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Rous-Associated Virus 1-Induced Erythroleukemic Cells Exhibit a Weakly Transformed Phenotype In Vitro and Release c-*erbB*-Containing Retroviruses Unable to Transform Fibroblasts

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Avian leukosis viruses induce erythroblastosis in chicks by integrating into the c-*erbB* gene and thus activating c-*erbB* transcription. We characterized Rous-associated virus 1-induced leukemic erythroblasts in vitro and showed that they mostly resemble erythropoietin-independent but otherwise normal erythroid progenitors. Some leukemic cells, however, were able to both differentiate and proliferate extensively in vitro. All 14 leukemias studied expressed high levels of *erbB*-related proteins that were 5 to 10 kilodaltons larger but otherwise very similar to the gp74^{erbB} protein of avian erythroblastosis virus ES4 with respect to biosynthesis, glycosylation, and cell surface expression. Two leukemias contained and released retroviruses that transduced *erbB*. Chicken embryo fibroblasts fully infected with these viruses expressed high levels of *erbB* RNA and protein but retained a normal phenotype. Our results suggest that certain forms of c-*erbB*, activated by long terminal repeat insertion or viral transduction, are capable of inducing erythroleukemia but unable to transform fibroblasts.

Leukemia-inducing avian retroviruses have been subdivided into two main groups (for a review, see reference 12). The acute leukemia viruses, which contain cell-derived oncogenes, rapidly transform specific types of hematopoietic cells in vivo and in vitro (6, 10, 15, 26). In contrast, the avian leukosis viruses (ALVs), which lack oncogenes, induce a wide variety of leukemias and other neoplasms in chickens but do not transform cells in vitro (22, 35).

One important step in the development of avian B-cell lymphomas is the activation of c-*myc* transcription by integration of an ALV provirus juxtaposed to the oncogene (18, 24). Similarly, erythroleukemic cells induced by ALVs in certain strains of inbred chickens carry an ALV provirus next to the c-*erbB* locus and express greatly enhanced levels of c-*erbB* RNA (9, 25a). Thus, both the broad oncogenic spectrum of ALVs and their long latency period could be explained by the hypothesis that these viruses can activate certain cellular oncogenes by integration either next to or within them (9).

Although certain molecular events leading to the activation of the c-*myc* gene by ALV promoter insertion have been elucidated, it is still unclear how this event converts a normal avian lymphoid precursor into a leukemic B-lymphoma cell. Further study of this question is difficult, since neither normal nor leukemic chicken B-lymphoblasts can be easily grown in culture and the available v-*myc*-containing avian retroviruses do not transform lymphoid cells in vitro.

Several reasons suggest that ALV-induced chicken erythroleukemia might provide a system for studying how insertional activation of the c-*erbB* gene leads to erythroblast transformation. First, both normal and leukemic erythroid precursors can be grown and analyzed in vitro (5, 28, 29). Second, avian erythroid precursors can be transformed in vitro by retroviruses that contain the v-*erbA* and v-*erbB* oncogenes or the v-*erbB* gene alone (3b, 8, 14, 20). Erythroblasts transformed by v-*erbB* or other oncogenes of the *src* family proliferate independently of the erythroid differentiation hormone erythropoietin (Epo), but also undergo spontaneous differentiation into erythrocytes with a certain frequency (3b, 3c, 5, 20). v-*erbA* acts in concert with v-*erbB* by fully arresting the differentiation of the infected erythroid progenitors and enabling them to grow in standard tissue culture media (8, 13). Finally, leukemic cells containing an activated c-*erbB* gene should express *erbB*-related proteins at their cell surface, permitting them to be distinguished from uninfected precursors at the single-cell level (3, 17).

Although molecular studies on numerous Rous-associated virus 1 (RAV-1)-induced leukemias have unraveled two possible mechanisms of c-*erbB* activation, i.e., provirus insertion and c-*erbB* transduction (9, 25a, 30a), no attempts have been made to characterize the leukemic cells in vitro to determine whether c-*erbB* activation leads to elevated expression of *erbB* gene products and causes a truly leukemic phenotype (25).

In this paper, we demonstrate that pure populations of RAV-1-induced erythroleukemic cells could be explanted and studied in vitro. These cells resemble hormone-independent erythroid progenitors and expressed *erbB*-related cell surface glycoproteins at high levels. Some leukemias contained new c-*erbB*-transducing retroviruses, which efficiently replicated in chicken embryo fibroblasts without transforming them.

MATERIALS AND METHODS

Viruses, chickens, and induction of erythroleukemia. Cloned stocks of RAV-1 (9) were obtained from L. Crittenden, East Lansing, Mich. Chickens of the inbred strain L15-1 were injected with RAV-1 in East Lansing as described previously

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(9). In a second series of experiments, L15-1 chicks were hatched from embryonated eggs shipped to Heidelberg and were injected via the leg vein with 0.1 ml of undiluted RAV-1 supernatant (10^6 to 10^7 infectious units/ml). They were monitored for leukemia development by inspection of blood smears as described previously (20). During a preleukemic phase of 40 to 60 days, low numbers of partially mature and immature erythroid cells appeared in the peripheral blood of 8 of 10 chicks. Shortly afterwards, four chicks rapidly developed lethal erythroleukemia, in which the buffy coat-containing leukemic blasts represented up to 30 to 50% of the total blood cell volume.

Purification of leukemic cells. Leukemic cells obtained from moribund chicks by heart puncture were washed twice in phosphate-buffered saline, suspended at 10×10^7 cells per ml in CFU-E medium without anemic serum (25), and centrifuged through a layer of Percoll (density, 1.072 g/cm^3 [3]). The immature cells banding at the interphase represented essentially pure leukemic cells, since more than 90% of them expressed markers of erythroid cells as well as *erbB* proteins at their surface, as revealed by fluorescent staining with *erbB*-specific antisera (3).

Cells and cell culture. For in vitro culture experiments, purified leukemic cells were seeded at 10×10^6 to 20×10^6 cells per ml in CFU-E medium with or without anemic serum and supplemented with 1 μg of insulin (Actrapid; Bayer, Leverkusen, Federal Republic of Germany) per ml. Two days later, erythrocytes and dead cells were removed by centrifugation through Ficoll (4), and cells were reseeded at 2×10^6 to 5×10^6 cells per ml in the same medium. Cultures were fed daily by the addition of fresh medium, and cell numbers were kept above 5×10^5 cells per ml by reducing culture size. Cultures were considered as nongrowing when all immature cells initially present had differentiated into erythrocytes without apparent massive cell death. Proliferating leukemic cultures were kept at cell densities between 1×10^6 and 4×10^6 cells per ml.

Chicken embryo fibroblasts were prepared from 11-day-old SPAFAS embryos as described previously (11) and cultivated in standard growth medium (Dulbecco modified Eagle medium supplemented with 8% fetal calf serum, 2% chicken serum, and 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.3). Normal bone marrow cells were prepared from 1- to 4-week-old chicks as described previously (11). The origin and cultivation of erythroblasts transformed by avian erythroblastosis virus (AEV)-ES4 (LSCC HD3) and by AEV S13, which contains a previously undetected oncogene rather than v-*erbB* (3a), have been described earlier (1, 3b).

Plasma clot colony assay. The plasma clot colony assay was performed essentially as described earlier (5). Briefly, 10^5 cells were seeded into differentiation medium with or without anemic serum (3) supplemented with 20% Methocel in Iscoves Dulbecco modified Eagle medium–2 mg of fibrinogen per ml–0.1 U of thrombin. After clot formation at 37°C and 5% CO_2, cultures were kept at 41°C and 2% CO_2 and processed either 3 or 6 days later as described previously (5).

