear-

FIG. 4. Structural features and sequence analysis of the 9134 E1 and 9134 S3 proviruses. These proviruses were cloned as described in the accompanying report (27). The extent of the viral and *c-erbB*-related sequences was determined by restriction enzyme mapping and Southern analysis. The 5' and 3' viral-*erbB* junction sequences are described by Raines et al. (27). The sequences defining the boundaries of the 139-amino-acid deletion in 9134 S3 are shown in panel B, below the diagram. The positions of two potential splice acceptors (SA), a cryptic splice donor (SD), and the first and second polyadenylation signals of *c-erbB* (pA1 and pA2) are also indicated. Abbreviations: B, *Bam*HI sites; LTR, long terminal repeat. Symbols: □, viral sequences; ▨, *erbB* intron sequences; ■, *erbB* coding sequences; ▩, 3' untranslated region of *erbB*.

rangement), since they protect a smaller band of 110 nucleotides. The size of this protected fragment allowed us to define a preliminary 3' boundary of the deletion point, 110 nucleotides upstream of the 3' *Bam*HI site.

We also labeled the same *Bam*HI fragment at its 3' end (Fig. 3, probe C) to define the 5' boundary of the deletion in the viral S3 samples. Fully protected fragments are again seen in samples E1, E2, and the F3 series, confirming the above mapping analysis (Fig. 3C). Liver samples infected with AEV-R, a virus known to carry an internal deletion in this region, were used as a positive control; a 436-nucleotide band was protected, in complete agreement with the reported site of the internal deletion in AEV-R (Fig. 3C). Curiously, S3a and S3b samples displayed two shorter bands, of 91 and 190 nucleotides in length. This raised the possibility that the S3a and S3b samples were infected with two acutely transforming *erbB* viruses. Immunoprecipitation of *erbB* protein products from infected CEF further indicated that a mixture of transducing *erbB* viruses was present (data not shown). To test this possibility, we infected fibroblasts with extracts from the S3a and S3b sarcomas and isolated these viruses by soft-agar cloning. Individual clones were picked and analyzed for *erbB* expression. When RNA from the individual clones was hybridized with probe C (Fig. 3D), two viral components were separable, and the one responsible for transformation is likely to be the species represented by the 190-nucleotide fragment, since this component was the only one expressed in all of the soft-agar colonies.

On the basis of the S1 analysis described above, a tentative structure of the carboxy-terminal domain of the transducing *erbB* products was established (see Fig. 6). The

leukemogenic E1 and E2 *erbB* protein products were predicted to be C-terminally intact. In contrast, the sarcomagenic *erbB* products are structurally altered in this region. The viral S3 *erbB* product contains an internal deletion of approximately 417 nucleotides, whereas the viral F3 *erbB* gene has sustained a short (70-nucleotide) carboxy-terminal truncation.

Molecular cloning of *erbB* transducing proviruses. To obtain precise structural information and to reveal possible point mutations accumulated in the *erbB* genes of these viruses, we proceeded to clone the proviruses for 9134 E1 and 9134 S3. The E1 provirus was cloned directly from a tumor sample, whereas the S3 provirus was derived from infected fibroblasts. The cloning and overall structure of the 9134 E1 and 9134 S3 proviruses are described in the accompanying report (27). In this communication, we have focused on defining the transformation potential of these viruses and on characterizing their *erbB* coding sequences.

The *erbB* gene of the 9134 E1 provirus was sequenced in its entirety and found to be completely identical to the proviral insertional activated *c-erbB* (1A *c-erbB*) gene without a single base change (Fig. 4). This virus retains both the splice donor and splice acceptor sites necessary to generate a *gag-erbB* fusion product. However, unlike the transcripts from the insertional activated *c-erbB* locus, the *gag-env-erbB* fusion product cannot be made owing to extensive deletions in the *env* sequences of the viral genome (Fig. 4).

