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Proviral-Activated c-*erbB* Is Leukemogenic but Not Sarcomagenic: Characterization of a Replication-Competent Retrovirus Containing the Activated c-*erbB*

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Avian leukosis virus (ALV) induces erythroblastosis in chickens by integrating its DNA into the host c-*erbB* locus and by activating expression of truncated c-*erbB* transcripts. Although there is a 100% correlation of c-*erbB* activation with ALV-induced erythroblastosis, direct evidence that the activated c-*erbB* is oncogenic has not been established. We have constructed a replication-competent retrovirus containing the activated c-*erbB* to investigate its transforming potential. The Rous c-*erbB* virus (REB-c) was constructed by inserting the activated c-*erbB* cDNA into a Rous sarcoma virus vector in place of *src*. When transfected into transformed quail fibroblasts (QT6), the REB-c construct stably integrates and expresses c-*erbB*-specific transcripts and produces infectious virus. The REB-c retrovirus produces short-latency polyclonal erythroblastosis in chickens. However, in contrast to avian erythroblastosis virus which contains v-*erbB*, the REB-c construct does not transform chicken embryo fibroblasts in vitro, nor does the REB-c virus produce sarcomas when injected into the wing web of chickens. Our results provide the first direct evidence that the activated c-*erbB* which lacks the amino-terminal extracellular domain but which retains the entire carboxy-terminal sequences is leukemogenic but not sarcomagenic.

Avian leukosis virus (ALV), a nonacute oncogenic avian retrovirus, can induce a variety of neoplasms, including B-cell lymphomas, erythroblastosis, and fibrosarcomas, in chickens (for a review, see references 3 and 24). Previous evidence suggests that ALV induces erythroblastosis by proviral insertion within the host c-*erbB* locus (6, 12, 16), the gene coding for the putative chicken epidermal growth factor receptor (5). Our analysis of 37 erythroblastosis samples demonstrated that proviral integration was clustered within a few hundred base pairs upstream of the VB-1 exon, the first c-*erbB* exon with homology to v-*erbB* (the oncogene of the avian erythroblastosis virus [AEV]). Of those proviruses analyzed, all were inserted in the same transcriptional orientation as c-*erbB* and elevated expression of truncated c-*erbB* mRNA was consistently observed (12, 14, 16). The provirus uses its 5′ long terminal repeat (LTR) promoter to generate a readthrough transcript encompassing both viral sequence and the downstream *erbB* sequence (14). The primary transcript is then processed by two in-frame splicing schemes, one using the *gag* splice donor (SD) site and the VB-1 exon splice acceptor (SA) site and the other involving a double splicing using the *gag* splice donor, as well as the envelope (*env*) splice acceptor and a cryptic splice donor. As a consequence, the activated c-*erbB* proteins are either *gag-erbB* or *gag-env-erbB* fusion proteins.

Although there is a complete correlation between proviral insertional activation of the c-*erbB* gene and the development of ALV-induced erythroblastosis, direct evidence that the activated c-*erbB* is oncogenic is lacking. In addition, since c-*erbB* is homologous to the human epidermal growth factor receptor which has a broad distribution in tissue, we wished to investigate whether the activated c-*erbB* gene has oncogenic potential in diverse tissues as does v-*erbB*. To this end, we have constructed a retroviral vector carrying the activated c-*erbB* cDNA. We reasoned that if the activated c-*erbB* gene were oncogenic, a retrovirus carrying the activated c-*erbB* should behave like an acute retrovirus, i.e., inducing polyclonal neoplasms with a short latency. To avoid possible complications due to helper virus, we have used a replication-competent retroviral vector. The construction scheme is detailed in Fig. 1. Briefly, a Rous sarcoma virus-based vector, RCAN (replication-competent ALV with no splice acceptor) was used (10). In this vector, *src* and the *src* splice acceptor site were replaced by a *Cla*I restriction site. The activated c-*erbB* cDNA was inserted into the *Cla*I site via a cloning plasmid Cla-12N. Before its insertion, the cDNA was modified by replacing the *gag* sequences with a genomic splice acceptor site. This was accomplished by ligating an 230-base-pair (bp) fragment of DNA from pAEV-11R (a plasmid carrying the AEV-R genome [23]) containing the preceding intron and the splice acceptor (SA) site of the VB-1 exon, to a fragment of the activated c-*erbB* cDNA, which contains the remaining *erbB* sequence and a 110-bp segment of the 3′-untranslated region before the poly(A) signal. Thus, the c-*erbB* insert contains the natural splice acceptor site, 3 nucleotides of v-*erbB* (which are identical to c-*erbB*), and all the downstream c-*erbB* coding sequences. The proviral construct retains all appropriate splice donor and acceptor sites necessary to generate *gag-erbB* and *gag-env-erbB* messages found in the original leukemic cells (Fig. 1). This proviral construct was designated REB-c (for Rous c-*erbB* virus).

