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Characterization of Hematopoietic Cells Transformed In Vitro by AEV-H, a *v-erbB*-Containing Avian Erythroblastosis Virus

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A. Introduction

Avian erythroblastosis virus ES4 (AEV-ES4) is a replication-defective avian leukemia virus that causes erythroleukemia and fibrosarcomas in chicks and transforms bone marrow cells and fibroblasts in vitro (for review, see [7]). The 5.5 kb genome of AEV-ES4 contains two oncogenes, *v-erbA* and *v-erbB* [1, 5, 21] which code for a *gag*-related cytoplasmic protein, $p75^{gag\text{-}erbA}$, and for an integral membrane glycoprotein, $gp74^{erbB}$, respectively [10–12, 18]. Studies using deletion mutants in either *v-erbA* or *v-erbB* indicate that *v-erbB* is sufficient to transform fibroblasts and erythroblasts, whereas *v-erbA* does not transform by itself but cooperates with *v-erbB* in erythroblasts to generate a more transformed phenotype [6, 7, 20].

Recently, another strain of avian erythroblastosis virus (AEV-H) was isolated [13] and shown to contain *v-erbB* as its only oncogene [23]. In vivo, this strain induced both erythroblastosis and sarcomas which were anatomically and histologically indistinguishable from the respective diseases induced by AEV-ES4 [13]; in vitro, it induced foci of transformed fibroblasts (Hihara et al., 1983). Here we show that AEV-H is also able to transform bone marrow cells in vitro using specific culture conditions. The transformed erythroblasts undergo self-renewal as well as spontaneous differentiation into erythrocytes and do not require exogenous erythropoietin for either process. They express an *erbB*-coded cell surface glycoprotein ($gp79^{erbB}$) which is 5 kilodaltons larger than $gp74^{erbB}$, the *erbB* gene product of AEV-ES4.

B. Materials and Methods

I. Viruses

Avian erythroblastosis virus strain H was obtained as a gift from Dr. Toyoshima, Tokyo. Its origin has been described elsewhere [13]. The origin of avian erythroblastosis virus strains AEV-ES4 and AEV193 has been described earlier [8, 12].

II. Cells and Cell Culture

Bone marrow cells from 3- to 14-day-old chicks were prepared, infected with virus, and seeded into Methocel under standard or CFU-E (Colony-Forming Unit Erythroid) conditions as described earlier [9, 19]. Colonies were isolated 6–8 days later with a drawn-out Pasteur pipette and expanded in either standard growth medium (AEV-ES4, 193 strains) or CFU-E medium (AEV-H strain). These media were prepared as described by Radke et al. [19].

III. Assays for Erythroid Differentiation Markers

The morphology and hemoglobin content of AEV-H-transformed erythroblasts were

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analyzed by staining cytospin preparations with neutral benzidine plus histological dyes as described earlier [4]. Alternatively, a more sensitive staining for hemoglobin content using acid benzidine was done according to Orkin et al. [17]. Detection of erythroid-specific cell surface antigens by indirect immunofluorescence using antisera to mature erythroid cells and to immature erythroblasts (anti-Ery and anti-Ebl; [3]) was carried out as described previously [4]. The possible presence of myeloid cells in the transformed cultures was assessed using the myeloid-specific monoclonal antibody MC51-2 [15].

IV. Percoll Fractionation of AEV-H Erythroblasts

Erythroblasts ($10–20 \times 10^6$) from a single clone (ID4) were loaded on a discontinous Percoll gradient (prepared as described by Beug and Hayman [2]; densities from top to bottom: 1.070, 1.072, 1.075 and 1.085 g/cm^3) and centrifuged for 10 min at 2000 *g*. The least-dense cells (fraction I, 1.070) and the cells banding at 1.072 and 1.075 g/cm (fraction II) were retrieved, seeded in CFU-E medium containing either normal or anemic chicken serum, and analyzed by cytocentrifugation and staining as described above. Alternatively, cells from the least-dense fraction were seeded into plasma clot cultures, which were processed and stained 3–4 days later as described earlier [4].

