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# High-Frequency Transduction of c-*erb*B in Avian Leukosis Virus-Induced Erythroblastosis

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**Twenty-one cases of Rous-associated virus type 1-induced erythroblastosis have been analyzed for novel restriction endonuclease fragments of c-*erb*B. Twenty of the erythroleukemias contained novel c-*erb*B fragments; 10 of these were found to contain a proviral insertion in c-*erb*B, and 10 were found to have a new transduction of c-*erb*B. Each of the proviral insertions was in the same transcriptional orientation as c-*erb*B, and most appeared to have retained both long terminal repeats as well as 5′ viral sequences that signal packaging of RNA into virions. Each of the new c-*erb*B transducing viruses had a characteristic *Eco*RI fragment that contained a spliced form of c-*erb*B sequences. When inoculated into 1-week-old chickens, the new transducing viruses caused rapid-onset erythroblastosis.**

Transduction, or the carrying by a virus of host chromosomal DNA from one cell to another, was originally observed in phage-infected bacteria. Phage such as P1 transduce fragments of bacterial DNA that have become randomly packaged into phage particles, whereas phage such as lambda transduce host genes that have recombined with the DNA of the phage. In 1976 it was realized that retroviruses have the potential of transducing eucaryotic cell DNAs (39). In retroviral transductions, the transduced host sequences have recombined with a retroviral genome. In most instances, the transduction is recognized by the ability of the transduced gene to cause the malignant growth of recipient cells.

The transduction of host sequences by retroviruses has been considered a relatively rare event; fewer than 45 transductions have been documented in the 73 years since the isolation of Rous sarcoma virus (2, 3, 5, 6, 11, 16, 19, 20, 22–24, 30, 37). In this paper we report 12 new transductions of the chicken gene c-*erb*B. These were observed in 21 cases of Rous-associated virus type 1 (RAV-1)-induced erythroblastosis. This unexpected finding indicates that retroviral transduction of some host sequences is far from rare.

Before this study, two transductions of c-*erb*B had been identified. One of these is carried in the ES4 and R strains of avian erythroblastosis virus (AEV-R) (10, 26), whereas the second is carried in AEV-H (16). AEV-R has transduced sequences from two host genes, c-*erb*A and c-*erb*B (45), whereas AEV-H has transduced sequences only from c-*erb*B (49). The c-*erb*B sequences in AEV-R and AEV-H are very similar, both viruses have transduced about 2 kilobases (kb) of exon sequences from over 18 kb of chromosomal DNA (26, 34, 45, 46, 49, 50). Before this study it had also been shown that erythroblastosis could be caused by proviral insertions in c-*erb*B and that certain cases of RAV-1-induced erythroblastosis contained DNAs that appeared to be spliced forms of c-*erb*B (13).

## MATERIALS AND METHODS

**Viruses.** RAV-1 was obtained from L. B. Crittenden at the Regional Poultry Research Laboratory, East Lansing, Mich. RAV-1 is a replication-competent retrovirus that was isolated from a stock of Bryan Rous sarcoma virus (33). RAV-1 has the *gag* (core proteins), *pol* (RNA-directed DNA polymerase), *env* (envelope glycoprotein), and long terminal repeat (LTR) sequences that are characteristic of retroviruses. RAV-1 does not contain a transduced host sequence.

**Chickens.** $15_1$ or $(K28\times15_1)\times K28$ chickens were used for inoculations. Line $15_1$ chickens are highly susceptible to the induction of erythroblastosis by RAV-1 (1), whereas K28 chickens are not (31). $15_1$ is an inbred line that is maintained at the Regional Poultry Research Laboratory in East Lansing, Mich. K28 is a random-bred line of chickens that is maintained at the Worcester Foundation for Experimental Biology. All matings were performed by artificial insemination.

**Induction of tumors.** Erythroleukemias were induced by intravenous inoculation of 1-day-old $15_1$ or $(K28\times15_1)\times K28$ chickens with $\sim10^6$ infectious units of RAV-1. Beginning at 6 weeks post-inoculation, hematocrits and blood smears of infected birds were examined at least twice a week for immature erythroid cells. When erythroblastosis was detected, tumor (buffy coat, bone marrow, spleen) and control tissues (bursa) were harvested from moribund birds and quickly frozen in liquid nitrogen.

**Analysis of tumor DNA.** DNA was harvested from tumor and control tissues by homogenizing minced tissue in 100 mM NaCl–10 mM EDTA–10 mM Tris (pH 7.5). Homogenates were digested with proteinase K (0.2 mg/ml) in the presence of 0.5% sodium dodecyl sulfate for 1 to 2 h at 37°C and then extracted twice with phenol and twice with chloroform-isoamyl alcohol (40:1). DNA was next precipitated by adding 2.5 volumes of isopropyl alcohol and then dissolved in 0.1 mM EDTA–1 mM Tris (pH 8.0). Restriction endonucleases were purchased from a variety of companies and used according to the directions of the suppliers. Restriction endonuclease cleavage of genomic DNAs was monitored by examining the extent of digestion of 0.5 μg of lambda DNA by a sample of the reaction mixture. Blots were performed by the procedure of Southern (36), with the exceptions that a high-salt buffer (40 mM Tris, 50 mM sodium acetate, 10 mM EDTA, pH 7.9) was used during gel electrophoresis, 10× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) was used during transfer of denatured DNA from gels to nitrocellulose paper, and nitrocellulose papers containing transferred DNA were not allowed to air dry before baking. Pre-hybridizations (2 to 4 h of incubation in