To generate infectious viruses, REB-c DNA was transfected into QT6 cells, a chemically transformed quail cell line (13). RCAN vector and SRA-2, a plasmid carrying Schmidt-Ruppin A viral DNA (4), were similarly transfected to serve

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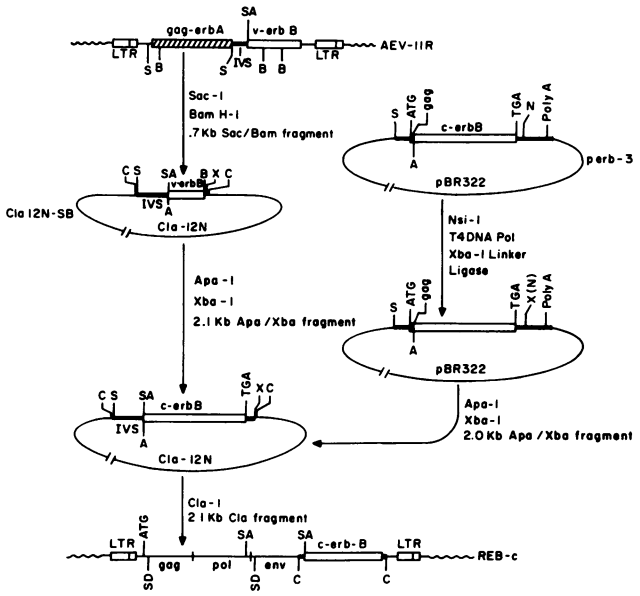

FIG. 1. Molecular construction of REB-c provirus DNA containing the activated c-*erbB* gene. The AEV-11R plasmid carrying the nonpermuted AEV-R genome was obtained from J. M. Bishop, University of California, San Francisco (22, 23). Briefly, pAEV-11R was digested with *Sac*I and *Bam*HI and the purified 0.7-kb fragment containing the intervening sequence (IVS) and the v-*erbB* splice acceptor (SA) site was religated with T4 DNA ligase into the *Sac*I and *Bam*HI sites in the polylinker of the plasmid Cla-12N (10), resulting in the plasmid Cla-12N-SB. The activated c-*erbB* coding sequence was prepared from cDNA clone p*erb*-3 (14) first by digestion with *Nsi*I (Nsi-1) (which is located in the 3′ untranslated region), blunt-end conversion with T4 DNA polymerase, and ligation with *Xba*I linkers. The activated c-*erbB* sequence was then excised by *Apa*I and *Xba*I digestion and ligated into the similarly treated Cla-12N-SB plasmid by replacing the corresponding *Apa*I-*Xba*I v-*erbB* fragment. The resulting c-*erbB* sequence, with the SA site attached, was removed by *Cla*I digestion and was ligated into the virus RCAN (10) at the unique *Cla*I site. The resulting virus contains the natural splice acceptor site, 230 bp of intervening sequence from pAEV-11R and 3 bp of v-*erbB* coding sequence (identical to c-*erbB*), as well as the intact c-*erbB* coding sequence with 110 bp of 3′-untranslated region. ATG: initiation codon. TGA, termination codon; SA, splice acceptor site; SD, splice donor site. Restriction enzyme sites: A and Apa-1, *Apa*I; B and Bam H-1, *Bam*HI; C and Cla-1, *Cla*I; N and Nsi-1, *Nsi*I; S and Sac-1, *Sac*I; X and Xba-1, *Xba*I.

