Haematology and Blood Transfusion Vol. 29
Modern Trends in Human Leukemia VI
Edited by Neth, Gallo, Greaves, Janka

Characterization of the Number of Carbohydrate Chains on the Avian Erythroblastosis Virus *erb* B Gene Product and Their Role in Transformation

M. J. Hayman[1], J. A. Schmidt[1], G. Kitchener[1], J. Marshall[1], and H. Beug[2]

A. Introduction

Avian erythroblastosis virus, AEV, is a replication-defective avian acute leukemia virus that causes acute erythroid leukemia and sarcomas on injection into young chicks. Analysis of the genome of AEV revealed that it was 5.0 kilobases in length and that the gene order was 5′-*gag-erb-env-poly* A-3′ where *gag* and *env* represent partially deleted structural genes and *erb* is a 3.25 kilobases sequence of nonviral origin [2, 13]. Subsequently, sequences homologous to *erb* could be found in cellular DNA from all vertebrate species tested [16, 19]. This evidence suggested that the *erb* sequences represented the viral oncogene of AEV, and led to studies attempting to define the products of this gene.

Studies rapidly revealed that AEV contained an oncogene capable of coding for two gene products. Consequently, *erb* was divided into two regions known as *erb* A and *erb* B. The first gene product identified was the *erb* A protein, a 75 000 molecular weight protein, $p75^{erb\,A}$ that could be shown to be composed of NH_2 terminal *gag* sequences plus approximately 50 000 daltons of *erb* A sequences [10]. Recently, the *erb* B protein has also been identified in AEV-transformed cells and was shown to be a membrane glycoprotein [9, 14]. This protein has a molecular weight of 62 500 in the nonglycosylated state, but is modified by glycosylation first to a 66 000 molecular weight form, $gp66^{erb\,B}$ and then through a 68 000 molecular weight phosphorylated form, $gp68^{erb\,B}$ to a plasma membrane form, $gp74^{erb\,B}$ [8, 9].

Interest in the *erb* B protein was heightened by several recent observations. First, site-directed mutagenesis experiments on the AEV genome demonstrated that it was primarily the *erb* B gene that was responsible for the transformation of both erythroid cells and fibroblasts [6, 18]. The *erb* A gene did, however, play some role in the development of the fully transformed erythroblast phenotype. Second, characterization of a new isolate of AEV, termed AEV-H, which was capable of causing both erythroblastosis and fibrosarcoma, revealed that the only *erb*-related sequence it contained in its genome was *erb* B [11, 23]. Finally, it was demonstrated that avian leukosis virus-induced erythroblastosis was associated with insertion of the viral long terminal repeat, LTR, into the cellular *erb* B locus, c-*erb*, resulting in the elevation of c-*erb* B transcription [7]. Thus, the central role of *erb* B in erythroblasosis and fibrosarcoma induction has encouraged us to characterize the product of this gene in greater detail.

Digestion of the v-*erb* B protein with the enzyme endoglycosidase H, endo-H, had previously shown that both $gp66^{erb\,B}$ and $gp68^{erb\,B}$ were sensitive to digestion with this enzyme [9], whereas $gp74^{erb\,B}$ was resistant [8]. This enzyme specifically cleaves

1 Imperial Cancer Research Fund Laboratories, St. Bartholomew's Hospital, Dominion House, Bartholomew Close, London EC1A 7BE, England
2 EMB Laboratories, Postfach 10, 2209, Meyerhofstrasse 1, 6900 Heidelberg, FRG

off the high mannose core sugars which are associated with the immature forms of glycoproteins [20]. Therefore, these results were consistent with gp66 and gp68 being the immature forms and gp74 being the mature form of the *erb* B gene product. Recently, analysis of mutants of AEV which are temperature sensitive for cell transformation have shown that the mutation blocks the processing of gp68 into gp74 and hence there is no surface expression of *erb* B [1]. Consequently, we have examined the number of carbohydrate units and their role in the function of the *erb* B protein.