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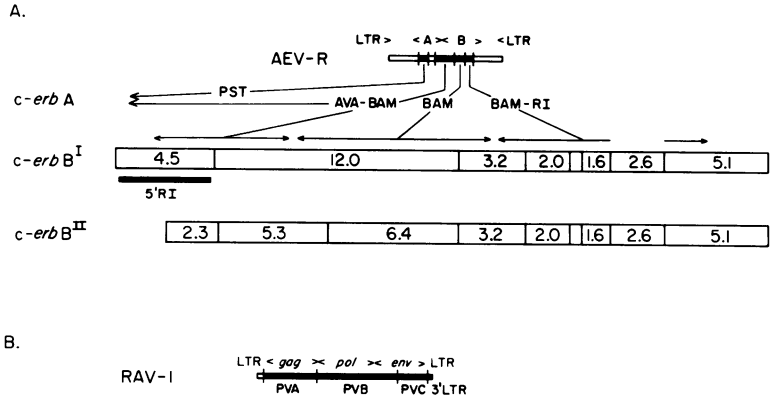


FIG. 1. Restriction endonuclease sites and probes used to identify viral and c-*erb*B sequences. In c-*erb*B$^{I}$, the 12.0-kb fragment has also been reported as 12.1 kb; the 3.2-kb fragment has been reported as 3.1 and 3.0 kb, the 2.0-kb fragment has been reported as 1.9 kb, and the 1.6-kb fragment has been reported as 1.3 kb (13, 34, 45). Boxes indicate *Eco*RI fragments of the indicated cellular DNAs. The numbers indicate fragment sizes in kb. Heavy lines indicate probes used to detect c-*erb*A, c-*erb*B, and proviral sequences.

6× SSC–10× Denhardt solution–0.1 mg of fish DNA per ml–0.1% sodium dodecyl sulfate) as well as hybridizations (overnight incubation with pre-hybridization buffer plus $10^6$ to $10^7$ cpm of $^{32}$P-labeled probe per ml) were carried out at 67°C. Hybridization probes were prepared by nick translation of DNAs with all four $^{32}$P-labeled nucleotide triphosphates to specific activities of $>10^8$ cpm/μg. Probes were used within 1 to 2 days of preparation. After hybridization, filters were washed over a 2-h period with at least five changes of 3× SSC–0.1% sodium pyrophosphate–0.1% sodium dodecyl sulfate at 67°C. Autoradiographs were obtained with X-Omat film (Eastman Kodak Co.) in the presence of an intensifying screen.

**DNAs used for hybridization probes.** The origins of the DNA fragments used for probes are depicted in Fig. 1. Most probes for RAV-1 sequences were obtained from pRAV-1 DNA. pRAV-1 is a derivative of pBR322 that contains RAV-1 DNA cloned at the *Sac*I site at base 255 of the viral genome. LTR sequences were obtained by using an enzyme that has only one recognition site within the LTR to cleave a permuted LTR from the tandem LTRs in pRAV-1. The PVA fragment contained sequences from base 255 (*Sac*I site) to base ~2300 (*Eco*RI site), the PVB fragment contained sequences from base ~2300 (*Eco*RI site) to base ~6100 (*Eco*RI site), and the PVC fragment contained sequences from base ~6100 (*Eco*RI site) to base ~7700 (*Eco*RI site) of the RAV-1 genome. The LTR, PVA, PVB, and PVC fragments were isolated from *Eco*RI-digested pRAV-1 DNA that had been fractionated on low-melting-point agarose. DNAs used for the LTR, PVB, and PVC probes contained only viral sequences. The PVA fragment contained pBR322 as well as viral sequences. The 3′ LTR probe contained the ~150 base pairs (bp) 3′ of the *Eco*RI site in the LTR. 3′ LTR sequences were obtained from p53. p53 is pBR322 with a cDNA clone of 3′ viral sequences cloned in its *Pst*I site (25). 3′ LTR sequences were obtained from p53 by preparing an *Eco*RI pop-out plasmid, digesting the pop-out plasmid with *Pst*I and *Eco*RI, and separating the ~150-bp 3′ LTR fragment from the ~3-kb pBR fragment on a sucrose gradient. Fragments of v-*erb*A and v-*erb*B were obtained from cloned AEV-R DNA (pAE) (46). PST is the ~450-bp *Pst*I fragment of v-*erb*A. AVA-BAM is the ~1.1-kb *Ava*I-*Bam*HI fragment of the 3′ v-*erb*A and 5′ v-*erb*B sequences. BAM is the ~500-bp *Bam*HI fragment of v-*erb*B. BAM-RI is the ~500-bp *Bam*HI-*Eco*RI fragment of v-*erb*B. Each of the pAE fragments was subcloned in pBR322. The PST, AVA-BAM, and BAM-RI fragments (but not the BAM fragment) were isolated from their cloning vectors before being used to prepare probes. The probe designated 5′ RI is the most 5′ *Eco*RI fragment of c-*erb*B$^{I}$ cloned into pBR327.

## RESULTS

**Restriction endonuclease maps for two alleles of c-*erb*B: c-*erb*B$^{I}$ and c-*erb*B$^{II}$.** The chickens used for the induction of erythroblastosis were segregating for two different alleles of c-*erb*B: c-*erb*B$^{I}$ and c-*erb*B$^{II}$ (referred to as types I and II by Fung et al. [13]). *Eco*RI restriction endonuclease sites in c-*erb*B$^{I}$ and c-*erb*B$^{II}$ were mapped by hybridizing blots of *Eco*RI-digested DNAs from chickens that were homozygous for c-*erb*B$^{I}$ or c-*erb*B$^{II}$ with probes for the v-*erb*B sequences of AEV-R (Fig. 1). In instances where chromosomal fragments contained sequences homologous to only one of the probes, fragments were subordered according to maps for molecularly cloned c-*erb*B$^{I}$ sequences (34, 45). The 2.6-kb fragment near the 3′ end of c-*erb*B$^{I}$ and c-*erb*B$^{II}$ was not detected by the BAM-RI probe. It is included in the map because of its presence in cloned c-*erb*B$^{I}$ DNAs. The 3′ 5.1-kb fragment has not been mapped by other laboratories. This fragment could represent the 3′ ends of c-*erb*B$^{I}$ and c-*erb*B$^{II}$. Alternatively, this fragment could be from a third v-*erb*B-related sequence.