V. Protein Analysis

Using methionine-free differentiation medium [2], $5–10 \times 10^6$ cells were labeled with 100–250 μCi ^{35}methionine for 2 h. They were then lysed and immunoprecipitated with anti-*erbB* serum according to published procedures and analyzed on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) [10–12].

C. Results and Discussion

I. In Vitro Transformation of Bone Marrow Cells by AEV-H

Since AEV-H was negative in standard bone marrow transformation assays (T. Graf and H. Beug, unpublished observations) we tested whether bone marrow cells could be transformed under conditions (CFU-E-Methocel; Radke et al., 1982) which had been successfully employed to obtain erythroblasts transformed by AEV-ES4 mutants that carry a deletion in the *erbA* gene (AEV-A$^-$B$^+$; [6]). Bone marrow cells were infected with AEV-H and, in controls, with two other AEV strains (AEV-ES4 and AEV193, both containing *erbA* and *erbB* oncogenes). The infected cells were then seeded into Methocel under both standard [9] and CFU-E conditions. Numerous colonies were obtained from AEV-H-infected bone marrow in CFU-E Methocel, whereas no transformed colonies were seen with this virus under standard conditions. In contrast, the two other AEV strains tested yielded comparable numbers of colonies under standard and CFU-E conditions.

Several of the AEV-H-induced colonies were isolated and then cultivated in CFU-E medium. Most clones grew in this medium for 20–35 generations, exhibiting doubling times of 24 h or less. When transferred to standard growth medium, however, the AEV-H cells ceased proliferating, developed large vacuoles, and disintegrated within 4–7 days. In contrast, AEV-ES4- and AEV193-derived clones exhibited similar growth rates and life spans in standard and CFU-E media.

II. AEV-H-Transformed Cells Consist of Erythroid Precursors at Different Stages of Maturation

To determine the lineage and degree of maturation of bone marrow cells transformed in vitro by AEV-H, the AEV-H clones were analyzed for a set of erythroid differentiation markers [3, 4] as well as for the myeloid-cell-specific antigen 51-2 [15]. Table 1 and Fig. 1 show that all AEV-H clones tested consisted entirely of erythroid cells, representing a mixture of immature erythroblasts and more mature reticulocytes and erythrocytes. In contrast, AEV-ES4- and AEV193-transformed clones consisted exclusively of immature erythroblasts.

Table 1. Characterization of erythroblast clones transformed by AEV-H

AEV-H clone No.	Hemoglobin-positive cells (%)[a]	Cells classified as LR+E (%)[b]	Cells stained with		
			α Erb (%)[c]	α Erb (%)	α Mbl (%)
I B 4	78	11	40 (10)[d]	80	ND[f]
I B 6	27	0.9	10 (3)	>95	<0.1
I C 1	100	68	[e]	[e]	ND
I C 2	71	10	55 (30)	50	ND
I C 6	63	3.2	40 (10)	80	<0.1
I D 1	95	46	[e]	[e]	ND
I D 4	89	17	80 (60)	40	ND

[a] Determined by staining with acid benzidine (Beug et al. 1982)
[b] Cell types were defined by neutral benzine plus histological staining (Beug et al. 1982). LR, late reticulocytes; E, erythrocytes
[c] The characterization of these antisera has been described elsewhere (Beug et al. 1979; Kornfeld et al. 1983). α Erb, anti-erythroblast serum; α Ery, anti-erythrocyte; α Mbl, anti-myeloblast monoclone MC 51/2
[d] Figures in parenthesis; cells strongly stained in a ring-like fashion (Beug et al. 1979)
[e] These clones differentiated into erythrocytes before sufficient cell numbers for fluorescent staining were built up
[f] ND: not determined

The data in Table 1 also indicate that the AEV-H clones tested varied with respect to the proportion of mature cells. Furthermore, an inverse relationship was seen between the frequency of mature cells in a particular clone and its growth rate. A few clones could not be propagated more than 7–10 days after isolation because all cells differentiated into erythrocytes.