as controls. After 5 passages, the concentrated medium was collected and the virus content was assayed by RNA dot-blot analysis (Fig. 2C). Whereas the *erbB* hybridization confirms the presence of c-*erbB* sequence in REB-c, the LTR hybridization reveals that all three viruses were produced at similar concentrations. To further verify the structure of the viruses, high-molecular-weight DNA was isolated from the transfected cultures. *Eco*RI-digested DNA was blotted and hybridized with either a 5′ or a 3′ *erbB* probe (probe A or B, Fig. 2E). When hybridized with probe A (Fig. 2A), the predicted 2.0-kilobase-pair (kb) fragment was detected in REB-c. The 4.5-kb band represents the endogenous quail c-*erbB* locus. Hybridization with probe B confirms the C-terminal structure of REB-c provirus. There was no evidence of deletions or rearrangements of the inserted c-*erbB* sequence. These data demonstrate that REB-c propagates well and is stable in the initial transfection and passage in vitro.

We then asked whether the *erbB* messages encoded by REB-c were correctly spliced. S1 nuclease mapping analysis was used according to a previously described procedure (8). This procedure entails the use of a cDNA fragment encompassing the *gag-env-erbB* junctions (Fig. 2F) as an S1 probe. Transcripts containing *gag-env-erbB* sequences would be expected to protect 620 bp of the probe, whereas *gag-erbB* messages would protect 470 bp. The data demonstrates that both splicing schemes were used (Fig. 2B). The relative proportions of *gag-erbB* and *gag-env-erbB* messages are similar to those found in the ALV-induced erythroblastosis (8). It is noteworthy that this is the first time that the *env* splice donor site has been shown to be involved in splicing an internal viral gene. It invites speculation that the Rous sarcoma virus may also use this *env* splice donor site in generating a small fraction of the *src* messages. The recent isolation of a recovered *src* virus in which *src* and *env* are joined via the same *env* splice donor and *src* splice acceptor site supports this contention (19).

To test the stability of the REB-c virus, serial infections of QT6 cells were performed. QT6 cells transfected with either RCAN vector or REB-c viral DNA were grown for 3 weeks without selection to allow uniform infection of the cell cultures (primary infection). Medium that had been conditioned for 6 h was then collected from confluent cultures and was used to infect fresh QT6 cells (secondary infection) at a low multiplicity of infection (<0.1) to maximize reinfection. The secondary culture was passaged for 10 days to ensure full infection before the procedure was repeated for the third (tertiary) infection. High-molecular-weight DNA was isolated from the three serially infected cell cultures, digested with *Eco*RI and subjected to Southern analysis. Hybridization to probe A revealed the expected 2.0-kb *erbB* band in the cultures infected with REB-c, but not RCAN (Fig. 2D). No evidence for gross deletion or alteration of the *erbB* sequences was found. The relative intensity of the 2.0-kb *erbB* insert and the 4.5-kb endogenous *erbB* band gives a measure of the extent of infections in each culture. To provide further evidence that the insert region is not particularly prone to alteration (compared with other viral genes), a probe (probe C; Fig. 2E) that simultaneously detects the viral *env* fragment and a fragment linked to the *erbB* insert was used. The 3.8-kb band represents the *env* fragment, common to both RCAN and REB-c viruses (Fig. 2D, bottom panel). The 0.75-kb band was specific for the *erbB* insert and was present only in the REB-c-infected cultures. The intensity ratio of the 0.75- and 3.8-kb bands did not vary significantly upon passages. Likewise, the intensity of the 2.0-kb band detected by probe A on a duplicate filter (top panel) also correlated well with that of the 3.8-kb band. These data, taken together, provide strong evidence for the stability of the entire *erbB* insert in REB-c. We, therefore, conclude that the REB-c virus is genetically stable for at least three serial passages, each representing multiple rounds of infections and spanning at least 30 cell generations.