**Two classes of novel c-*erb*B fragments in tumors.** DNAs from tumor as well as control tissues of chickens with RAV-1-induced erythroblastosis were digested with *Eco*RI and analyzed by the Southern technique (36) for novel fragments related to the v-*erb*A or v-*erb*B genes of AEV-R (Fig. 1); 20 of 21 tumor DNAs exhibited novel v-*erb*B-related fragments, whereas none of the tumors exhibited novel v-*erb*A-related fragments.

To determine which c-*erb*B sequences were present in novel fragments, blots of *Eco*RI-digested DNAs were hybridized with probes for three different regions of v-*erb*B (Fig. 1). These hybridizations revealed two different classes of tumor-associated fragments. Fragments in the first class hybridized with only one of the probes for v-*erb*B, whereas fragments in the second class hybridized with each of the three probes for v-*erb*B. Further analyses revealed that the fragments in the first class represented proviral insertions in c-*erb*B, whereas the fragments in the second class repre-

TABLE 1. Proviral insertions in c-*erb*B: size and sequence content of novel *Eco*RI fragments

| Chicken | c-*erb*B alleles[a] | Fragment size (kb) | Hybridization with probe[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | PST | AVA-BAM | BAM | BAM-RI | 3′ LTR | 5′ RI | LTR |
| Line $15_1$ | | | | | | | | | |
| B867 | I/I | 3.7 | − | + | − | − | + | + | + |
| | | 1.4 | − | − | − | − | − | + | ? |
| B835 | I/I | 3.6 | − | + | − | − | + | + | + |
| | | 1.9 | − | + | − | − | − | + | + |
| B832 | I/II | 0.85 | − | + | − | − | + | + | + |
| | | 1.8 | − | + | − | − | − | + | + |
| $(K28\times15_1)\times K28$ | | | | | | | | | |
| NB3 | I/II | 2.1 | − | + | − | − | + | + | + |
| | | 1.4 | − | − | − | − | − | + | − |
| 4563 | II/II | 1.5 | − | + | − | − | + | + | + |
| | | 1.3 | − | − | − | − | − | + | + |
| B917 | II/II | 1.4 | − | + | − | − | + | + | ? |
| 5065 | I/II | 1.2 | − | + | − | − | + | + | + |
| | | 1.45 | − | − | − | − | − | + | + |
| 4880 | I/II | 1.1 | − | + | − | − | + | + | + |
| | | 1.7 | − | − | − | − | − | + | + |
| 4705 | II/II | 0.9 | − | + | − | − | + | + | + |
| | | 1.75 | − | − | − | − | − | + | + |
| 4888 | II/II | 0.8 | − | + | − | − | + | + | + |
| | | 1.9 | − | + | − | − | − | + | + |

[a] I, c-*erb*B$^{I}$; II, c-*erb*B$^{II}$. The c-*erb*B allele thought to be the target of the proviral insertion is listed second.
[b] +, Hybridization band in the tumor, but not the control DNA; −, did not hybridize with tumor-specific fragment; ?, tumor-specific fragment comigrated with an endogenous virus band or an internal fragment of RAV-1.

sented new transductions of c-*erb*B. Of the 21 cases of erythroblastosis, 10 contained proviral insertions in c-*erb*B, and 10 contained new transductions of c-*erb*B.

**Proviral insertions in c-*erb*B.** Each of the erythroblastosis-inducing insertions in c-*erb*B was initially detected as a novel *Eco*RI fragment that hybridized with the AVA-BAM probe, but not with the BAM or BAM-RI probe, for v-*erb*B (Table 1). Of the 10 tumors caused by proviral insertions, 7 displayed one novel AVA-BAM-related fragment, whereas 3 displayed two novel AVA-BAM-related fragments. In all, 13 novel AVA-BAM-related fragments were observed.

To map proviral and c-*erb*B sequences in the 13 novel AVA-BAM-related fragments, *Eco*RI-digested DNAs were blotted and hybridized with probes for the 5′ *Eco*RI fragment of c-*erb*B (5′ RI), the viral LTR, and sequences 3′ to the *Eco*RI site in the viral LTR (3′ LTR) (Fig. 1). Hybridizations with the 5′ RI probe determined whether insertions had occurred in the most 5′ *Eco*RI fragment of c-*erb*B$^{I}$ or c-*erb*B$^{II}$ and, if they had, identified novel fragments that represented the 5′ and 3′ junction fragments of proviral and host sequences. Hybridization with the LTR probe determined whether LTR sequences might be present in both junction fragments, whereas hybridization with the 3′ LTR probe identified 3′ junctions of proviral and host sequences. Table 1 summarizes the results of these analyses, and Fig. 2 presents data and a restriction endonuclease map for the insertion in chicken 4888.