III. AEV-H Erythroblasts Proliferate and Differentiate in an Erythropoietin-Independent Fashion

To study how the heterogeneity of cells within single erythroblast clones was generated and maintained during culture, cells from a clone (ID4) containing about 20% mature cells were fractionated according to density using a discontinuous Percoll gradient [2]. The individual fractions were then seeded in CFU-E medium in the absence or presence of anemic chicken serum as a source of erythropoietin [4] and aliquots analyzed for their stage of differentiation during the following few days. As illustrated in Fig. 2A, four fractions were obtained from the Percoll gradient; the top fraction (I) and the pooled intermediate-density fractions (II) were analyzed further. Fraction I contained exclusively immature erythroblasts that grew rapidly and regenerated the initial proportion of mature cells within 3 days, regardless of whether or not erythropoietin was present (Fig. 2B and data not shown). In contrast, fraction II, which contained partially mature, hemoglobinized cells ceased proliferating after 2 days, at which time essentially all cells exhibited an erythrocyte-like phenotype. The most dense fraction consisted of erythrocyte-like cells with no in vitro proliferative capacity.

Similar results were obtained by seeding cells from the above Percoll-density fractions into plasma clot cultures [4]. Whereas the immature cells from fraction I frequently grew into large colonies consisting of either immature or a mixture of immature and mature erythroid cells, CFU-E-like erythrocyte colonies and single erythrocytes were formed exclusively by the cells from fraction II. Similar numbers