Immunoprecipitation analysis. Fresh and cultivated RAV-1-induced leukemic cells (10×10^6 to 20×10^6) were labeled with [^{35}S]methionine (250 μCi) or [^{3}H]glucosamine (200 μCi) as described earlier (3). Preparation of lysates, immunoprecipitation with rat antiserum to *erbA* plus *erbB* (*erbA* + *B*) proteins or specific for *erbB* protein, and analysis of immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were done as described previously (16, 17). Treatment of cells with tunicamycin during metabolic labeling was done as described previously (17).

Analysis of DNA and RNA. DNA and RNA from cells and tissues was purified and analyzed essentially as described previously (8, 33). The first exon probe was made from a subcloned 4.5-kilobase (kb) *Eco*RI fragment that encodes the first exon of c-*erbB* hybridizing with v-*erbB* (33). The *erbA* + *B* probe was made from a subcloned 2.5-kb *Pvu*II fragment from AEV (34). The *erbB* 3′ probe represents sequences present in a 570-nucleotide *Bam*HI-*Eco*RI fragment of v-*erbB* (34). The long terminal repeat (LTR) probe was made from a 1.5-kb *Eco*RI-*Bam*HI fragment of AEV that contains parts of *env*, LTR, and 5′-untranslated sequences of AEV.

Infection of chicken embryo fibroblasts with *erbB*-containing retroviruses released by leukemic cells. Cultivated RAV-1 erythroblasts (BH02, BH03) (10×10^6) were seeded into 60-mm dishes containing CFU-E medium plus insulin. After the addition of 10^6 chicken embryo fibroblasts freshly trypsinized from minced embryos (11), the cultures were incubated for 2 days at 39°C. Thereafter, the dishes were carefully rinsed with standard growth medium to remove nonadherent cells, and the adherent fibroblasts were propagated in standard growth medium with daily rinses to remove any nonadherent cells. This procedure effectively removed all leukemic erythroblasts, which do not survive in standard growth medium. After an additional five to seven passages in standard growth medium, fibroblasts were frozen in liquid nitrogen or used for analysis.

Assays for fibroblast transformation parameters. Immunofluorescent staining for actin cables and fibronectin protein network were done as described previously (23). Hexose uptake was measured as described previously (27). Undiluted, filtered supernatants from either leukemic cells or infected fibroblasts were tested for focus formation on chicken embryo fibroblasts as described previously (8).

Immunofluorescence. Staining of viable leukemic erythroblasts with fluorescent *erbB*-specific antibodies and differentiation-specific antibodies was done as described previously (3, 6). To assay *erbB* protein expression at the surface of infected fibroblasts, cells were detached from the dish by EDTA treatment and stained in suspension as described above (17).

RESULTS

Characterization of RAV-1-induced erythroleukemia cells from leukemic animals and after culture in vitro. Purified leukemic cells from moribund, RAV-1-infected chicks (see above) were first characterized for their state of erythroid differentiation. The leukemic cells consisted almost exclusively of erythroid cells at various stages of maturation, about 60% of which resembled immature erythroblasts (Table 1).

To test whether the RAV-1-induced erythroleukemic cells could be grown in tissue culture, we seeded the purified leukemic cells in CFU-E medium, since erythroid cells transformed by v-*erbB* alone proliferate well in this complex medium but quickly die in standard growth medium (8, 25). Three of eight leukemias were cultured successfully. In these cultures, total cell numbers decreased during the first 5 to 7 days. However, clumps of immature cells persisted among the increasing fraction of erythrocyte-like cells (Fig. 1B). Thereafter, the immature cells started to proliferate and continued to grow for up to 3 weeks (Fig. 1B) with doubling times of about 24 h. In contrast, the leukemic cells from the other five animals tested survived in culture for the first 4 to 7 days, but then they all differentiated into erythrocytes. As

TABLE 1. Characterization of leukemic cells

Cell type	% Cells classified as[a]:			% Cells stained with[b]:		
	Ebl	ER	LR + E	αEry	αEbl	αMbl
Fresh leukemic cells						
BH02	70	17	13	53	61	5
BH03	ND[c]	ND	ND	61	66	3
HM27237[d]	77	17	6	ND	ND	ND
Peripheral blood	0	1	>95	>95	0	5
Leukemic cells after 10 days in CFU-E medium						
BH02	83	2	15	52	70	<1
BH03	82	9	9	ND	ND	ND
HM27237	95	2	3	ND	ND	ND
AEV-ES4[e]	>99	<0.1	<0.1	<1	99	<1

[a] Cell types were defined by benzidine (at neutral pH) plus histological staining (5). Ebl, Erythroblasts; ER, early reticulocytes; LR, late reticulocytes; E, erythrocytes.

[b] Characterization of these antisera has been described elsewhere (6). αEry, Antierythrocyte; αEbl, antierythroblast; αMbl, monoclonal antibody 51/2 directed against myeloblasts.

[c] ND, Not determined.

[d] Smears prepared from frozen cells immediately after thawing.

[e] *ts*167 AEV clone E3 (3).

expected, the leukemic cells fom all animals died after 2 to 3 days in standard growth medium.

When characterized for differentiation parameters, the cultured RAV-1-induced erythroleukemic cells again consisted of a mixture of erythroblasts and more mature erythroid cells (Table 1; Fig 1C). Thus, both fresh and cultivated RAV-1-induced erythroleukemic cells resemble erythroblasts transformed by v-*erbB* or *src* (3b, 20).

RAV-1-induced erythroleukemia cells proliferate and differentiate independent of exogeneous Epo. The above findings prompted us to study in more detail how RAV-1 erythroleukemic cells differ from normal, late erythroid progenitors (CFU-E cells) in their ability to self-renew and differentiate in vitro and in their dependence on exogeneously added Epo. Purified leukemic cells of chicks BH02 and BH03 were seeded into plasma clot cultures in the presence or absence of anemic serum as a source of chicken Epo (5). Normal bone marrow cells were tested as controls. After 3 days of incubation, about 14% of the leukemia-derived colonies consisted entirely of immature erythroid cells (type III, Fig. 1A), a colony type which was absent from the controls. About 30% of the colonies contained partially mature erythroid cells (type II, Fig. 1A), whereas more than half of the leukemia-derived erythroid colonies were indistinguishable from the normal CFU-E colonies (type I, Fig. 1A), obtained almost exclusively in the normal bone marrow controls (Table 2; data not shown).

Table 2 also demonstrates that RAV-1-induced erythroleukemic cells grew into undifferentiated as well as differentiated colonies with similar efficiencies in the presence and absence of anemic serum, whereas normal CFU-E colonies were stimulated more than 20-fold by the addition of anemic serum.

When the plasma clot cultures of RAV-1-induced erythroleukemic cells were incubated for 6 instead of 3 days before analysis, the mature and partially mature colonies had disintegrated. However, about 20% of the undifferentiated, type III colonies seen at day 3 had grown into large colonies containing between 2,000 and 10,000 cells. About half of these colonies were completely undifferentiated, while the other half consisted of undifferentiated cells as well as more differentiated cells. Taken together, these results indicate that the majority of the RAV-1-induced erythroleukemic cells resembled Epo-independent but otherwise normal CFU-E precursors, while a minority of the cells exhibited a sustained self-renewal capacity in vitro.

RAV-1-induced erythroblasts express high levels of an *erbB*-related glycoprotein at their surface. To study whether the RAV-1-induced erythroblasts expressed *erbB*-related proteins, fresh leukemic cells from one chicken (BH02) were labeled with [^{35}S]methionine and immunoprecipitated with anti-*erb* sera (16, 17). Antisera reactive either with both v-*erbA* and v-*erbB* or with v-*erbB* alone immunoprecipitated a group of 74- to 76-kilodalton (kDa) proteins (Fig. 2A, lanes 1 to 3) probably representing rough endoplasmic reticulum precursors of *erbB*-like proteins (16, 17). In contrast, antibodies to viral structural proteins or antibodies reacting with the *gag* or v-*erbA* domains of p75$^{gag\text{-}v\text{-}erbA}$ did not immunoprecipitate the 74- to 76-kDa proteins (Fig. 2A, lanes 4 and 5; data not shown).