Several generalizations about erythroblastosis-inducing insertions in c-*erb*B can be drawn from the data summarized in Table 1. First, all of the insertions were in the most 5′ *Eco*RI fragment of c-*erb*B$^{I}$ or c-*erb*B$^{II}$. This is evidenced by the detection of each of the 13 novel AVA-BAM-related fragments by the 5′ RI probe. Second, most of the insertions had retained both of their LTRs. In all except three tumors (B867, NB3, and B917), both tumor-associated junction fragments appeared to contain LTR sequences. Third, most of the insertions did not involve deletions or rearrangements in c-*erb*B. In all but three tumors (B835, NB3, and B917), the sizes of the two junction fragments were consistent with the fragments being comprised of one ~0.3-kb LTR (*Eco*RI cuts within the proviral LTR) plus the sequences present in the 2.3-kb *Eco*RI fragment of c-*erb*B$^{II}$ or the 4.5-kb *Eco*RI fragment of c-*erb*B$^{I}$. Fourth, seven insertions were such that v-*erb*B (AVA-BAM)-related sequences were 3′ of the inserted provirus (hybridized with the 3′ LTR probe), whereas three had occurred within v-*erb*B-related sequences (both novel 5′ RI fragments hybridized with the AVA-BAM probe).

The orientation and location of the proviruses within c-*erb*B were deduced from the mapping of novel 3′ LTR-, AVA-BAM-, and 5′ RI-related sequences in *Eco*RI-, *Sac*I-, and *Kpn*I-digested DNAs. These mapping studies indicated that the insertions were in the same transcriptional orientation as c-*erb*B (Fig. 2). Knowing that the proviruses were in the same transcriptional orientation as c-*erb*B, the sizes of the novel 3′ *Eco*RI junction fragments (Table 1) could be used to map the insertions in c-*erb*B. The eight insertions in c-*erb*B$^{II}$ resulted in 3′ junction fragments that ranged from 0.8 to 2.1 kb in size. Two of these (the 0.8- and 0.85-kb fragments) appear to be within sequences transduced by the AEV-R, whereas the remainder (0.9- to 2.1-kb fragments) appear to be 5′ of the c-*erb*B sequences transduced by AEV-R. The two insertions in c-*erb*B$^{I}$ generated novel 3.6- and 3.7-kb 3′ junction fragments. The insertion with the 3.6-kb fragment appears to have been just within c-*erb*B sequences transduced by AEV-R, whereas the insertion with the 3.7-kb fragment appears to have been just upstream of c-*erb*B sequences transduced by AEV-R. Thus the erythroblastosis-inducing insertions occurred just within as well as immediately 5′ (within ~1 kb) of the c-*erb*B sequences transduced by AEV-R.

**Most erythroblastosis-inducing proviruses retain sequences for the packaging of RNA into virions and appear to be complete proviruses.** Since transducing retroviruses are thought to result from proviral insertions generating virus-

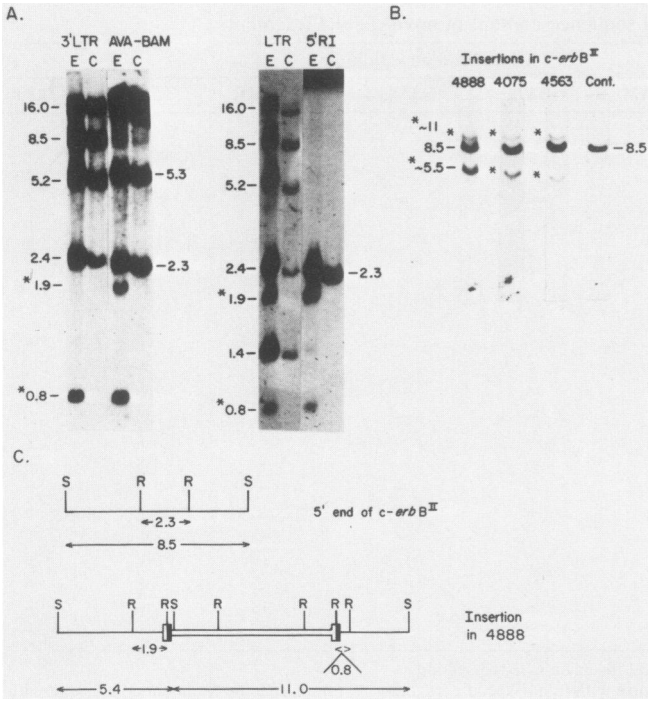


FIG. 2. Mapping of a proviral insertion in c-*erb*B$^{II}$. (A) *Eco*RI fragments of the proviral insertion in chicken 4888. Data are presented from two blots. One blot was hybridized with the 3′ LTR probe and then the AVA-BAM probe. The second blot was hybridized with the LTR and then the 5′ RI probe. In between hybridizations, probes were melted from the blots. Lanes designated E contain DNA from the erythroleukemia (bone marrow). Lanes designated C contain DNA from a normal tissue. Sizes of fragments are indicated in kb. Asterisks indicate novel fragments. (B) *Sac*I fragments of the proviral insertions in c-*erb*B$^{II}$ in chickens 4888, 4075, and 4563. The blot was hybridized with the 5′ RI probe. Lanes designated 4888, 4075, and 4563 contain DNA from the erythroleukemias (bone marrow) in the indicated chickens. The lane designated Cont contains DNA from an uninfected K28 chicken. Each of the ~11- and ~5.5-kb tumor-associated fragments is of a slightly different size. (C) Restriction endonuclease map of the insertion in chicken 4888. Only the most 5′ *Eco*RI fragment of c-*erb*B$^{II}$ is included in this map. Data for the mapping was taken from panels A and B as well as from data on tumor associated *Kpn*I fragments. R, *Eco*RI site; S, *Sac*I site; ——, cellular sequence; ═, proviral sequence; □, proviral LTR.

host transcripts that can be packaged into virions (4, 40, 44), erythroblastosis-inducing insertions were tested for the *Sac*I site that resides in (or near) sequences with signals for the packaging of RNA into virions (35). This *Sac*I site is the only cleavage site for *Sac*I in RAV-1 proviral DNA. If a provirus retained this site, the insertion should result in a tumor containing two novel *Sac*I fragments. If the provirus did not retain this site, the insertion should result in a tumor containing only one novel *Sac*I fragment. Each of the tumors tested displayed two novel *Sac*I fragments (Fig. 2B). This result suggests that erythroblastosis-inducing insertions tend to retain signals for the packaging of RNA into virions.