Having demonstrated that the REB-c provirus construct expressed *erbB* appropriately and stably, we then tested its in vivo oncogenicity. Viruses released from transfected QT6 cell cultures were first clarified, concentrated in an ultracentrifuge, and then used to inject $15_1 \times I_4$ 1-day-old birds. In general, we found that QT6 cells release 10 to 20 times less ALV than chicken embryo fibroblasts (CEF). The concentration step was included to ensure that the viral titer was comparable with that produced by CEF. After 4 to 6 weeks, 16 of 18 birds inoculated with REB-c developed erythroblastosis, which was acutely fatal. The erythroblastosis

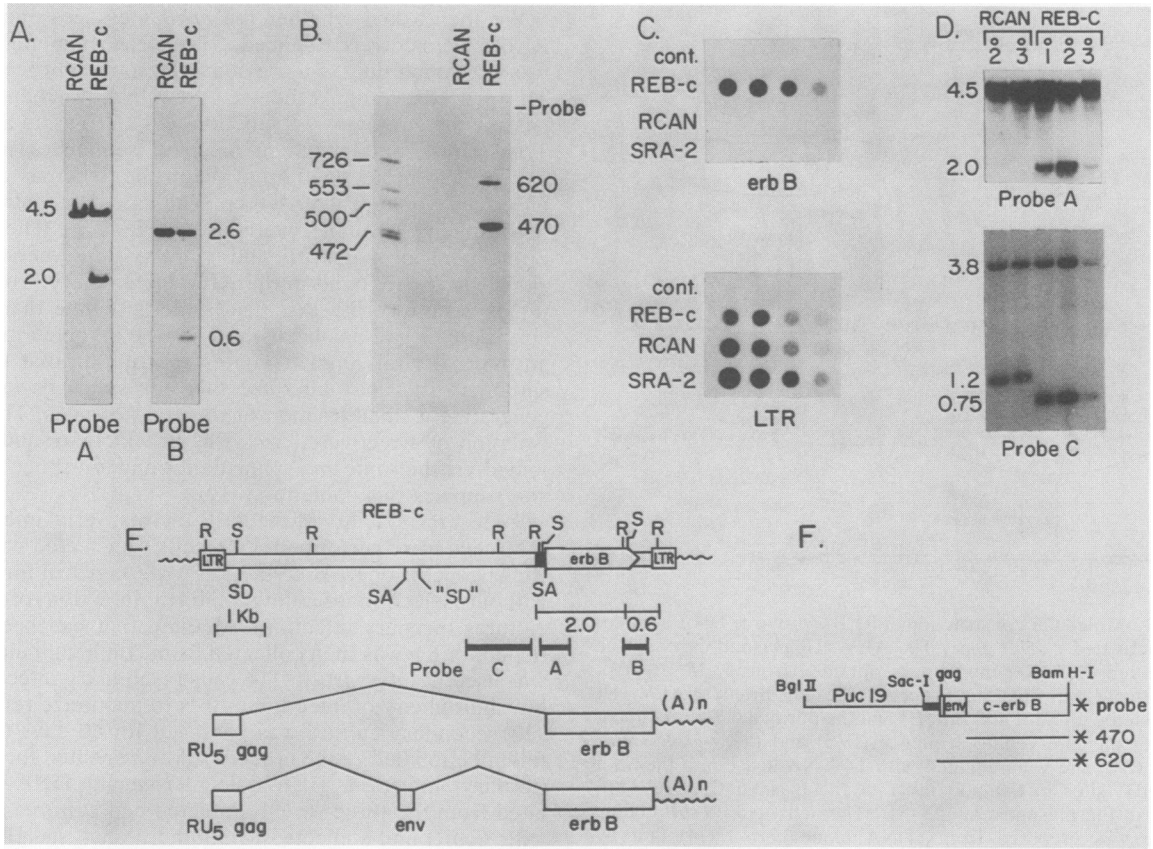

FIG. 2. Analysis of REB-c-transfected QT6 cultures. (A) Autoradiograms of Southern blot analysis of transfected QT6 cells. Genomic DNA (20 μg), isolated from QT6 cultures transfected with either RCAN or REB-c viral DNA, were digested with *Eco*RI, separated by electrophoresis on 0.7% agarose, and transferred to nitrocellulose filters as previously described (16). Filters were hybridized to ^{32}P-labeled nick-translated *erbB*-specific DNA. Probe A is a 4.5-kb *Eco*RI genomic fragment derived from pEB-R4.5 (6) and hybridizes to a single 4.5-kb fragment corresponding to the quail c-*erbB* locus. Probe B is a 360-bp *Eco*RI-*Nsi*I fragment derived from p*erb*-3 (14), which hybridizes to a 2.6-kb endogenous c-*erbB* genomic fragment. Regions of REB-c that hybridize to probes A and B are diagrammed in panel E. Fragment sizes are given in kilobases (kb). (B) S1 nuclease mapping of RNA isolated from QT6 cells. Total cellular RNA was prepared from transfected QT6 cultures by a guanidinium isothiocyanate procedure as previously described (14). RNA (50 μg) was hybridized with the S1 probe (described in panel F) for 16 h at 48°C and digested with S1 nuclease as described previously (8). Nuclease-resistant fragments were analyzed on 8 M urea–6% acrylamide gels, dried, and autoradiographed. (C) Dot blot hybridization analysis of viral supernates from infected cells. Total RNA was prepared from QT6 culture conditioned medium collected from one 490-cm^2 roller bottle by first clarifying media at 5,000 × *g* for 10 min, followed by pelleting the virions at 110,000 × *g* for 2 h. RNA was extracted from viral pellets with guanidinium isothiocyanate as described above. Total RNA was initially suspended in 50 μl of H_2O and denatured in 12× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–6% formaldehyde at 95°C for 10 min (300-μl volume), and duplicate 1:2 serial dilutions were performed in denaturing buffer. Five dilutions of each sample were then blotted