When leukemic cells from 12 other RAV-1-infected chicks were analyzed, proteins of slightly differing sizes were immunoprecipitated by *erbB*-specific serum. Four of the leukemias contained 74- to 76-kDa proteins (Fig. 2B, lane 4), whereas two groups of three animals each displayed smaller proteins of 72 to 74 kDa (lane 3) and 70 to 72 kDa (lanes 1 and 2), respectively. The leukemic cells of one animal expressed two distinct protein species of 69 to 71 kDa and 74 to 76 kDa (Fig. 2B, lane 5). In all cases in which the leukemic cells could be grown in culture, the proteins detected in the fresh leukemic cells were indistinguishable from those found in the cells from the same animal after 10 days of in vitro culture (Fig. 2C, lanes 1 and 2).

Leukemic cells from chick BH02 were labeled with [^{3}H]glucosamine and immunoprecipitated to determine whether their *erbB*-related proteins were glycosylated. A protein of 83 kDa was detected which probably represented the mature cell surface form of the 74- to 76-kDa *erbB*-related proteins found in these cells after [^{35}S]methionine labeling (Fig. 3A). When the cells were labeled with [^{35}S]methionine in the presence of tunicamycin, a 72-kDa nonglycosylated form of the gp83^{erbB} protein could be detected (Fig. 3B). These results indicate that the RAV-1 erythroblasts expressed an *erbB* protein that was 9 to 10 kDa larger but otherwise closely related to the gp74^{erbB} protein of AEV-ES4 erythroblasts. This was confirmed by two-dimensional peptide-mapping studies and by immunoprecipitation of *erbB* proteins from RAV-1-induced erythroblasts with an antiserum to a peptide of the human epidermal growth factor (EGF) receptor (21; data not shown).

Finally, we tested whether fresh and cultivated erythroleukemic cells expressed *erbB*-related proteins at their surface. Virtually all immature erythroblast-like cells from both fresh and cultivated leukemic cells were strongly stained by *erbB*-specific antibodies (Fig. 4A; data not shown), while the mature cells apparently underwent down-regulation of *erbB* protein expression as seen in differentiated temperature-sensitive AEV erythroblasts (Fig. 4B) (3).

Two RAV-1-induced leukemias contain retrovirus-transduced *erbB* genes. Previous studies have shown that in a large number of RAV-1-infected leukemic chicks the ALVs had integrated close to the first c-*erbB* exon homologous to v-*erbB*, leading to highly elevated levels of c-*erbB* transcripts (9, 25a). As will be shown below, two of the ALV-induced leukemias studied here (BH02 and BH03, Table 1)

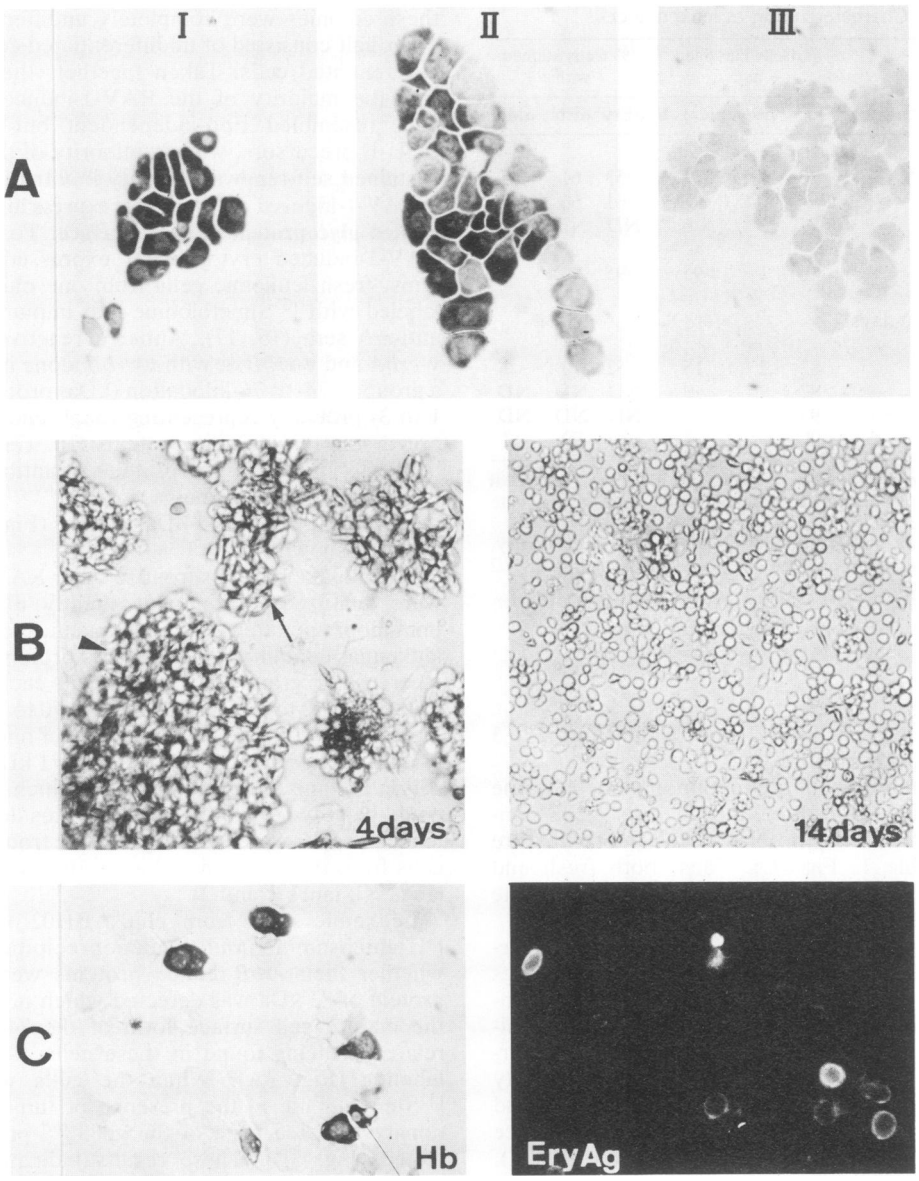

FIG. 1. In vitro characterization of RAV-1-induced leukemic cells. (A) Purified leukemic cells of chicken BH02 were seeded into plasma clot cultures without anemic serum. Three days later, clots were processed as described previously (5). Shown are photographs of mature (I), partially mature (II), and immature (III) colonies taken under blue light (5) to reveal staining for hemoglobin. (B) Photographs of leukemic cells from chicken BH02 are shown after 4 and 14 days of in vitro culture. Note clumps of mature cells (arrow) and immature cells (arrowhead) visible after 4 days. (C) Leukemic cells from chicken BH02 were cytocentrifuged onto slides and stained with neutral benzidine for hemoglobin (Hb) or stained with antierythrocyte serum (EryAg; 6) by indirect immunofluorescence. The culture consists of a mixture of immature and more mature cells, as revealed by both markers.

contained unmodified c-*erbB* genes as well as retrovirus-like elements that had transduced exons but not introns of c-*erbB*. These elements were present at multiple integration sites, suggesting an oligoclonal or polyclonal origin of the leukemias.