In most tumors, the sizes of the erythroblastosis-associated *Sac*I fragments added up to the size of one provirus plus the size of the acceptor c-*erb*B *Sac*I fragment. The sizes of erythroblastosis-associated *Kpn*I fragments (*Kpn*I has only one recognition site within proviral DNA) also tended to add up to the size of one provirus plus the size of the *Kpn*I acceptor fragment (data not shown). Thus most erythroblastosis-inducing proviral insertions appear to be complete proviruses.

**Viral transductions of c-*erb*B.** Ten of the tumors displayed novel c-*erb*B-related sequences that hybridized with probes for the AVA-BAM, BAM, and BAM-RI regions of v-*erb*B; eight of these displayed one novel c-*erb*B-related fragment, whereas two displayed two novel c-*erb*B-related fragments. In all, 12 novel fragments that are indicative of new transductions of c-*erb*B were observed. The sizes and sequence content of these fragments are summarized in Table 2. Figures 3A and 4A present autoradiographs of two of the fragments, and Fig. 3B and 4B present possible genomes for the viruses with these fragments.

Several predictions can be made about the new transductions from the data in Table 2. First, the transduced host sequences contained spliced forms of chromosomal DNA. Each of the transducing viruses had a relatively small *Eco*RI fragment that hybridized with exon sequences residing in over 18 kb of chromosomal DNA (Table 2, Fig. 1). Second, the transductions were generated by recombinations that took place at many sites in the viral genome. Of the six novel fragments analyzed for proviral as well as c-*erb*B sequences, one contained sequences detected by the PVA probe, two contained sequences detected by the PVB probe, one contained sequences detected by the PVB and PVC probes, and one contained sequences detected by the PVC probe (Table 2, Fig. 3 and 4). Fourth, none of the new transducing viruses contained both c-*erb*A and c-*erb*B sequences. Thus the incorporation of c-*erb*A and c-*erb*B into an erythroblastosis-inducing virus has been observed only once, in AEV-R.

**Induction of rapid onset erythroblastosis by the new transductions of c-*erb*B.** Strong evidence that candidate transduced sequences did, indeed, represent viral transductions of c-*erb*B was obtained by demonstrating that these sequences could be transmitted by a filtrable agent (32). Homogenates (10%, wt/vol) of tumors with candidate transduced sequences were filtered through 0.22-μm membrane filters (Millipore Corp.) and inoculated intravenously into 1-week-old ($K28\times15_1$)×K28 chicks. Within 6 weeks, ca. 50% of the inoculated birds had developed erythroblastosis (Table 3).

To verify the rapid-onset erythroblastosis was caused by the candidate transducing viruses, DNAs were prepared from tumor and control tissues of birds that had been inoculated with filtered homogenates. Blot analyses of these DNAs revealed novel *Eco*RI fragments of the same size as the fragment in the inducing tumor (Fig. 5).

At this point the new transductions were named AEVs and designated for the chicken in which they had originated (i.e., AEV-5005, AEV-4920). In chickens with two new transductions, A was added to the designation of the virus with the larger of the novel *Eco*RI fragments (AEV-B854A), and B was added to the virus with the smaller of the novel *Eco*RI fragments (AEV-B854B).

**Most erythroleukemias caused by the new AEVs are polyclonal.** Avian leukosis viruses integrate at many sites in the chromosomal DNA of their host (17). If an avian leukosis virus-induced tumor is a clonal outgrowth, each tumor cell contains junctions of viral and host sequences that existed in the founder cell. These junctions can be visualized as novel LTR-related fragments on autoradiographs of Southern blots (Fig. 2A and 6A). However, if the tumor resulted from the abnormal growth of many infected cells, the junctions that occur in individual infected cells cannot be detected in blots of genomic DNA.

TABLE 2. New transductions of c-*erb*B: size and sequence content of novel *Eco*RI fragments

| Chicken | c-*erb*B alleles[a] | Fragment size, (kb) | Hybridization with probe[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PST | AVA-BAM | BAM | BAM-RI | 3′ LTR | PVA | PVB | PVC |
| Line15$_1$ | | | | | | | | | | |
| B854 | I/I | 2.2 | − | + | + | + | NT | NT | NT | NT |
| | | 3.5 | − | + | + | + | NT | NT | NT | NT |
| B864 | I/II | 3.1 | − | + | + | + | NT | NT | NT | NT |
| B838 | I/I | 3.6 | − | + | + | + | NT | NT | NT | NT |
| B845 | I/I | 4.3 | − | + | + | + | NT | NT | NT | NT |
| | | 5.0 | − | + | + | + | NT | NT | NT | NT |
| (K28×15$_1$)×K28 | | | | | | | | | | |
| 4920 | II/II | 2.1 | − | + | + | + | − | − | − | − |
| 4883 | I/II | 2.2 | − | + | + | + | − | − | ft | + |
| 5009 | II/II | 3.0 | − | + | + | + | − | − | ft | − |
| 4890 | II/II | 3.5 | − | + | + | + | ? | ? | + | * |
| 5040 | II/II | 4.2 | − | + | + | + | − | − | − | +,* |
| 5005 | II/II | 4.3 | − | + | + | + | + | + | − | − |

[a] See footnote *a* of Table 1.