onto duplicate GeneScreen filters (New England Nuclear Corp.) and were hybridized to ^{32}P-labeled nick-translated probes. DNA probes specific for c-*erbB* (*Apa*I to *Eco*RI, 1.6-kb fragment of p*erb*-1 [14]) or LTR (*Eco*RI to *Sac*I, 1.1-kb fragment from RCAN) were used. (D) Autoradiograms of Southern blot analysis of serially infected QT6 cells. The procedures for serial infections are described in the text. Probe C is a 1.4-kb *Xba*I-*Cla*I fragment derived from RCAN viral DNA which hybridizes to a 3.8-kb *Eco*RI and a 1.2-kb *Eco*RI fragment in the RCAN vector. In REB-c, because of the presence of an additional *Eco*RI site in the insert, a 0.75-kb (rather than the 1.2-kb) fragment is detected. (E) Map of REB-c provirus and the predicted splicing schemes of *erbB* transcripts. Only *Eco*RI (R) and *Sac*I (S) cleavage sites are shown. R, small terminal repeat; U_5, unique 5′ region; $(A)_n$, Poly(A) sequence; other abbreviations as in the legend to Fig. 1. (F) Schematic diagram of S1 nuclease probe used in 2B. The S1 probe was derived from a 780-bp *Sac*I-*Bam*HI fragment of the cDNA clone p*erb*-1 (14), which was subcloned into the *Sac*I and *Bam*HI polylinker sites of pUC19 (vector sequences present in the S1 probe). The plasmid containing the *Sac*I-*Bam*HI fragment was cleaved with *Bam*HI, phosphatased, and 5′ end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. After secondary cleavage with *Bgl*II, the 1.4-kb labeled fragment was gel purified. The asterisk denotes the labeled end. The predicted length of the probe protected by RNA hybridization is given in nucleotides. RNA transcripts with *gag-erbB* and *gag-env-erbB* splicing should protect 470- and 620-nucleotide fragments, respectively.

resulted in enlargement of the liver and spleen, and the blood and bone marrow histology was indistinguishable from those of ALV-induced erythroblastosis. However, the latency was significantly shorter. None of the birds inoculated with RCAN vector developed neoplasms during that period. To demonstrate that the leukemic induction was caused by REB-c infection, high-molecular-weight DNA from erythroblast-infiltrated livers was analyzed by restriction enzyme digestions, confirming the structure of REB-c in tumor DNA. Representative digestions of five tumor samples by *Eco*RI are shown in Fig. 3A. When hybridized with probe B, the digests contain a 0.6-kb signature band consistent with an *Eco*RI digestion fragment generated between the downstream *Eco*RI site in *erbB* and an *Eco*RI site in the LTR (the 2.6-kb band is due to endogenous *erbB* sequences). In this and other analyses, we found no additional *erbB*-specific