The first 5′ exon of the c-*erbB* gene with homology to v-*erbB* represents about 300 nucleotides encoded in a 4.5-kb (c-*erbB* α allele) or 2.3-kb (c-*erbB* β allele) *Eco*RI fragment (25a). Since ALV LTRs contain *Eco*RI sites, integration of ALV into either of these fragments would interrupt them and thus generate two smaller fragments that can be detected by using the subcloned 4.5-kb *Eco*RI fragment of the c-*erbB* α allele as a probe (first exon probe). Chick BH02 was homozygous for the α allele, and both alleles appeared to be intact in the BH02 leukemic cells (Fig. 5A). In addition, a single novel, weakly hybridizing, 3.8-kb fragment was detected with this probe. The same 3.8-kb fragment hybridized strongly with a v-*erbA* + *B* probe (which encodes all of v-*erbB*) and with a probe representative for the carboxy-terminal part of v-*erbB* (Fig. 5B and C). A second tumor-specific fragment of 2.2 kb was also detected with these probes, but it did not hybridize to the first exon probe. Finally, the additional *erbB* sequences in the leukemic cells were contained in a DNA segment similar in size to those in v-*erbB*, since double digestion with *Apa*I and *Eco*RI (which cut at the extreme ends of the AEV-ES4 v-*erbB* gene) generated a fragment of 1.7 kDa in the leukemic-cell DNA (Fig. 5D) which is of the same size as the corresponding

TABLE 2. Effect of Epo on RAV-1-induced erythroleukemic cells

Cell type analyzed	Plasma clot colonies							
	+Epo				−Epo			
	Colonies/10^5 cells	% Type[a]:			Colonies/10^5 cells	% Type:		
		I	II	III		I	II	III
Fresh BH02								
Leukemic cells	2,550	58	29	13	2,580	56	30	14
Normal bone marrow	1,150	92	8	0	50	80	20	0
AEV-ES4[b]	TMTC[c]	<0.1	1	99	TMTC	<0.1	1	99

[a] Types of colonies (I, differentiated colonies; II, partially differentiated or mixing colonies; III, colonies containing immature erythroblasts) are as shown in Fig. 1.
[b] *ts*167 AEV clone E3 (3).
[c] TMTC, Too many to count.

v-*erbB* fragment. Analysis of leukemic cells after 14 days of in vitro culture gave the same results as obtained with the fresh leukemic cells (data not shown).

A parallel analysis performed on the *erbB* gene of the BH03 leukemic cells (Table 1) gave similar results as above, except that these cells were heterozygous for the α and β *erbB* alleles and that the novel *erbB* fragment, which hybridized weakly with the first exon probe but strongly with the *erbA* + *B* and *erbB* 3′ probes (Fig. 5B plus C), had a different size (3.4 kb) than in leukemia BH02.

In conclusion, the above results show that both leukemic-cell DNAs contain 5′ and 3′ v-*erbB* sequences in a fragment of similar size as in v-*erbB*, suggesting that *erbB* is present in these leukemias as a transduced sequence lacking introns. An analysis of leukemic-cell DNA from both leukemias after digestion with *Sac*I gave results supporting this hypothesis (data not shown).

We then studied whether the leukemic cells were polyclonal, as a result of spread of a c-*erbB*-containing retrovirus, or clonal, as a consequence of reverse transcription and subsequent reintegration of c-*erbB* mRNA. Leukemic-cell DNA was digested with restriction enzymes that do not

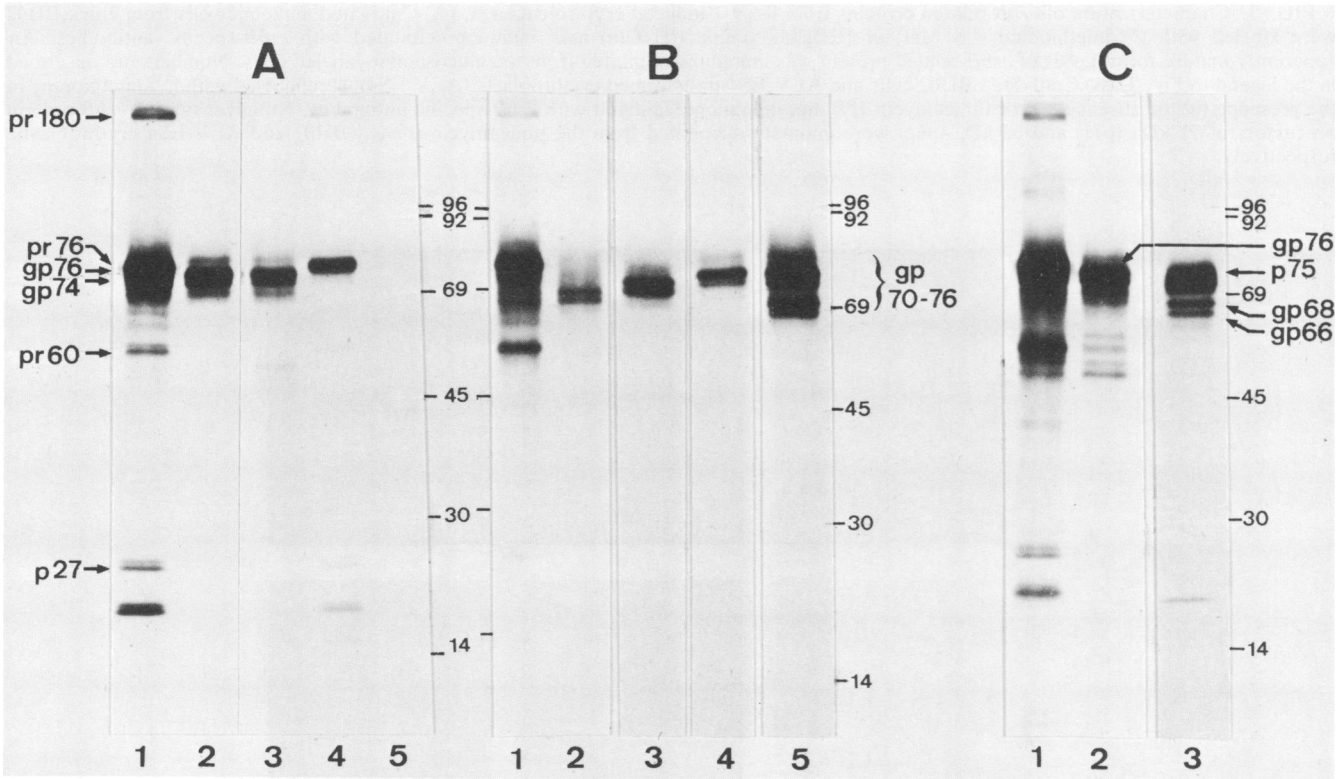

FIG. 2. RAV-1-induced erythroleukemias express *erbB*-related proteins. (A) Purified leukemic cells from chick BH02 were labeled with [^{35}S]methionine, and extracts were immunoprecipitated with anti-*erbA* + *B* serum (17) (lane 1), the same serum preincubated with virus lysate (2) (lane 2), anti-*erbB* serum (16) (lane 3), or anti-*gag-erbA* serum without (lane 4) or with (lane 5) competing virus. The positions of *erbB*-related proteins (gp74, gp76, probably representing rough endoplasmic reticulum precursors) are indicated. Small numerals indicate molecular weight markers (×10^3). (B) Leukemic cells from chicks BH03 (lanes 1 and 2), HM29393 (lane 3), HM27235 (lane 4), and HM8641 (lane 5) were immunoprecipitated with anti-*erbA* +*B* serum without (lane 1) or with (lanes 2 to 5) competing virus. (C) Leukemic cells from chick BH02 were cultivated for 14 days and then labeled and immunoprecipitated with anti-*erbA* + *B* serum without (lane 1) or with (lane 2) competing virus. The positions of *erbB*-related proteins (gp76) and of the AEV-ES4-encoded proteins (lane 3) p75$^{gag\text{-}erbA}$ (p75) and gp74^{erbB} precursors (gp66, gp68) are indicated.