[b] Intense hybridization band in the tumor, but not the control DNA; ft, faint hybridization band in the tumor, but not the control DNA; −, did not hybridize with tumor-specific fragment; ?, novel c-*erb*B fragment comigrated with endogenous virus band or internal fragment of RAV-1; NT, not tested; *, novel fragment that did not comigrate with novel c-*erb*B fragment.

To determine whether the erythroleukemias caused by the new c-*erb*B transducing viruses were clonal outgrowths, blots of *Eco*RI-digested DNAs were analyzed for novel provirus-host junction fragments. Autoradiographs of 9 of 10 tumors induced by the new AEVs did not display novel fragments (Fig. 3A and 6B). Thus nine of the erythroleukemias induced by new AEVs were polyclonal tumors. Interestingly, the one erythroleukemia that did display novel provirus-host junction fragments had an unusually long latent period. Thus the clonal or oligoclonal nature of this

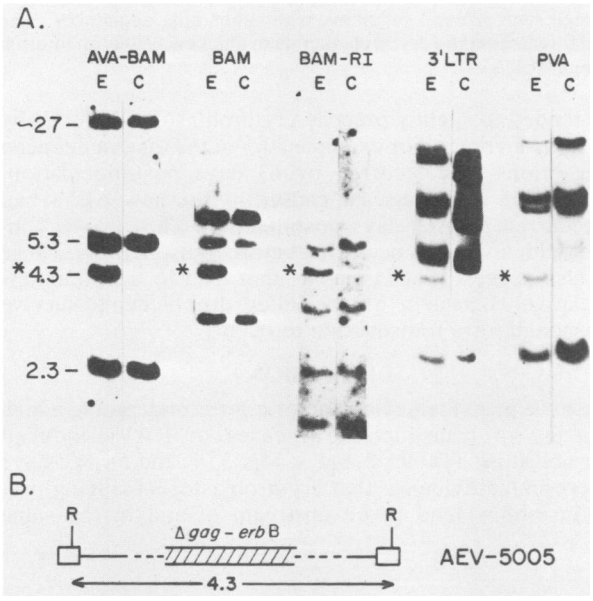


FIG. 3. *Eco*RI fragment of transduced c-*erb*B sequences in chicken 5005. (A) Autoradiographs of Southern blot analyses of *Eco*RI-digested DNAs from chicken 5005. Lanes designated E contain tumor DNA (bone marrow), and lanes designated C contain control DNA (bursa). Hybridization probes are indicated above each pair of lanes. Numbers indicate sizes of fragments in kb. The asterisks indicate the fragment of transduced c-*erb*B sequence. (B) Possible organization of the genome of AEV-5005. R, *Eco*RI site; □, proviral LTRs; ▨, transduced sequence. Both AEV and AEV-H have an *Eco*RI site near the 3′ boundary of transduced c-*erb*B sequences. This site is not portrayed in the schematic since we could not detect a novel PVC-related fragment in *Eco*RI-digested 5005 tumor DNA.

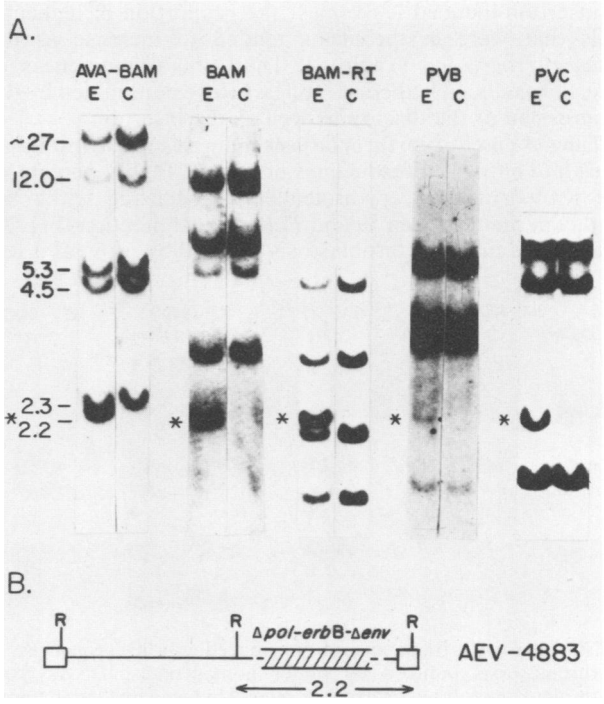


FIG. 4. *Eco*RI fragment of transduced c-*erb*B sequences in chicken 4883. (A) Autoradiographs of Southern blot analyses of *Eco*RI-digested DNA from chicken 4883. Lanes designated E contain tumor DNA (bone marrow), and lanes designated C contain control DNA (bursa). Hybridization probes are indicated above each pair of lanes. Sizes of fragments are given in kb. Asterisks indicate the fragment of transduced c-*erb*B sequence. (B) Possible organization of the genome of AEV-4883. R, *Eco*RI site; □, proviral LTR; ▨, transduced sequence.

TABLE 3. Induction of rapid-onset erythroblastosis by filtered homogenates of tumors containing candidate transductions of c-*erb*B[a]

| Tumor homogenate | No. with erythroblastosis/ no. inoculated |
|---|---|
| 4920 | 1/8 |
| 4883 | 4/6 |
| 5009 | 2/7 |
| 4890 | 5/5 |
| 5040 | 4/7 |
| 5005 | 4/6 |

[a] Homogenates (10%, wt/vol) of tumors (spleens) with candidate transductions were passed through 0.22-μm filters and inoculated intravenously into 1-week-old (K28×15$_1$)×K28 chickens. Erythroblastosis occurred between 2 and 6 weeks post-inoculation.

tumor could have resulted from progeny of one or a few AEV-transformed cells having a slight growth advantage over other AEV-transformed cells.