Sequence analysis of the 9134 S3 *erbB* gene confirms the S1 mapping data; there is an in-frame deletion of 417 nucleotides (Fig. 4). No other nucleotide differences were observed. The 5' boundary of the deletion is 16 amino acids

FIG. 5. Immunoprecipitation analysis of *v-erbB* protein products. Quail fibroblasts (QT6) transfected with 9134 E1 and uninfected CEF, or CEF infected with 9134 S3, 9134 F3, AEV-R, or AEV-H were metabolically labeled for 90 min with [35 S]methionine, and cell lysates were immunoprecipitated with an anti-*erbB* serum as described previously (Maihle et al., submitted) and analyzed by 0.1% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide). The apparent molecular sizes of the viral *erbB* products are indicated in kilodaltons.

away from the 3' end of the kinase domain. This deletion removes over 50% of the sequence in the carboxy-terminal domain, including the putative P3 autophosphorylation site.

Immunoprecipitation of the viral *erbB* products. To verify the predicted structure of the viral *erbB* genes, we have analyzed the viral *erbB* protein products of the cloned viral isolates. QT6 cells and CEF were transfected with the molecularly cloned 9134 E1 provirus or infected with soft-agar-cloned viral isolates (9134 S3 or 9134 F3). When transfected, pSV2neo was cotransduced as a selectable marker, and stable transformants were isolated by culturing in the presence of G418. These cells were metabolically labeled with [35 S]methionine, and cell extracts were immunoprecipitated with an anti-*erbB* serum directed against the kinase domain (Maihle et al., submitted). In 9134 E1-infected cells, two prominent *erbB* products, gp 82 and gp 84 , are expressed (Fig. 5). These products comigrate with the products of the *gag-erbB* transcript produced in ALV-induced erythroblastosis (Maihle et al., submitted). This observation substantiates the prediction that the *v-erbB* product of 9134 E1 is C-terminally intact. The *v-erbB* proteins produced by the S3 provirus, in contrast, have M_r 62,000 and 64,000 only, consistent with the presence of an internal deletion of 139 amino acids. The F3 provirus codes for two polypeptides, of M_r 81,000 and 83,000, which migrate slightly faster than the corresponding products of 9134 E1, but more slowly than the viral *erbB* products of AEV-H, which has previously been demonstrated to have sustained a 34-amino-acid carboxy-terminal truncation (Fig. 5). These data lend further support to the postulated 23-amino-acid truncation at the carboxy terminus of the viral *erbB* product of 9134 F3.

Oncogenic potential of the *erbB* transducing viruses. The above data strongly suggested that the leukemia- and sarco-

TABLE 1. Disease potential of clone-purified virus stocks

Virus	Route of injection ^a	No. of chickens diseased ^b /no. injected		
		Ery	Sarc	Hem
9134 E1	i.p.	9/9	0/9	0/9
	w.w.	1/5	0/5	0/5
9134 S3	i.p.	0/10	6/10	10/10
	w.w.	0/5	5/5	3/5
9134 F3	i.p.	9/10	10/10	0/10
	w.w.	5/5	5/5	0/5

^a i.p., Intraperitoneal; w.w., wing web.

^b Ery, Erythroblastosis; Sarc, fibrosarcoma; Hem, hemangioma.