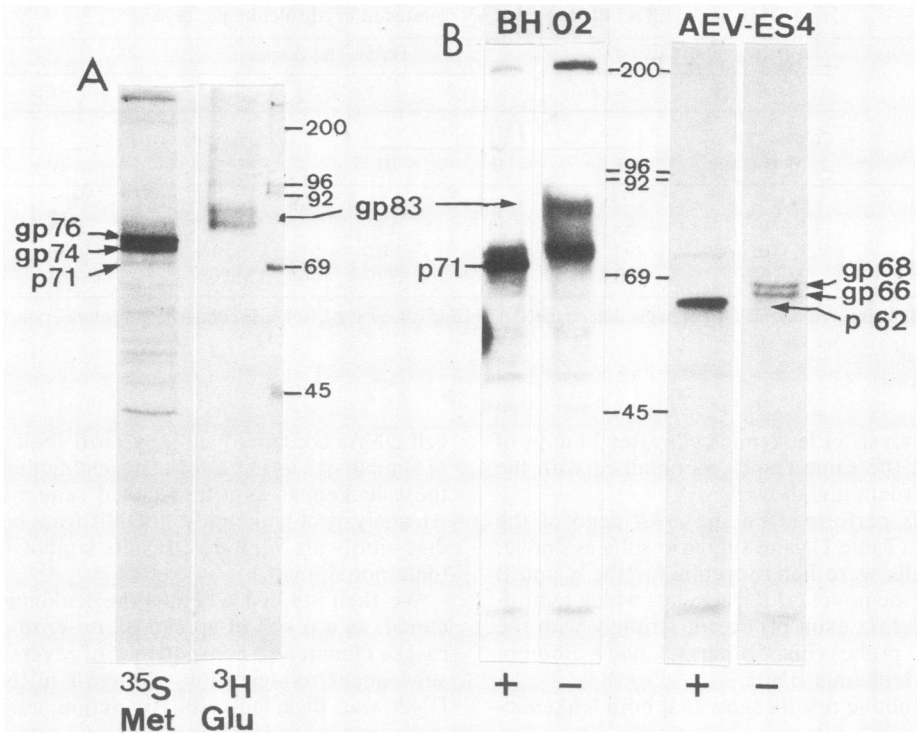

FIG. 3. Characterization of *erbB*-related proteins from RAV-1-induced erythroleukemia. (A) Cultivated leukemic cells from chick BH02 were labeled with [^{35}S]methionine (^{35}S Met) or [^{3}H]glucosamine (^{3}H Glu) and immunoprecipitated with *erbB*-specific antibodies. An apparently mature form (gp83) of *erbB*-related protein was immunoprecipitated from the glucosamine-labeled cells. Numbers are described in the legend to Fig. 2. (B) Cultivated BH02 cells and AEV-ES4-transformed erythroblasts (AEV-ES4) were labeled with [^{35}S]methionine in the presence (+) or absence (−) of tunicamycin (17) and immunoprecipitated with *erbB*-specific antibodies. Nonglycosylated *erbB* protein precursors of 71 kDa (p71) and 62 kDa (p62) were immunoprecipitated from the tunicamycin-treated BH02 and AEV-ES4 erythroblasts, respectively.

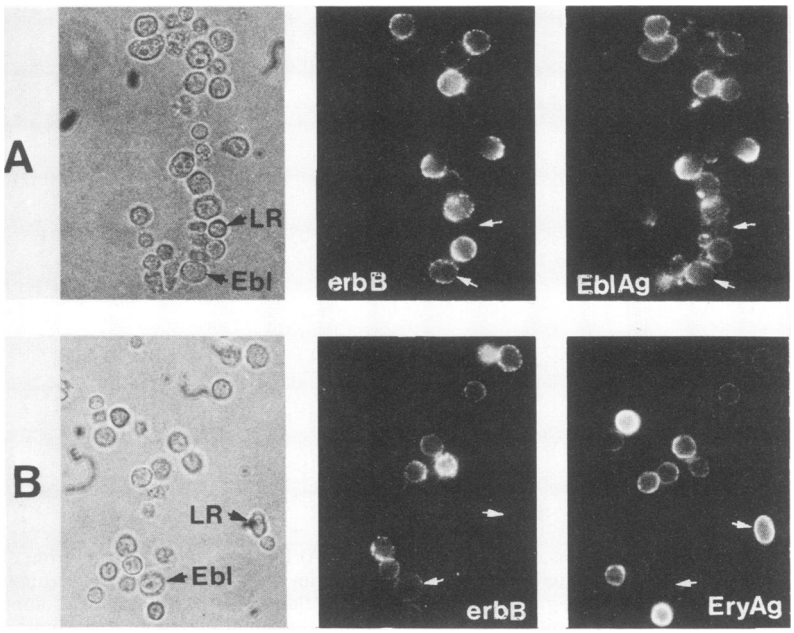

FIG. 4. *erbB*-related proteins of RAV-1-induced leukemias are expressed at the cell surface. Purified leukemic cells from chick BH02 were double labeled with anti-*erbB* serum (*erbB*) plus antierythroblast serum (A; EblAg) or antierythrocyte serum (B; EryAg) (6) in indirect immunofluorescence as described earlier (3). Photographs of the same cells viewed with bright-field illumination (left panels), fluorescein isothyocyanate-conjugated fluorescence (*erbB*, middle panels), or rhodamine fluorescence (EblAg, Ery-Ag; right panels) are shown. Erythroblasts (Ebl) and late reticulocytes (LR) are marked with arrows.

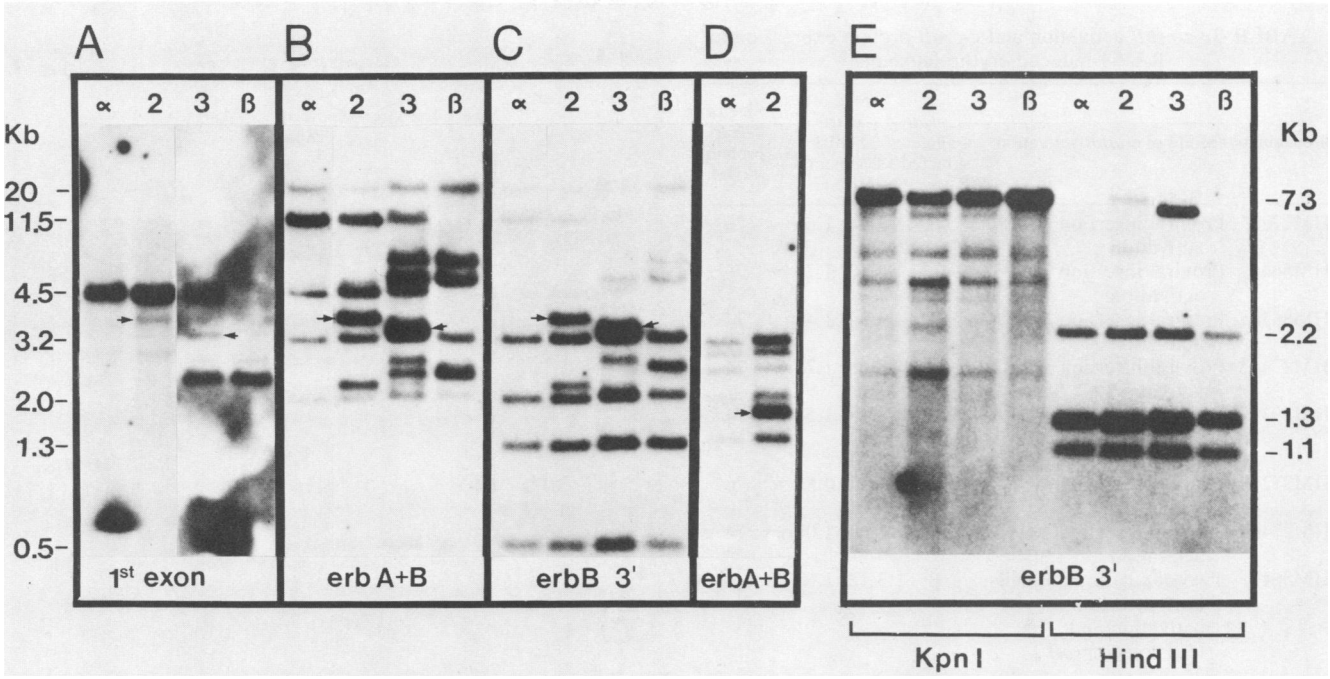

FIG. 5. Identification of transduced *erbB* sequences in two RAV-1-induced leukemias. DNA was purified from the in vitro-cultivated leukemias BH02 and BH03 and subjected to analysis by the Southern transfer technique (29a). The restriction patterns of the *erb* sequences in the leukemic cells were compared with those of normal chicken DNAs homozygous for either the α or β allele of *erbB*. The arrows indicate novel *erbB*-specific fragments. (A) The DNAs were cut with *Eco*RI and hybridized with a probe representative for the first exon of c-*erbB* homologous to v-*erbB*, as described in the text. (B and C) The *Eco*RI-digested DNAs were hybridized with probes representative for v-*erbA* + *B* and the v-*erbB* 3′-specific probe, respectively. (D) DNA from BH02 was digested with *Apa*I and *Eco*RI and hybridized with the v-*erbA* + *B* probe. (E) The DNAs were cut with *Kpn*I or *Hin*dIII and hybridized with the v-*erbB* 3′-specific probe. The sizes of the major c-*erbB*-specific fragments are indicated to the left and right.