**Erythroleukemias induced by proviral insertions and viral transductions of c-*erb*B contain cells at several stages of differentiation.** Each of the erythroleukemias consisted of polychromatic erythrocytes as well as erythroblasts. Some of the new AEVs caused erythroleukemias that consisted mainly of erythroblasts, whereas others caused erythroleukemias that consisted mainly of polychromatic erythrocytes (Table 4). The proportion of erythroblasts in these leukemias did not change as the disease progressed. Thus the primitiveness of most AEV-induced leukemias appears to be determined by sequences in the inducing AEV. In contrast, in insertion-induced leukemias, the proportion of leukemic cells that were erythroblasts tended to increase as the disease progressed (Table 4). Thus, the primitiveness of insertion-induced leukemias appears to be determined by the progression of the disease as well as the insertion.

**Time of onset of erythroblastosis induced by proviral insertions into or viral transductions of c-*erb*B.** Eighty percent of the RAV-1-induced erythroleukemias occurred within an 18-day period between 59 and 77 days postinfection (Fig. 7). During this time, erythroblastosis induced by proviral insertions tended to slightly precede erythroblastosis induced by viral transductions. For example 50% of the disease induced by insertions had occurred by 63 days post-inoculation, whereas 50% of the disease caused by the new AEVs had not occurred until 68 days postinfection. This data is consistent with insertions occurring before transductions. These data also suggest that insertions that lead to transductions are relatively benign in that they allow the chicken to survive long enough for a transduction to occur.

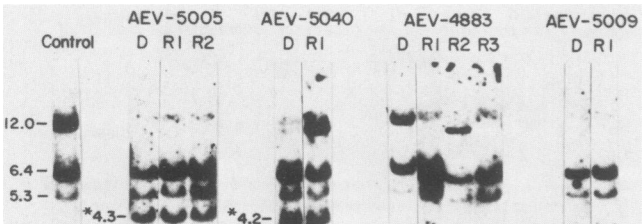


FIG. 5. *Eco*RI fragments of transduced c-*erb*B sequences in erythroblastosis induced by tumor homogenates. DNAs from erythroleukemias (bone marrow or spleen) induced by filtered tumor homogenates were *Eco*RI digested and analyzed for fragments that hybridized with the BAM probe. The viruses indicated over the lanes designate the AEV that originated in the donor tumor (D) and was present in the tumors of recipient chicks (RI, R2, etc.). The control lane displays DNA from a bird that was heterozygous for c-*erb*B$^{I}$ and c-*erb*B$^{II}$. Numbers with asterisks indicate the sizes in kb of *Eco*RI fragments of the new AEVs, whereas numbers without asterisks indicate the sizes of *Eco*RI fragments of c-*erb*B$^{I}$ and c-*erb*B$^{II}$.

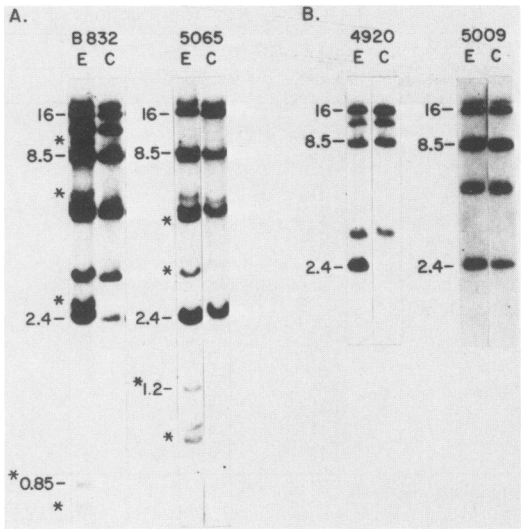


FIG. 6. Erythroleukemias induced by the new AEVs are polyclonal. Autoradiographs displaying LTR related sequences in DNAs from erythroleukemias caused by proviral insertions into c-*erb*B (A) or viral transductions of c-*erb*B (B). Autoradiographs are of Southern blots of *Eco*RI-digested DNAs from chickens B832, 5065, 4920, and 5009. Blots were hybridized with the 3′ LTR probe. Lanes designated E contain DNA from an erythroleukemia (bone marrow or spleen), and lanes designated C contain DNA from a control tissue (bursa). Numbers beside lanes give sizes in kb of *ev* 1 fragments and internal fragments of RAV-1 proviral DNA. Numbers with an asterisk designate fragments that represent proviral junctions with c-*erb*B (Tables 1 and 2). Asterisks designate fragments that represent proviral junctions with other host sequences. The 2.4-kb fragment in the erythroleukemia in chicken 4920 is an internal fragment of RAV-1.