ma-inducing viruses differed not only in their viral RNA structures but also in their *erbB* sequence contents. However, to establish the oncogenic potential of these viruses individually, it was important to demonstrate that the viral clones from which the *v-erbB* structures were derived were actually responsible for the original neoplasms observed. To this end, viruses were rescued from the transfected and/or infected cells described above in the presence of RAV-1 helper virus (Fig. 1). They were injected into the wing webs or peritoneums of newly hatched chickens. Consistent with transformation by acute transforming viruses, apparent diseases developed within a short period (3 to 6 weeks) with all viral stocks. However, the disease spectrum of the individual viruses varied significantly (Table 1). The 9134 E1 virus produced only erythroblastosis, with no indication of sarcoma development, even when injected into the wing web. The 9134 S3 virus, in contrast, was potentially sarcomagenic, irrespective of the injection route. Wing web injection of this virus resulted in the rapid development of sarcomas at the site of injection. Intraperitoneal injection led to disseminating fibrosarcomas of the liver and omentum adjacent to the site of injection. The development of these sarcomas, however, was not lethal to the injected birds. Instead, the birds typically succumbed to another disease, hemangioma (5), which is characterized by infiltration of the liver, spleen, and kidney with blood cysts. Numerous blood cysts ranging in size from 1 to 20 mm in diameter were observed in these tissues. A large hemorrhagic cyst associated with the spleen was routinely observed and was believed to be the apparent cause of death. This disease is developmentally distinct from the angiosarcomas reported previously by Tracy et al. (32) in its latency (1 to 2 weeks) and in the primary organs affected. Both diseases, however, appear to be of endothelial origin (5; A. Fadley, personal communication). Interestingly, the 9134 F3 virus that expresses a *v-erbB* gene product nearly identical to the 9134 E1 *v-erbB* gene product, with the exception of a short carboxy-terminal truncation, can efficiently induce both sarcomas and erythroblastosis. These data implicate different *erbB* protein domains in the regulation of different disease potentials.

DISCUSSION

Induction by 9134 E1 of erythroblastosis but not sarcomagenesis. In this report, we describe the isolation and characterization of three *erbB* transducing viruses, newly released during ALV-induced erythroblastosis. ALV-induced erythroblastosis provides a rich source for the study of transducing viruses and lends itself to the study of the mechanisms involved in *erbB*-mediated oncogenesis as well as

oncogene transduction (20, 27). A number of novel *erbB* viruses previously analyzed were studied by using uncloned (or not rigorously cloned) viral stocks, and the *erbB* structures of these viruses were only partially characterized. We therefore undertook both molecular and biological approaches to purify the viruses described in this report in order to unambiguously correlate alterations in disease potential with changes in structural domains of the *erbB* protein. We have found that the original 9134 extract released at least three transforming viral isolates. These isolates vary not only in *erbB* content but also in their viral *erbB* junction sequences (27; unpublished observations), suggesting that they are products of independent recombination events. Whether they share a common ancestry prior to recombination (i.e., from a single proviral insertion) is unclear. In the original leukemic sample, expression of the leukemogenic 9134 E1 viral component predominates. On the basis of Southern and S1 analyses, the S3 and F3 components, if they exist, cannot exceed 2% of the total viral population. We do not know exactly when the S3 and F3 viruses were generated. The fact that two independent sarcomas (S3a and S3b) induced by the secondary E2 extracts harbor identical S3 components argues for the preexistence of the S3 component in the E2 extract.

We have provided convincing evidence that 9134 E1 is a leukemic virus that is likely to be responsible for the induction of the original leukemia. Our evidence is threefold: (i) predominance of this virus in the original leukemic sample (the normal *c-erbB* alleles were not altered), (ii) its persistence in leukemias during subsequent passage, and (iii) the ability of the molecularly cloned virus to induce leukemia (and only leukemia). The E1 viral genome is 4.5 kb long. It has a short 5' ALV sequence, encompassing only 161 base pairs of the *gag* coding region; however, the *gag* splice donor and the *erbB* splice acceptor sites are retained. The E1 *erbB* protein is likely to be generated from an in-frame spliced mRNA, and thus the mature *v-erbB* product is identical to the *gag-erbB* fusion product of the ALV insertionally activated *c-erbB* locus. An alternate possibility, i.e., that the E1 *erbB* protein is directly translated from the genomic RNA, resulting in a fusion protein carrying additional *gag* sequences, is inconsistent with both the size of the 9134 E1 *erbB* protein product and the sequence data, which reveal multiple termination codons between the *gag* and *erbB* coding sequences.