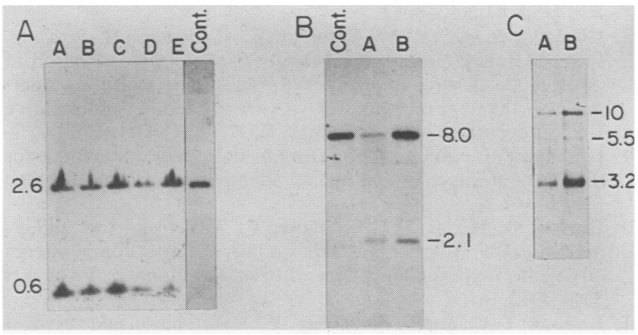

FIG. 3. Analysis of REB-c-induced erythroblastosis samples. (A and B) Southern analyses of leukemic samples. High-molecular-weight DNAs were prepared from erythroblast-infiltrated livers from chickens infected with REB-c virus (lanes A to E). Genomic DNAs were prepared, digested with *Eco*RI (panel A) or *Sac*I (panel B), and subjected to Southern blot analysis as described in the legend to Fig. 2. Filters were hybridized to probe B, as indicated in Fig. 2E. This probe hybridizes to an endogenous 2.6-kb *Eco*RI fragment and an 8.0-kb *Sac*I fragment found in normal uninfected birds (lane labeled Cont.). (C) Northern blot analysis of *erbB*-specific transcripts in leukemic samples. Total cellular RNA was extracted from erythroblast-infiltrated liver samples. Polyadenylated RNA was prepared and fractionated on a 1.0% denaturing formaldehyde agarose gel and was transferred to GeneScreen as previously described (14). Polyadenylated RNA (5 μg) was analyzed in each lane. ^{32}P-labeled nick-translated probe B (Fig. 2E) was used in hybridizations. Sizes of transcripts were determined relative to the mobility of ^{32}P-labeled denatured *Hin*dIII-digested lambda DNA and are given in kilobases.

bands generated, again indicative of the stability of the provirus. To test the clonality of these tumors, genomic DNA was digested with *Sac*I and was analyzed for clonal insertion sites (Fig. 3B). Probe B detected an 8.0-kb band corresponding to the endogenous *erbB* locus and a 2.1-kb band specific for the internal region of REB-c (Fig. 2E). No additional bands representing the terminal fragments of the proviruses were observable, indicating heterogeneous proviral insertion sites in tumor cells which was consistent with a polyclonal cell population. Further evidence that REB-c virus was responsible for transformation comes from Northern analysis of RNA isolated from the leukemic samples (Fig. 3C). The 10-, 5.5-, and 3.2-kb transcripts detected by an *erbB* probe represent, respectively, the genomic RNA, the subgenomic *env*, and spliced *erbB* messages. Hybridization to *env* probe (probe C) confirmed such an assignment (data not shown). Interestingly, in REB-c-infected samples, we have consistently observed a low level of 5.5-kb *env* message and a high level of 3.2-kb *erbB* message (including both the directly spliced *gag-erbB* and the doubly spliced *gag-env-erbB* forms). Among other possible explanations, this suggests that the acceptor sequence for the *erbB* VB-1 exon may be an efficient splice site, which is much more active than the corresponding *src* acceptor associated with various Rous sarcoma virus isolates (for an example, see reference 20). The relatively low level of *env* message apparently did not affect the production of a REB-c virus titer sufficient for leukemogenesis.

The above experiment demonstrates that the REB-c virus can efficiently induce erythroblastosis. To test the transforming potential of REB-c in nonhematopoietic tissues, an in vitro approach was chosen. CEF cells were transfected with the REB-c plasmid DNA by a calcium phosphate precipitation technique (9). Positive control cells were transfected with the *src* containing SRA DNA, or cloned DNA of two avian erythroblastosis strains (AEV-R and AEV-H [23, 25]) in the presence of helper RCAN. Additional cells were transfected with helper virus alone or with salmon sperm DNA. Transfected cells were passaged for 10 days to allow virus spread before being assayed for anchorage-independent growth in soft agar (18). SRA and AEV-R induced a significant number of colonies capable of adherent independent growth (Table 1). In contrast, REB-c-transfected cells produced only rare small colonies equal to the negative controls.