## DISCUSSION

We have presented evidence for c-*erb*B undergoing a high frequency of transduction in cases of RAV-1-induced erythroblastosis (Tables 2 and 3; Fig. 3, 4, and 5). We have also presented evidence that erythroblastosis-inducing proviral insertions tend to be upstream of and in the same

TABLE 4. Stages of leukemic cells during the progression of erythroblastosis[a]

| Inititating event | Diseased chicken | % Erythroblasts in leukemic cells | | |
|---|---|---|---|---|
| | | −6, 7 days | −3, 4 days | Work-up |
| Proviral insertion | 4888 | NA | 38 | 85 |
| | 5065 | TFTC | 57 | 98 |
| Viral transduction | 4920 | TFTC | 92 | 95 |
| | 5005 | TFTC | 10 | 13 |

[a] Erythroblasts and polychromatic erythrocytes were counted in blood smears harvested at the indicated times. NA, Not available; TFTC, too few to count.

transcriptional orientation as c-*erb*B (Table 1, Fig. 2). Our results indicate that most of these insertions have retained both LTRs as well as sequences that are critical to the packaging of RNAs into virions (Table 1, Fig. 2). We think that the position, orientation, and sequence content of erythroblastosis-inducing proviral insertions is key to the high-frequency transduction of c-*erb*B.

**Transduction of host sequences by retroviruses.** Analyses of the nucleotide sequences of transducing viruses and transduced host genes indicate that 5′ virus-host junctions occur in either intron or exon sequences of the transduced gene (18, 40, 41, 47), whereas 3′ host-virus junctions tend to occur within exons of the transduced gene (18, 47). Thus 5′ junctions appear to involve recombination of viral sequences with chromosomal DNAs, whereas 3′ junctions may involve recombination of viral sequences with spliced RNAs (4, 44). Both of these junctions may form during normal steps of the virus life cycle, with 5′ junctions resulting from the integration of proviral DNA into chromosomal DNA and 3′ junctions being formed during the reverse transcription of RNAs that have been packaged together in a virion (14).

We think that transductions of c-*erb*B by RAV-1 are most likely initiated by erythroblastosis-inducing proviral insertions. Any such provirus that underwent deletion of 3′ sequences (but not 5′ LTR and 5′ packaging sequences) would promote read through transcripts containing packaging as well as c-*erb*B sequences. For transduction to occur, the read through transcript would have to (i) be polyadenylated and spliced so as to retain essential 5′ viral as well as c-*erb*B sequences, (ii) be packaged into a virion (using proteins provided by a helper virus), and (iii) undergo recombination with essential 3′ viral sequences during reverse transcription in the next round of infection.

At present our evidence for transduction occurring according to this model is circumstantial. Most erythroblastosis-inducing proviral insertions appear to have sites of insertion, orientations, and sequence contents that would allow them to initiate viral c-*erb*B transcripts that could be packaged into virions (Table 1) (13). Interestingly, most other series of virus-induced tumors that have exhibited cancer-associated proviral insertions have not had sites of insertion, orientations, and sequence contents that would have allowed them to initiate packageable viral c-*onc* transcripts (7, 8, 12, 27, 28, 38, 48). These tumors have not been associated with the high frequency of transduction of the target gene. Thus the association of insertions that could yield packageable virus-cell transcripts with a high frequency of transduction lends credence to the hypothesis that such insertions generate transductions.

**Differences in the erythroleukemias induced by proviral insertions and viral transductions of c-*erb*B.** Erythroleukemias induced by proviral insertions differed from those induced by the new AEVs in that insertion-induced leukemias tended to become more primitive as the disease progressed (Table 4). This may reflect the fact that insertion-induced erythroleukemias are clonal, whereas most AEV-induced leukemias are polyclonal (Fig. 2, 3, and 6). Erythroleukemias induced by proviral insertions represent at least 30 population doublings of the founder cell (leukemic chickens have at least $10^9$ leukemic cells). During the course of these doublings certain leukemic cells may acquire additional mutations that result in their not differentiating beyond the blast stage. In contrast, in AEV-induced leukemias, the infecting virus transforms many erythroid cells, and individual transformed cells may not undergo sufficient numbers of doublings for chance mutations to override the phenotype conferred by the AEV. Thus, in most AEV-induced leukemias the primitiveness of the leukemia appears to be determined by sequences in the AEV, not by the progression of the disease.

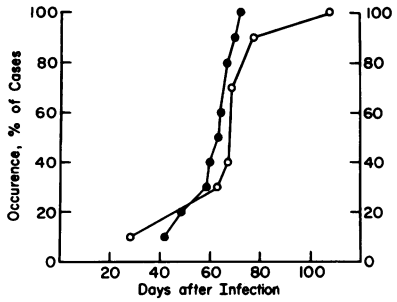


FIG. 7. Time of onset of erythroblastosis induced by proviral insertion into or viral transduction of c-*erb*B. Symbols: ●, erythroblastosis induced by a proviral insertion; ○, erythroblastosis induced by a viral transduction.

**Oncogenic potential of the new AEVs.** Like AEV-R and AEV-H, each of the new AEVs caused rapid onset erythroblastosis. However, unlike AEV-R and AEV-H (15, 16), the new AEVs do not appear to transform cultured cells or cause fibrosarcoma in chickens (42). Also, as we have begun to pass the new AEVs, we have discovered that stocks of two of the new AEVs contain angiosarcoma as well as erythroblastosis-inducing transductions of c-*erb*B (42; work in progress). Thus the new AEVs have revealed a new disease potential for c-*erb*B sequences.

Recent sequence data indicate that c-*erb*B encodes the transmembrane and cytoplasmic domains of the receptor for epidermal growth factor (9, 21, 29, 43, 50). RAV-1 infects a wide variety of chicken tissues. Presumably the transduced c-*erb*B sequences (which are packaged in proteins encoded by RAV-1 and expressed by transcriptional control elements acquired from RAV-1) also infect many cell lineages. However, AEVs appear to cause disease only in erythroblasts, fibroblasts, or angioblasts. This puzzling result suggests that only certain cell types respond to the transmembrane and cytoplasmic domains of the epidermal growth factor receptor in such a way that they can become transformed by c-*erb*B-encoded proteins.

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