cleave within v-*erbB* and that cut once (*Kpn*I) or twice (*Hin*dIII) in helper virus DNA. No discrete extra *erbB* fragments were detected in the DNA from either leukemia after digestion with *Kpn*I (Fig. 5E), as would have been expected if these leukemias had been of clonal origin. The polyclonal origin of the BH02 leukemic cells was confirmed by *Hin*dIII digestion, which showed a smear of *erbB*-hybridizing fragments with sizes between 5 and 7.5 kb (Fig. 5E). With BH03 leukemic cells, however, a discrete fragment of 7 kb was seen after *Hin*dIII digestion, suggesting that this *erbB*-transducing element contained two internal *Hin*dIII sites.

Last, the other 11 RAV-1-induced leukemias were analyzed for the presence of transduced c-*erbB* sequences and for a possible correlation of this event to a particular type of *erbB* protein (Fig. 2). No such correlation could be found (Table 3), because proteins of several sizes were produced both from leukemias exhibiting modified c-*erbB* alleles and from those containing transduced *erbB* sequences. Interestingly, one leukemia contained both an insertional activation and a transduction of *erbB*; consequently, these leukemic cells expressed *erbB* proteins of two different sizes (Fig. 2; Table 3).

RAV-1-induced leukemias with transduced *erbB* sequences express multiple species of *erbB* RNA at elevated levels. Polyadenylated RNA isolated from fresh BH02 leukemic cells as well as from in vitro-cultivated BH02 and BH03 erythroblasts was subjected to Northern analysis with an *erbA* + *B* probe and an LTR probe. One major 6.0-kb RNA hybridizing with both *erb* and LTR probes was seen in the fresh BH02 leukemic cells (Fig. 6A, 2f). After 14 days of in vitro culture, however, two additional *erb*- and LTR-positive RNAs of 7.8 and 5.1 kb were seen (Fig. 6A, 2c). As expected, both RNA samples contained 7.8-kb genomic and 2.8-kb subgenomic RNAs of RAV-1 virus. Similarly, the cultivated BH03 cells contained several RNA species positive with both *erb* and LTR probes (4.5, 3.6, and 3.2 kb [Fig. 6A, 3c]). Similar results were obtained with a v-*erbB*-specific probe (data not shown). This suggests that in vitro culture led to the selection of subpopulations of leukemic cells that contain *erbB*-transducing elements of similar size but have different modes of *erbB* transcription.

Next, we compared the level of *erbB* RNA transcription in RAV-1 leukemic cells with that in AEV-transformed erythroblasts and in normal chicken embryo cells. The level of *erbB* RNA in BH02 cells was about fivefold lower than in AEV-transformed erythroblasts, but still 100- to 200-fold higher than the levels of the 12- and 9-kb c-*erbB* mRNAs in chicken embryo cell RNA (Fig. 6B).

To rule out the possibility that high levels of c-*erbB* transcription are a common event in transformed erythroblasts, the RNA of erythroblasts transformed by oncogenes other than *erbB* was examined. Erythroblasts transformed by S13 virus (3a) contained no c-*erbB* RNA (Fig. 6). Erythroblasts transformed by E26 virus (25) contained low *erbB* RNA levels similar to those found in normal chicken embryo RNA, whereas RNA from normal bone marrow cells (containing erythroid precursors similar to the RAV-1-induced leukemic cells) was negative for c-*erbB* RNA (data not shown). However, c-*erbA* RNAs were found in all three samples, at levels comparable to those found in normal chicken embryonic cells (not shown).

TABLE 3. c-*erbB* activation and c-*erbB* protein expression in RAV-1-induced erythroleukemia

Leukemia	Mode of c-*erbB* activation[a]	c-*erbB* allele affected	Size of extra *Eco*RI fragments (kb)	Size of *erbB* protein (kDa)
HM27235	Proviral insertion activation	α	3.14; 1.66	76
HM8640	Proviral insertion activation	α	3.10; 1.70	76
HM8638	Proviral insertion activation	α	3.32; 1.48	74
HM27442	Proviral insertion activation	α	3.06; 1.74	74
HM29393	Proviral insertion activation	α	3.23; 1.57	74
HM27248	Proviral insertion activation	β	1.77; 0.83	76
HM27444	Proviral insertion activation	β	1.60; 1.00	74
HM8641	Two populations of cells; both insertion activation and transduced *erbB*	β	3.12; 1.68	76 and 70
HM8663	Transduced *erbB*	NR[b]		76
HM8669	Transduced *erbB*	NR		72
HM8660	Transduced *erbB*	NR		72
BH02	Transduced *erbB*	NR		76
BH03	Transduced *erbB*	NR		72

[a] Analysis of leukemic-cell DNA was performed as described previously (9, 25a).
[b] NR, Not relevant.

RAV-1-induced leukemias BH02 and BH03 produce infectious *erbB*-containing retroviruses that do not transform fibroblasts. To test whether the two leukemias containing transduced *erbB* sequences (BH02 and BH03) produce infectious, *erbB*-containing retroviruses, the leukemic cells were cocultivated with primary chicken embryo fibroblasts (see Materials and Methods). Analysis of these fibroblasts of *erbB*-specific RNA after cocultivation revealed that they expressed *erbB* RNAs of similar sizes as seen in the original erythroleukemic cells, although the relative abundance of the different RNA species seemed to be different in fibroblasts and leukemic cells (Fig. 7A). This suggests that *erbB*-containing retroviruses had been transmitted to the fibroblasts in both cases. For the virus released from BH03 cells (referred to as ERB-2 virus below) this could be confirmed by protein analysis, since the infected fibroblasts expressed the expected 70- to 72-kDa *erbB* proteins at levels that were somewhat lower than in the leukemic cells (Fig. 7B) but equivalent to levels seen in AEV-ES4-transformed fibroblasts (data not shown). In contrast, very little *erbB*-related protein could be immunoprecipitated from the fibroblasts cocultivated with BH02 erythroblasts.

To determine the proportion of virus-infected cells in the BH03 cocultivated culture, we tested the live fibroblasts for surface expression of *erbB* proteins with a fluorescent *erbB*-specific antiserum. Some 83% of the fibroblasts scored positive as compared with 86% in a control culture transformed with AEV-ES4 virus. The staining of the ERB-2-infected fibroblasts was stronger than that of the AEV-transformed control cells (data not shown), as was also observed with the corresponding erythroblasts (3).