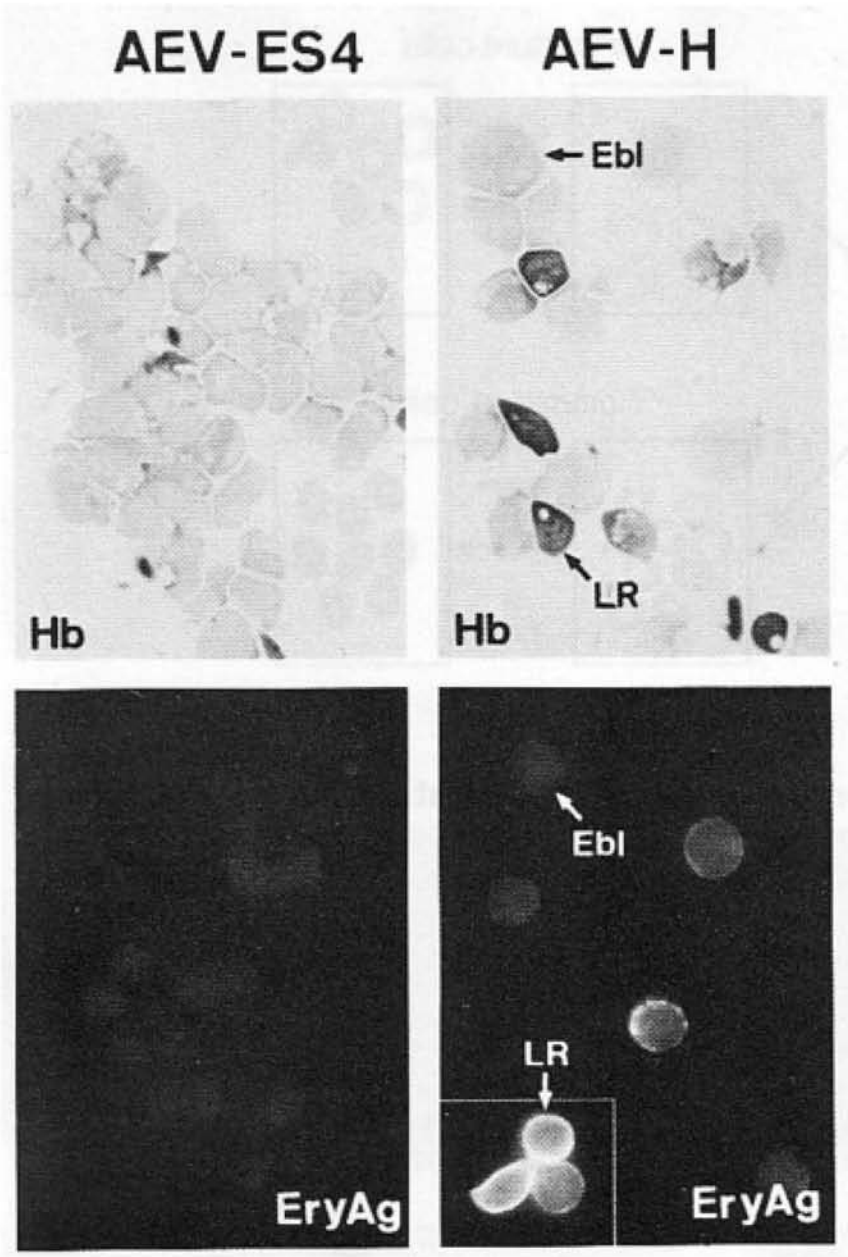

Fig. 1. Characterization of AEV-H-transformed erythroblasts. Cells from an AEV-H-transformed erythroblast clone (ID4) and from a culture of AEV-ES4-transformed erythroblasts were cytocentrifuged onto slides and stained with neutral benzidine (*Hb*). Live cells from the same preparations were also stained with anti-erythrocyte serum by indirect immunofloures-cence (*ErgAg*) (Beug et al., 1979). *Ebl,* cells classified as erythroblasts; *LR,* cells classified as late reticulocytes. (Beug et al., 1982)

and proportions of these colonies were obtained in the absence and presence of erythropoietin (data not shown). These results suggest that AEV-H-transformed erythroblasts can either self-renew or become committed to terminal differentiation with a frequency characteristic for the particular clone. In contrast to normal erythroid progenitors, AEV-H erythroblasts carry out both these functions in the absence of exogenous erythropoietin.

IV. In Vitro Transformed AEV-H Erythroblasts Express High Levels of an *erbB*-Coded Cell Surface Glycoprotein

To study expression of *erbB*-related proteins by AEV-H erythroblasts, cells were labeled with [^{35}S]methionine and immunoprecipitated with *erbB*-specific sera. Figure 3 shows that proteins of 72 and 74 kilodaltons were immunoprecipitated from AEV-H cell lysates. Thus, the AEV-H *erbB* gene products are approximately 5 kilodaltons larger than *erbB* gene products of AEV-ES4 but only slightly larger than the respective proteins expressed by AEV193 [12]. As expected, no *erbA*-related proteins could be immunoprecipitated from AEV-H cells [23].

The *erbB* protein produced by AEV-H erythroblasts is expressed at the cell surface, since live cells were strongly stained by *erbB*-specific sera by indirect immunofluorescence (Fig. 4; [12]). Interestingly, most if not all mature cells (as classified by staining with antierythrocyte