The failure of REB-c to transform CEF cells cannot be attributed to a lack of infection. Viral integration was confirmed by genomic DNA analysis (data not shown), and the viral expression was monitored by recovery of *erbB*-specific RNA in tissue culture supernatant (data not shown). Dot blot hybridization of viral RNA extracted from concentrated tissue culture medium indicated a similar magnitude of virus production between RCAN vector, REB-c and the positive controls AEV-R, AEV-H, and SRA. We therefore conclude that REB-c is not capable of inducing fibroblast transformation. This finding is compatible with previous studies involving *erbB*-transducing viruses recovered from some of the ALV-induced erythroblastosis samples (1, 21). These viruses, which presumably have captured the activated c-*erbB* with an apparently intact 3′-coding sequence, were also ineffective in transforming fibroblasts. All the variants that display fibroblast-transforming potential have apparently undergone further deletions (7). However, in these earlier reports, none of the transduced viral genomes have been cloned and sequenced. Since most transduced *erbB* viral isolates contain mixtures of viruses, it is difficult to assign oncogenic activity to a specific viral component. Furthermore, point mutations or subtle structural changes not detected by Southern analysis may have accumulated in the genomes of the transduced viruses, and the possibility that these changes might affect viral oncogenicity cannot be excluded. Our data therefore represents the first direct evidence that the activated c-*erbB* gene is incapable of fibroblast transformation and sarcoma induction. The major difference between the activated c-*erbB* and v-*erbB* contained in the fibroblast-transforming viral isolate, AEV-H (25), is the presence of the carboxy-terminal 34 amino acids

TABLE 1. In vivo oncogenicity and in vitro fibroblast transformation of *erbB*-containing viruses

Virus	Erythroblastosis[a]		No. of soft agar colonies[b] (per 10^5 cells)	Sarcoma incidence[c] (no. with tumor/no. injected)
	Incidence (no. with disease/ no. injected)	Avg latency (days)		
RCAN	0		3	NT
REB-c	16/18	37	7	0/12[d]
AEV-R	12/12	17	2,300	15/15
AEV-H	12/12	25	240	NT
SRA	NT		8,200	6/6

[a] 2-day-old chicks (line $15_1 \times I_4$) were injected intraperitoneally with 0.1 ml of virus-containing supernatant, clarified and concentrated as described in the legend to Fig. 2C. Viral pellets were suspended in 1 ml of sterile tissue culture media (m-199) and used for injection. Birds were monitored for erythroblastosis by peripheral blood smears twice a week. NT, Not tested.

[b] Infected CEF were trypsinized and suspended in 0.33% agar as described previously (18). Colonies with >100 cells were counted on days 14 through 17.

[c] 2-day-old chicks were injected with 0.1 ml of concentrated virus (isolated as described above) in the left wing web.

[d] 2-day-old chicks were injected in the wing web and failed to develop any nodules before succumbing to erythroblastosis between days 29 to 52.

which contain the putative major tyrosine autophosphorylation site (14). This stretch of peptides may represent a regulatory domain which influences the c-*erbB* protein activity in fibroblasts. Interestingly, the C-terminal sequence of c-*src* also seems to negatively control its fibroblast-transforming ability; removal or mutation of the C-terminal tyrosine (amino acid 527) activates the c-*src* gene (2, 11, 15). Likewise, mutation of the C-terminal tyrosine (amino acid 969) of c-*fms* also enhances the transforming ability of this oncogene (17). Thus, it seems that the presence of a C-terminal regulatory domain with a major tyrosine phosphorylation site represents a common feature of the tyrosine-kinase oncogene family. Nevertheless, it should be pointed out that whatever the negative influence of the C-terminal sequences on fibroblast transformation is, it does not seem to affect the erythroblast-transforming potential of the insertionally activated c-*erbB*.

In summary, we have demonstrated in this report the utility of a replication-competent retroviral vector to study the activated c-*erbB* gene. The recombinant virus replicates well and is genetically stable. It provides all the proper processing machinery to generate an oncogenic *erbB* gene. On the basis of these analyses, it was shown that the activated c-*erbB* with an intact carboxy-terminal sequence is leukemogenic but not sarcomagenic.

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