In ALV-induced erythroblastosis, expression of the *gag-erbB* product is always accompanied by expression of a tripartite *gag-env-erbB* fusion product which results from an alternatively spliced mRNA (22). The difference between the two *erbB* protein products resides in 53 amino acids of *env* sequence. This *env* sequence includes a hydrophobic stretch of 13 amino acids which has previously been shown to function as the signal sequence, active in translocation of the *env* gp⁸⁵ product (13). Since the signal peptide of the normal *c-erbB* product has been removed as a result of proviral insertion, an added signal peptide should facilitate transport of the truncated molecule to the cell surface, a step necessary for *erbB*-mediated transformation (3). Previously it was unclear whether expression of the *gag-env-erbB* protein was required for ALV-induced erythroblastosis. The data presented in this study suggest that the *gag-erbB* protein, without an amino-terminal signal peptide, is in itself sufficient to induce leukemia.

Correlation between changes in disease spectrum and alterations in specific *erbB* coding sequences. Several recent reports indicate that many of the *erbB* transducing viruses,

newly released during ALV-induced erythroblastosis, are leukemogenic but not sarcomagenic (4, 11, 20, 32). Recently, we have also demonstrated that expression of the insertionally activated *c-erbB* product in the context of a replication-competent retroviral vector results exclusively in leukemogenesis (23). Since the major differences between the proviral insertionally activated *c-erbB* products and the sarcomagenic *v-erbB* products of AEV-ES4 and AEV-H reside in the carboxy-terminal region, it has been postulated that the presence of an intact carboxy terminus may inhibit fibroblast transformation. The data reported here are, in general, consistent with this postulate: the C-terminally intact E1 *erbB* product is exclusively leukemogenic, whereas the sarcomagenic S3 and F3 *v-erbB* products have sustained deletions at or near the carboxy terminus (Fig. 6). Although we have not sequenced the F3 *erbB* gene to exclude the existence of other subtle mutations, the major alteration in this gene appears to be the removal of approximately 23 amino acids at the carboxy terminus. This carboxy-terminal truncation, which includes removal of the putative tyrosine autophosphorylation site P1, is smaller than any other fibrosarcomagenic *v-erbB* lesion thus far reported. Although F3 has acquired fibrosarcomagenic potential, it has not lost its leukemogenicity.

In contrast, the S3 virus, which has sustained an extensive internal deletion in the carboxy terminus near the kinase domain, is still fibrosarcomagenic, although it has apparently lost its ability to transform erythroblasts. Surprisingly, birds infected with S3 rapidly succumb to another disease, hemangioma (5), which is characterized by infiltration of the liver, spleen, and kidney with blood cysts. The target cells in this disease are of endothelial origin, and, interestingly, these cells are also the apparent target cells in hemangiomas induced by expression of the polyomavirus middle T antigen in transgenic mice (2). Since the *erbB* product exhibits protein kinase activity and since transformation by polyomavirus middle T antigen is postulated to be mediated by its interaction with cellular tyrosine kinases, common pathways may be involved in the development of these diseases (2, 17). Recently, Tracy et al. (32) have identified several acutely transforming *erbB* viral variants that induce angiosarcomas, another disease of endothelial origin, but which is histologically and developmentally distinct from the hemangiomas described here. Tracy et al. (32) have correlated angiosarcomagenic potential with the expression of a viral *erbB* gene which contains a shortened carboxy-terminal *BclI-HindIII* fragment. Unlike the S3 virus reported here, these viral isolates do not cause fibrosarcomas. It is therefore not clear whether the angiosarcoma- and hemangioma-inducing determinants of the *erbB* product precisely overlap or, perhaps, whether different injection routes contribute to these discrepancies. Gamett et al. have recently described a fibrosarcoma-inducing *erbB* virus, AFV-5014 (11). On the basis of restriction analysis, it also appears to carry an internal deletion. Whether this deletion is in frame has yet to be elucidated, as has the exact locations of the *erbB* deletion boundaries in this virus.