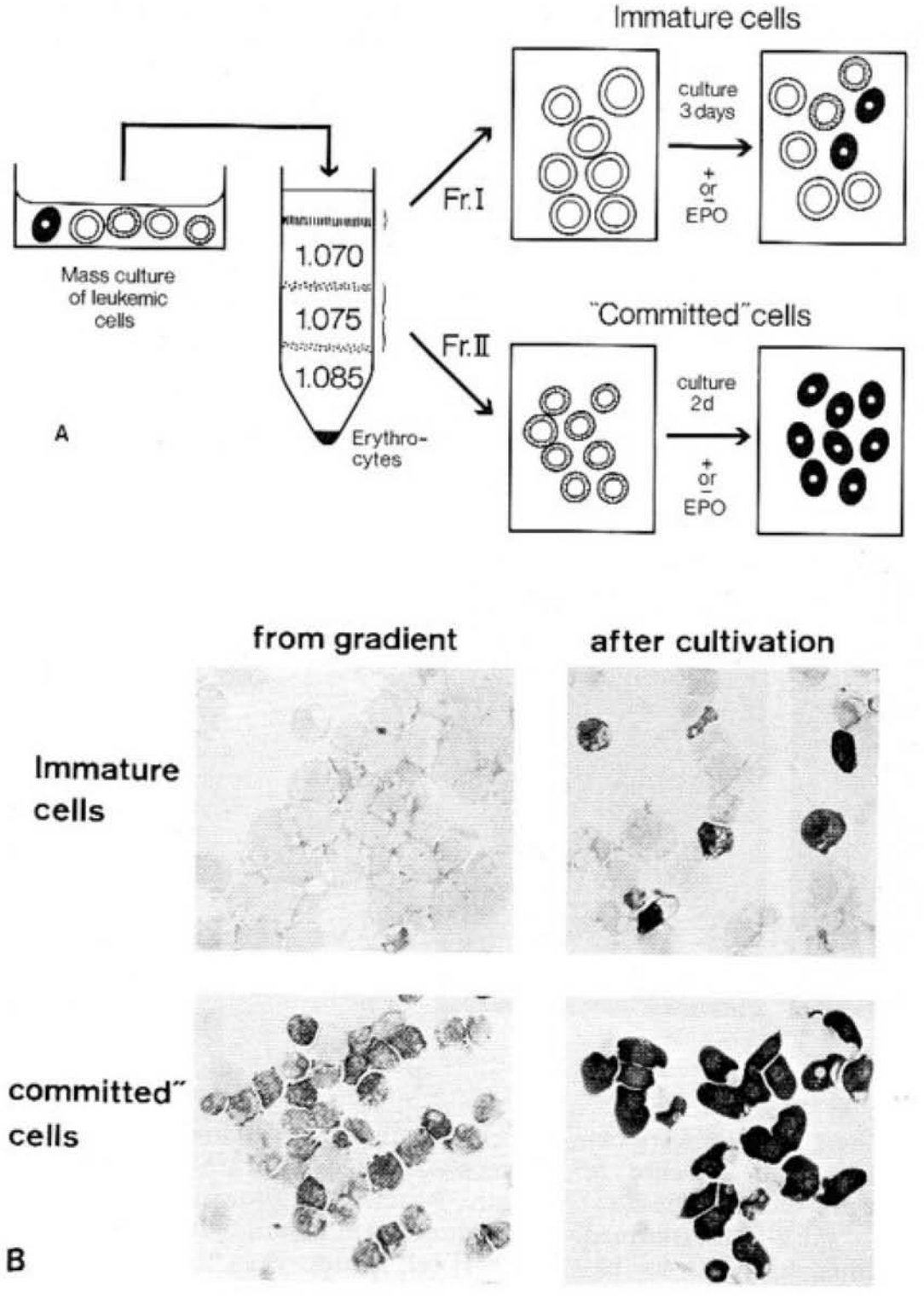

Fig. 2 A, B. Self-renewal and differentiation capacity of AEV-H erythroblasts fractionated according to density. **A** Schematic diagram showing Percoll fractionation of AEV-H-transformed erythroblasts and phenotypes of cells retrieved from the gradient before and after cultivation in the presence and absence of anemic chicken serum (*EPO*); **B** Cytospin preparations from the cell populations described in **A** were stained with neutral benzidine and then photographed

serum) were negative or only weakly positive with anti-*erbB* serum. This suggests a down-regulation of *erbB* protein expression during erythroid differentiation, as also seen in differentiating tsAEV erythroblasts [2].

V. What is the Role of *v-erbA* in Erythroid Transformation?

The results presented above clearly demonstrate that erythroblasts transformed by the