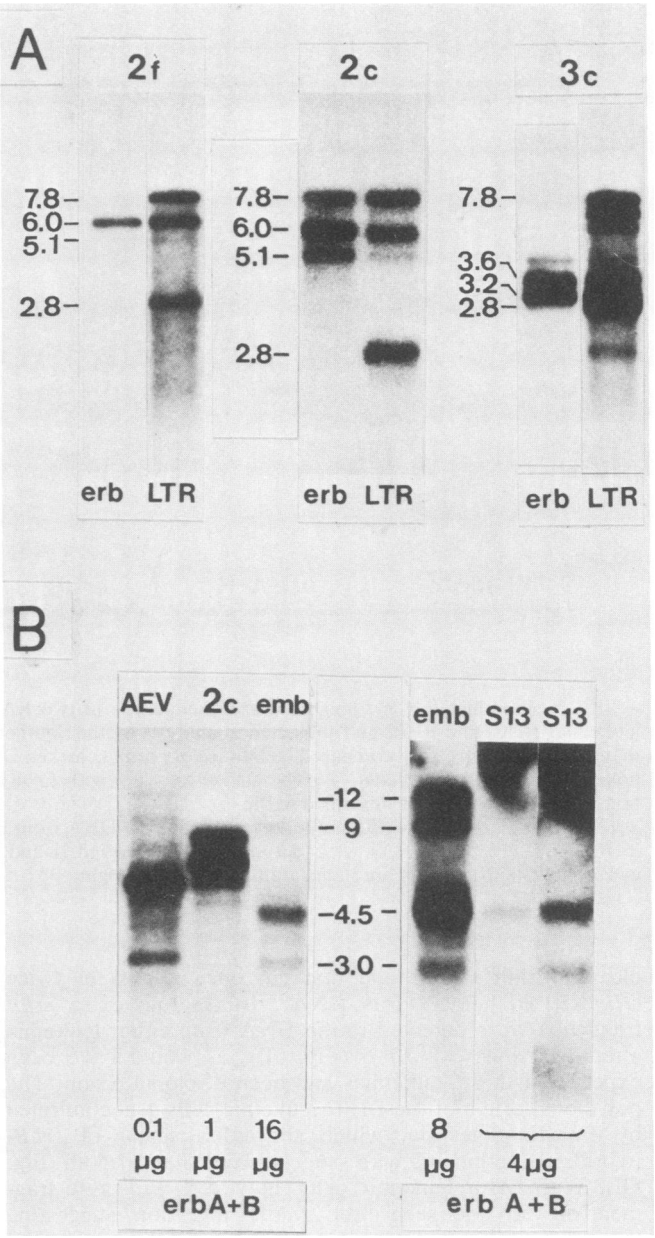

FIG. 6. Leukemias BH02 and BH03 contain abundant novel RNAs that hybridize with *erb* and retroviral sequences. (A) RNA was isolated from the fresh (f) or cultivated (c) BH02 and BH03 leukemic cells and analyzed by the Northern transfer technique. Hybridization was done with either a v-*erbA* + *B* probe (erb) or with a labeled DNA fragment from AEV-containing LTR sequences. (B) The abundance of *erb*-specific RNAs from AEV-ES4-transformed erythroblasts (AEV), BH03 leukemic cells, chicken embryonic cells (emb), and S13 virus-transformed erythroblasts (S13) was analyzed as described above. Two different exposures of the S13 RNA are shown. Hybridizing fragment sizes are shown in kilobase pairs in both panels.

By using both immunoprecipitation and immunofluorescence analysis, transmission of ERB-2 virus to fresh chicken embryo fibroblasts could also be demonstrated with filtered tissue culture supernatants from both leukemic cells and the ERB-2-producing fibroblasts generated by cocultivation (data not shown).

The ERB-2-infected cells retained a normal morphology (Fig. 7C), despite the fact that essentially all the cells

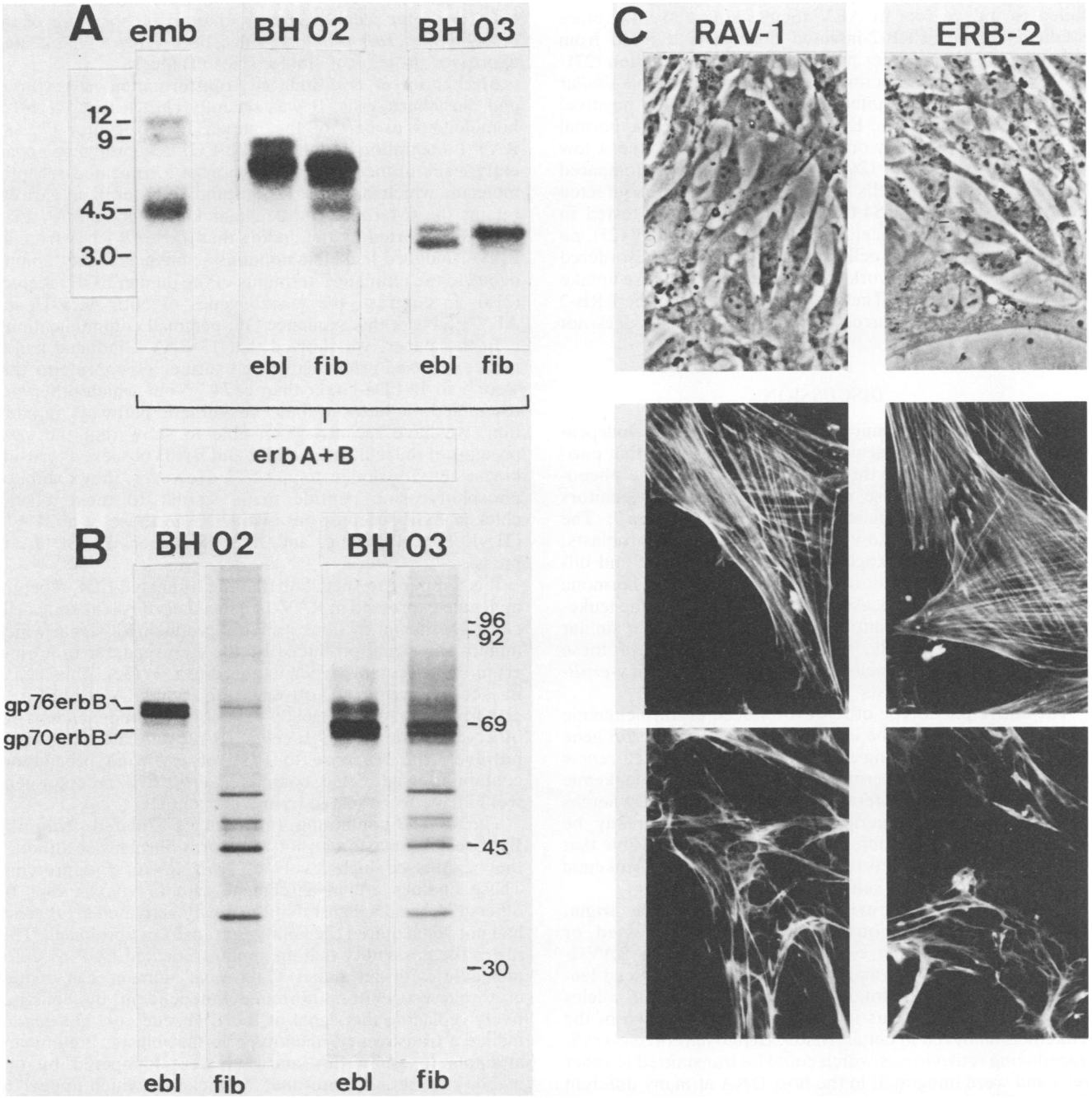

FIG. 7. Characterization of chicken embryo fibroblasts infected with *erbB*-containing virus from RAV-1-induced erythroleukemia. (A) Polyadenylated RNA from BH02 and BH03 leukemic erythroblasts (ebl) and from fibroblasts after cocultivation with the leukemic cells (fib) was analyzed by the Northern transfer technique with an *erbA + B* probe. Similar *erbB* RNA species are expressed in both cell types, which are clearly different from c-*erbB* RNA (9 and 12 kb) or c-*erbA* RNA (3 and 4.5 kb) present in normal chicken embryo RNA (emb). The BH03 RNA was subjected to autoradiography for 12 h and the BH02 RNA was autoradiographed for 48 h. Numbers on the left are in kilobases. (B) Leukemic erythroblasts (ebl) or fibroblasts after cocultivation with leukemic cells (fib) were labeled with [^{35}S]methionine and immunoprecipitated with *erb*-specific antibody. The same 70- to 72-kDa *erbB*-related proteins (gp70^{erbB}) are shown in BH03 erythroblasts and in fibroblasts after cocultivation. In contrast, little, if any, 76-kDa *erbB* protein (gp76^{erbB}) is detected in fibroblasts cocultivated with BH02 erythroblasts. Numbers on the right are in kilodaltons. (C) Fibroblasts cocultivated with BH03 leukemic cells (ERB-2) or infected with RAV-1 were assayed for their morphology by phase-contrast microscopy (top) or for expression of actin filament cables (middle) and fibronectin protein network (bottom) by double-label immunofluorescence as described earlier (23, 27).

expressed the *erbB* protein at their cell surface. In addition, virus harvested from the ERB-2 virus-producing BH03 leukemic cells or from the ERB-2-infected fibroblasts failed to induce foci in AEV focus assays. We therefore studied whether ERB-2-infected fibroblasts differed from normal cells in any other parameter of transformation (27).