The observation that the S3 virus appears to lack leukemogenic potential is also noteworthy, although rapid death due to hemangiomas could preclude the detection of erythroblastosis. The inability of the S3 virus to transform erythroblasts in vitro has been tested (M. G. Moscovici, unpublished data), and these results also indicate that the S3 virus is not capable of transforming erythroblasts in vitro. This suggests that the region encompassed by the S3 deletion is required for erythroblast transformation. This region, desig-

FIG. 6. Schematic comparison of viral *erbB* products displaying different oncogenic potentials. The ability of each virus to induce erythroblastosis (Ery), fibrosarcomas (Sarc), or hemangiomas (Hem) is indicated by a + or -. The transmembrane (■, TM) and protein kinase domains (▨, PK) are indicated, as well as the locations of the P1, P2, and P3 tyrosine residues homologous to the major autophosphorylation sites of the human epidermal growth factor receptor (8). The locations of deleted sequences are shown (.....). The predicted protein product is based on nucleotide sequence (7, 8, 34) analysis (except the structure of 9134 F3 v-*erbB*, which is based on analysis by S1 nuclease protection and immunoprecipitation). Abbreviations: aa, amino acid; NR, not reported. E is defined in the text.

nated as the E domain of *erbB* in Fig. 6 and bounded by the S3 and AEV-R 5' deletion points, appears to be most critical for erythroblast transformation. Mutants generated in other laboratories, with deletions extending into the E domain, e.g., *td130*, *td359*, and *dl82* (8, 14, 33) have the same phenotype as the S3 virus, i.e., they can transform fibroblasts but not erythroblasts (Fig. 6). Deletion mutants containing mutations that map downstream from the AEV-R 5' deletion point, including AEV-R, are capable of transforming erythroblasts. Since the E domain is close to the kinase domain, it is conceivable that this region is involved in recognition of a substrate that is erythroid specific.

Parallels between perturbation of carboxy-terminal sequences in c-*src* and c-*erbB* and activation of sarcomagenic potential. Since the major difference between the structure of the ALV insertionally activated c-*erbB* products, which are leukemogenic, and the viral *erbB* products of AEV-R and AEV-H, which are both leukemogenic and sarcomagenic, resides in the carboxy terminus, it has been postulated that the presence of an intact carboxy-terminus in *erbB* may inhibit fibroblast transformation. As mentioned above, the data reported here are, in general, consistent with this hypothesis. However, the finding that an internal deletion can also render *erbB* sarcomagenic indicates that the mere presence of the P1 autophosphorylation site in the carboxy terminus is not sufficient to suppress the fibrosarcomagenic potential. These data, taken together, suggest that interaction of this putative autophosphorylation site with an internal domain is also critical for oncogenesis. An interesting parallel can be found in the activation of the oncogenic potential of c-*src*. CEF transformation can be activated by truncation of the c-*src* carboxy terminus (via deletion of Tyr-527), or by point mutations in internal regions of c-*src* (6, 15, 16, 18, 24). These data invite the speculation that the phosphorylation of the P1 tyrosine of *erbB*, in analogy to phosphorylation of tyrosine 527 in the avian c-*src* product,

can suppress fibroblast transformation by inhibiting kinase activity.

In summary, characterization of naturally occurring acutely transforming *erbB* viruses has traditionally been useful in deciphering the disease determinants of the viral oncogenes. In this report we have discussed the isolation and characterization of three new *erbB* isolates which exhibit distinct disease potentials. Our data, together with those of others, strongly suggest that different structural domains of the *erbB* product confer distinct disease specificities. A corollary to this hypothesis is that there are tissue-specific pathways associated with *erbB*-mediated transformation. Identification of the substrates involved in *erbB*-mediated transformation should improve our understanding of the tissue specificity associated with transformation by this oncogene.

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