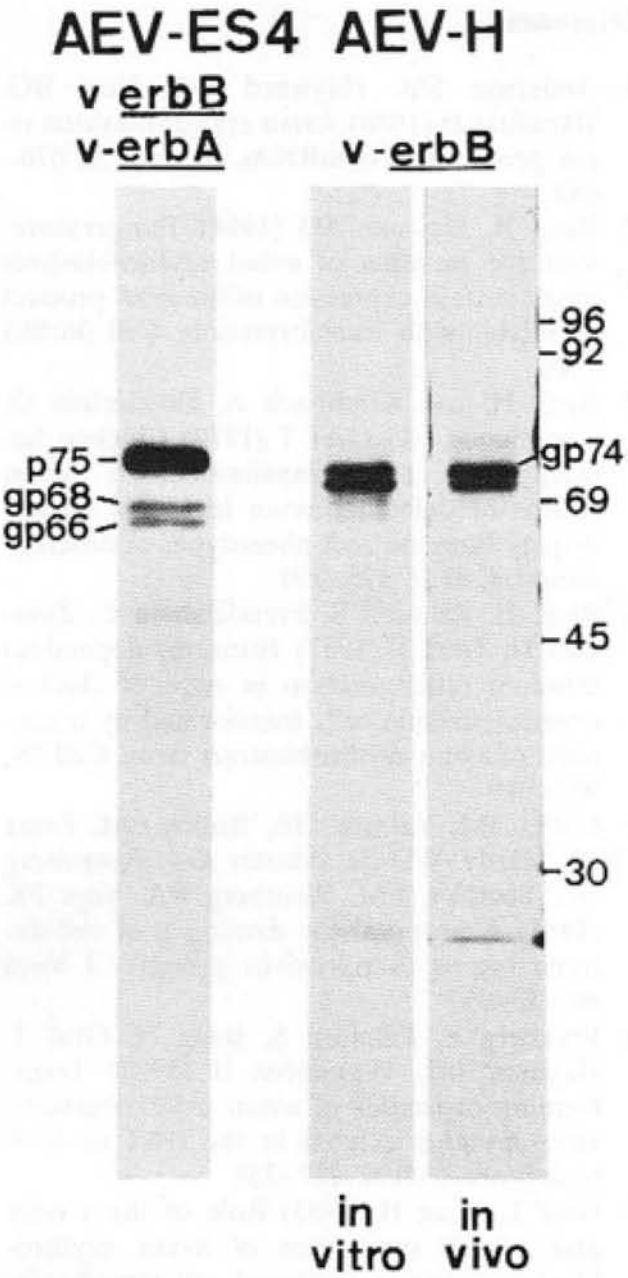

Fig. 3. Expression of *v-erbB*-encoded proteins in AEV-H-transformed erythroblasts; AEV-H erythroblasts (clone IB6, in vitro), erythroblasts grown from the blood of a leukemic chicken infected with AEV-H virus (Kahn et al., 1984; in vivo), and AEV-ES4 erythroblasts (as controls) were labeled with [^{35}S]methionine. Cell extracts were immunoprecipitated with anti-*erbA* + *B* serum followed by gel electrophoresis and autoradiography as described earlier (Hayman et al., 1979, 1983). *Gp66/68,* gp66/68^{erbB} from AEV-ES4; *p75,* p75$^{gag\text{-}erbA}$; *gp74,* gp72/74^{erbB} from AEV-H

v-erbB gene of AEV-H both self-renew and differentiate into erythrocytes, whereas erythroblasts transformed by two viruses that contain *v-erbA* in addition to *v-erbB* are restricted to the self-renewal pathway (Fig. 5). At the same time, *v-erbB*-transformed cells require specific growth conditions (including pH and ionic strength optima) similar to those required by normal erythroid progenitors, whereas erythroblasts transformed by both *v-erbB* and *v-erbA* grow in standard growth media. This suggests that *v-erbA* enhances the effect of *v-erbB* by abolishing two characteristics of normal erythroid precursors expressed by AEV-H erythroblasts, i.e., spontaneous differentiation into erythrocytes and specific growth requirements.

VI. The Phenotype of AEV-H Erythroblasts is Common Among Erythroid Cells Transformed by Other Avian Retroviruses

Recently, several avian retroviruses containing oncogenes other than *v-erbB* have been found to transform erythroid bone