The cells expressed actin cables (88% positive) to a similar extent as helper virus-infected control cells (92% positive) (Fig. 7C). In addition, ERB-2 cells expressed a normal fibronectin protein network (Fig. 7C) and exhibited a low rate of hexose uptake (26,000 cpm/10^6 cells as compared with 33,000 cpm/10^6 cells found with helper virus-infected control cells). AEV-ES4-transformed fibroblasts tested in parallel exhibited a typical, spindle-like morphology (25), no actin cables (13% of cells positive), a weak disordered fibronectin protein network (27), and elevated hexose uptake (80,600 cpm/10^6 cells). These results suggest that the ERB-2 virus contains a transduced c-*erbB* oncogene that does not transform fibroblasts.

DISCUSSION

RAV-1-induced leukemic cells resemble hormone-independent, late erythroid progenitors. We demonstrated that purified RAV-1-induced erythroleukemic cells exhibit a phenotype distinct from those of normal erythroid progenitors (CFU-E) and erythroblasts transformed by v-*erbB*. The leukemic cells, similar to v-*erbB*-transformed erythroblasts, were independent of exogeneous Epo for survival and differentiation, whereas normal CFU-E cells are hormone dependent for these processes. However, most of the leukemic cells differentiated into erythrocytes in a manner similar to normal CFU-E cells, while only a minority of these exhibited the sustained self-renewal characteristic of v-*erbB*-transformed erythroblasts.

The above phenotype of RAV-1-induced erythroleukemic cells is in accord with the idea that an activated c-*erbB* gene is less efficient in inducing erythroid precursors to self-renew in vitro than v-*erbB*. Alternatively, RAV-1-induced leukemic cells might mainly proliferate in the bone marrow, whereas progeny cells in the peripheral blood would already be committed to terminal differentiation. It is also possible that in vitro cultivation selects for a minor population of leukemic cells with an increased self-renewal ability.

c-*erb*-transducing retroviruses and their possible origin. Fung et al. (9) previously observed aa processed or transduced form of the c-*erbB* gene found in an RAV-1-induced leukemia. The majority of the RAV-1-induced leukemias studied here contained the expected c-*erbB* alleles activated by helper virus integration. However, two of the leukemias analyzed in detail (BH02, BH03) harbored c-*erbB*-transducing retroviruses which could be transmitted to other cells and were integrated in the host DNA at many different sites, suggesting an oligoclonal or polyclonal origin of these leukemias. Most likely, such c-*erbB*-transducing retroviruses arose as a secondary event by acquisition of the activated c-*erbB* sequences by the helper virus (30). Once generated, such retroviruses will continuously infect and transform new precursors, leading to rapid replacement of the primary clonal leukemia by a virus-induced polyclonal cell population. This notion is supported by our observation that the leukemic cells from one leukemia (HM8641) contained both a modified c-*erbB* allele and transduced *erb* sequences.

Owing to the small number of leukemias that can be grown into mass cultures, we do not know whether polyclonal leukemias containing transduced *erbB* sequences differ in their in vitro properties from those containing c-*erbB* alleles. Generation of such c-*erbB*-containing retroviruses, however, does not seem to grossly change the structure of the respective c-*erbB* proteins, since both types of leukemias expressed proteins of similar sizes (Table 3).

Mechanism of *erbB*-induced transformation of erythroid and fibroblastic cells. It was recently shown that v-*erbB* is homologous to part of the human EGF receptor (7, 32). RAV-1 integration into the c-*erbB*/EGF receptor gene apparently leads to increased expression of a truncated receptor molecule which lacks the ligand-binding N-terminal part but retains the C-terminal intracellular kinase domain (9, 25a). This is supported by the finding that the *erbB* RNA from an RAV-1-induced leukemia contains sequences highly homologous to the ultimate C terminus of the human EGF receptor (21a). In contrast, the v-*erbB* genes of both AEV-H and AEV-ES4 lack this sequence (36; personal communication).

In this paper, we showed that 13 RAV-1-induced leukemias expressed *erbB*-related cell surface glycoproteins that were 5 to 10 kDa larger than $gp74^{erbB}$ but which otherwise resembled the latter in their biosynthetic pathway. In addition, we have recently been able to show that the *erbB* proteins of the leukemias BH02 and BH03 possess a tyrosine kinase activity similar to $gp74^{erbB}$. However, they exhibited phosphotyrosine peptide maps similar to those of the chicken EGF receptor but distinct from those of $gp74^{v\text{-}erbB}$ (21; I. Lax, H. Beug, and J. Schlessinger, EMBO J., in press).

It is tempting to speculate that the truncated EGF receptor molecule expressed in RAV-1-induced erythroleukemic cells causes leukemia by constitutively producing a signal which mimics the signal produced by the Epo receptor in normal erythroid precursors when exposed to Epo (3c). This idea is in accord with our observation that RAV-1-induced erythroleukemic cells mostly resemble Epo-independent but otherwise normal CFU-E cells. To substantiate this notion, however, the response to EGF of erythroid progenitors containing an activated, complete c-*erbB*/EGF receptor gene would have to be tested by in vitro studies.

The c-*erbB*-containing retroviruses isolated from the BH03 erythroblasts did not transform fibroblasts, although they expressed high levels of *erbB* RNAs and proteins. These findings are in agreement with in vivo studies by others (30a) and suggest that the newly activated *erbB* genes had not yet acquired the ability to transform fibroblasts. This raises the possibility that an amino-truncated EGF receptor molecule with an intact C-terminal domain can render erythroid progenitors hormone independent by constitutively replacing the signal of the Epo receptor, but cannot induce a transformed phenotype in fibroblasts. Preliminary attempts to study this question were hampered by our inability to obtain more line 15 chickens, which appear to contain a dominant locus for susceptibility to c-*erbB*-transducing retroviruses (H. L. Robinson, submitted for publication). Although our studies on SPAFAS chickens showed that transformed erythroblast clones generated with ERB-2 virus supernatants from BH03 leukemic cells or infected fibroblasts expressed the 70- to 72-kDa *erbB* protein of the ERB-2 virus, we were unable to determine whether this protein was responsible for erythroblast transformation: all erythroblast clones expressed a second, *erbB*-transducing and erythroblast-transforming virus that was present in the original leukemia at levels too low to allow biochemical detection (ERB-2A virus; H. Beug et al., manuscript in preparation).

In this context, it is noteworthy that the v-*erbB*-containing erthyroblastosis viruses described previously caused erythroblastosis but not sarcomas when first isolated from a diseased chicken. Only after prolonged in vivo passage was a sarcomagenic potential acquired (19, 31). Since none of these earlier virus isolates is available anymore, c-*erbB*-transducing retroviruses from ALV-induced leukemias might be useful to identify discrete steps by which the normal EGF receptor is converted into an oncogenic protein.

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