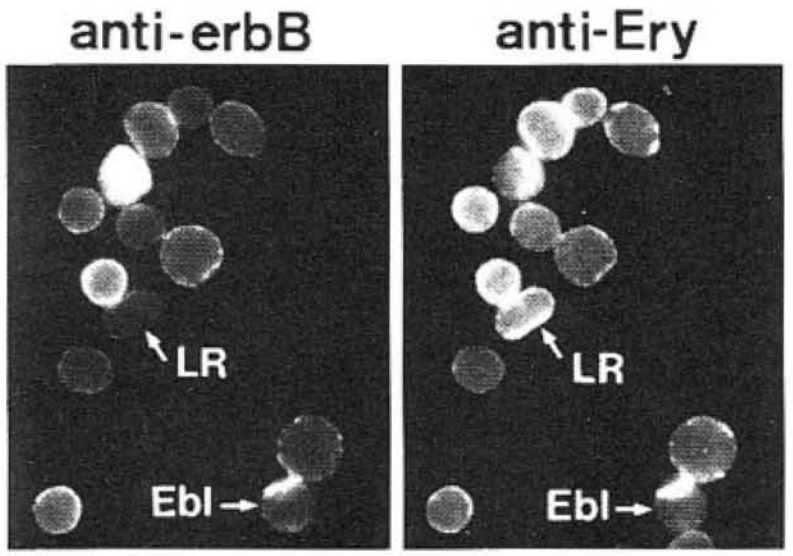

Fig. 4. AEV-H erythroblasts express *v-erbB* protein at the cell surface. AEV-H erythroblasts (clone ID4) were double stained by indirect immunofluorescence with anti-*erbB* serum (anti-*erbB*) and anti-erythroblast serum (anti-*Ery*) as described earlier (Beug and Hayman, 1984). Note absence of *erbB* fluorescence in some late reticulocytes (*LR*)

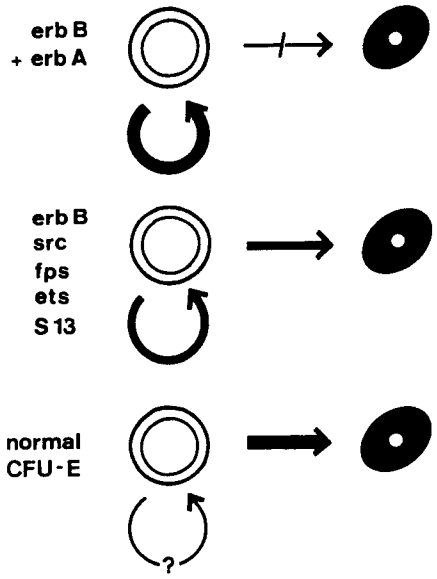

Fig. 5. Transformation phenotypes of erythroid cells containing various retroviral oncogenes. The diagram illustrates how different retroviral oncogenes modulate the self-renewal capacity (*circular arrows*) and the probability of undergoing terminal differentiation into erythrocytes (*straight arrows*). *Thick arrows* indicate high probability; *thin arrows* indicate low probability. *The bar across the straight arrow in the upper diagram* (*erbB*+*erbA*) indicates that these two oncogenes together completely arrest differentiation

marrow cells. Using growth conditions similar to those required by AEV-H erythroblasts, the *myb,ets*-oncogene-containing E26 virus [16, 19], the *src*-containing RSV (Rous sarcoma virus), the *fps*-containing FSV (Fujinami sarcoma virus) [14], and the S13 erythroblastosis virus (H. Beug et al., submitted for publication) were shown to induce transformed erythroblasts that closely resembled AEV-H erythroblasts (Table 1, Fig. 5). All these oncogenes cause two types of transformation-specific changes in the infected erythroid progenitors: they induce in them the ability to self-renew and render them independent of the erythroid differentiation hormone erythropoietin with respect to in vitro survival, self-renewal, and differentiation. It will be interesting to determine whether *v-erbA* can cooperate with these oncogenes in a similar manner as with *v-erb*B.

References

1. Anderson SM, Hayward WS, Neel BG, Hanafusa H (1980) Avian erythroblastosis virus produces two mRNAs. J Virol 36:676–683
2. Beug H, Hayman MJ (1984) Temperature-sensitive mutants of avian erythroblastosis virus: surface expression of the *erbB* product correlates with transformation. Cell 36:963–972
3. Beug H, von Kirchbach A, Doederlein G, Conscience J-F, Graf T (1979) Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18:375–390
4. Beug H, Palmieri S, Freudenstein C, Zentgraf H, Graf T (1982) Hormone-dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by *ts* mutants of avian erythroblastosis virus. Cell 28:907–919
5. Coffin JM, Varmus HE, Bishop JM, Essex M, Hardy WD Jr, Martin GS, Rosenberg NE, Scolnick EM, Weinberg RA, Vogt PK (1981) A proposal for naming host cell-derived inserts in retrovirus genome. J Virol 40:953–957
6. Frykberg L, Palmieri S, Beug H, Graf T, Hayman MJ, Vennström B (1983) Transforming capacities of avian erythroblastosis virus mutants deleted in the *erbA* or *erbB* oncogenes. Cell 32:227–238
7. Graf T, Beug H (1983) Role of the v-*erbA* and v-*erbB* oncogenes of avian erythroblastosis virus in erythroid cell transformation. Cell 34:7–9
8. Graf T, Royer-Pokora B, Schubert GE, Beug H (1976) Evidence for the multiple oncogenic potential of cloned leukemia virus: in vitro and in vivo studies with avian erythroblastosis virus. Virology 71:423–433
9. Graf T, von Kirchbach A, Beug H (1981) Characterization of the hematopoietic target cells of AEV, MC29 and AMV avian leukemia viruses. Exp Cell Res 131:331–343
10. Hayman MJ, Beug H (1984) Identification of a form of the avian erythroblastosis virus *erbB* gene product at the cell surface. Nature 309:460–462
11. Hayman MJ, Royer-Pokora B, Graf T (1979) Defectiveness of avian erythroblastosis virus: synthesis of a 75k *gag*-related protein. Virology 92:31–45
12. Hayman MJ, Ramsay GM, Savin K, Kitchener G, Graf T, Beug H (1983) Identification and characterization of the avian erythroblastosis virus *erbB* gene product as a membrane glycoprotein. Cell 32:579–588

13. Hihara H, Yamamoto H, Shimohira H, Arai K, Shimizu T (1983) Avian erythroblastosis virus isolated from chick erythroblastosis induced by lymphatic leukemia virus subgroup A. JNCI 70:891–897
14. Kahn P, Adkins B, Beug H, Graf T (1984) *Src-* and *fps*-containing avian sarcoma viruses transform chicken erythroid cells. Proc Natl Acad Sci USA 81:7122–7126
15. Kornfeld S, Beug H, Doederlein G, Graf T (1983) Detection of avian hematopoietic cell surface antigens with monoclonal antibodies to myeloid cells. Exp Cell Res 143:383–394
16. Leprince D, Gegonne A, Coll J, deTaisne C, Schneeberger A, Lagrou C, Stehelin D (1983) A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature 306:391–395
17. Orkin SH, Harosi FI, Leder P (1975) Differentiation in erythroleukemic cells and their somatic hybrids. Proc Natl Acad Sci USA 72: 98–102
18. Privalsky ML, Sealy L, Bishop JM, McGrath JP, Levinson AD (1983) The product of the avian erythroblastosis virus *erbB* locus is a glycoprotein. Cell 32: 1257–1267
19. Radke K, Beug H, Kornfeld S, Graf T (1982) Transformation of both erythroid and myeloid cells by E26, an avian leukemia virus that contains the *myb* gene. Cell 31:643–653
20. Sealy L, Moscovici G, Moscovici C, Bishop JM (1983) Site-specific mutagenesis of avian erythroblastosis virus: v-erbA is not required for transformation of fibroblasts. Virology 130:179–194
21. Sheiness D, Vennström B, Bishop JM (1981) Virus-specific RNAs in cells infected by avian myelocytomatosis virus and avian erythroblastosis virus: modes of oncogene expression. Cell 23:291–300
22. Vennström B, Fanshier S, Moscovici C, Bishop JM (1980) Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic virus by transfection of chicken cells. J Virol 36:575–585
23. Yamamoto T, Hihara T, Nishida T, Kawai S, Toyoshima K (1983) A new avian erythroblastosis virus, AEV-H, carries *erbB* gene responsible for the induction of both erythroblastosis and sarcomas. Cell 34:225–232