B. Results and Discussion

I. Endoglycosidase H Digestion of the *erb* B Protein

Previous analysis of the gp68 product by endo-H digestion had employed complete digestion in order to remove all the available high mannose glycan units. By using limited digestion with endo-H it is possible to remove these units sequentially and thus ascertain how many units there are on the protein. Figure 1 shows the results of such an experiment. The gp68 form of *erb* B is first converted into a gp66 form (lane 4) and then finally into the p62 form (lane 5), which is the same size as the in vitro translation product and the form synthesized in the presence of tunicamycin [9, 14]. As demonstrated previously, gp74 is resistant to digestion (lane 5). These data indicate that there are two *N*-linked carbohydrate chains present on the *erb* B protein which can be released with endo-H. Recent sequence analysis of the *erb* B gene of AEV-ES4 ([3] and our unpublished data), which is the strain used in our experiments, indicate that there are three consensus sequences for *N*-linked carbohydrate addition. Therefore, only two of the three possible sites for addition are being used. At the present time we are unable to say which these two sites are. However, one of the three sites is located only four amino acids from the presumptive membrane spanning region and therefore may not be available. Also, *erb* B has recently been reported to be highly homologous to the human epider-

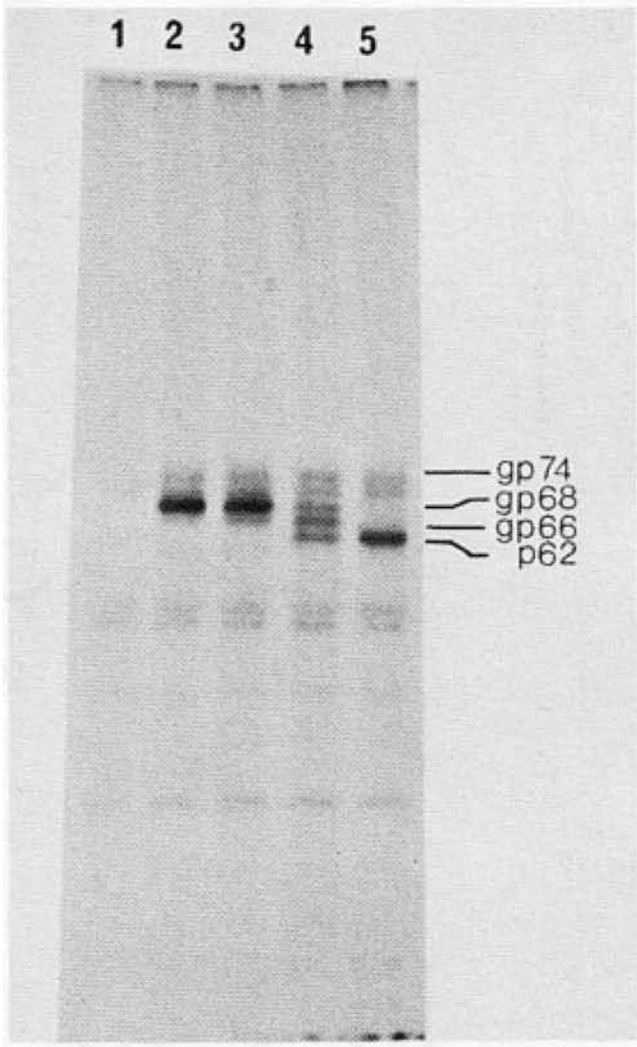

Fig. 1. Endoglycosidase H (endo-H) digestion of v-*erb* B. Erythroblasts transformed with *ts* 34 AEV were labelled with methionine ^{35}S under optimal conditions to label $gp68^{erb\,B}$ and $gp74^{erb\,B}$ [8]. Digestions of endo-H on immunoprecipitated v-*erb* B were performed as described [8]. Reactions contained either 0.01 mU (*lane* 3), 0.1 mU (*lane* 4) or 1 mU (*lane* 5) endo-H, in addition to a negative control (*lane* 2). *Lane* 1 is a normal rat serum immunoprecipitation of the same cell extract treated with 1 mU endo-H. Products were identified by fluorography after polyacrylamide gel electrophoresis

mal growth factor receptor, EGF-R [4, 22] and comparison of the glycosylation sites between *erb* B and EGF-R shows that this site is not conserved whereas the other two are. In summary, it appears as if the v-*erb* B protein contains two *N*-linked carbohydrate chains.

II. Effect of Inhibitors of Glycosylation on the Function of *erb* B

Recently, inhibitors of the processing of carbohydrate side chains have proved very

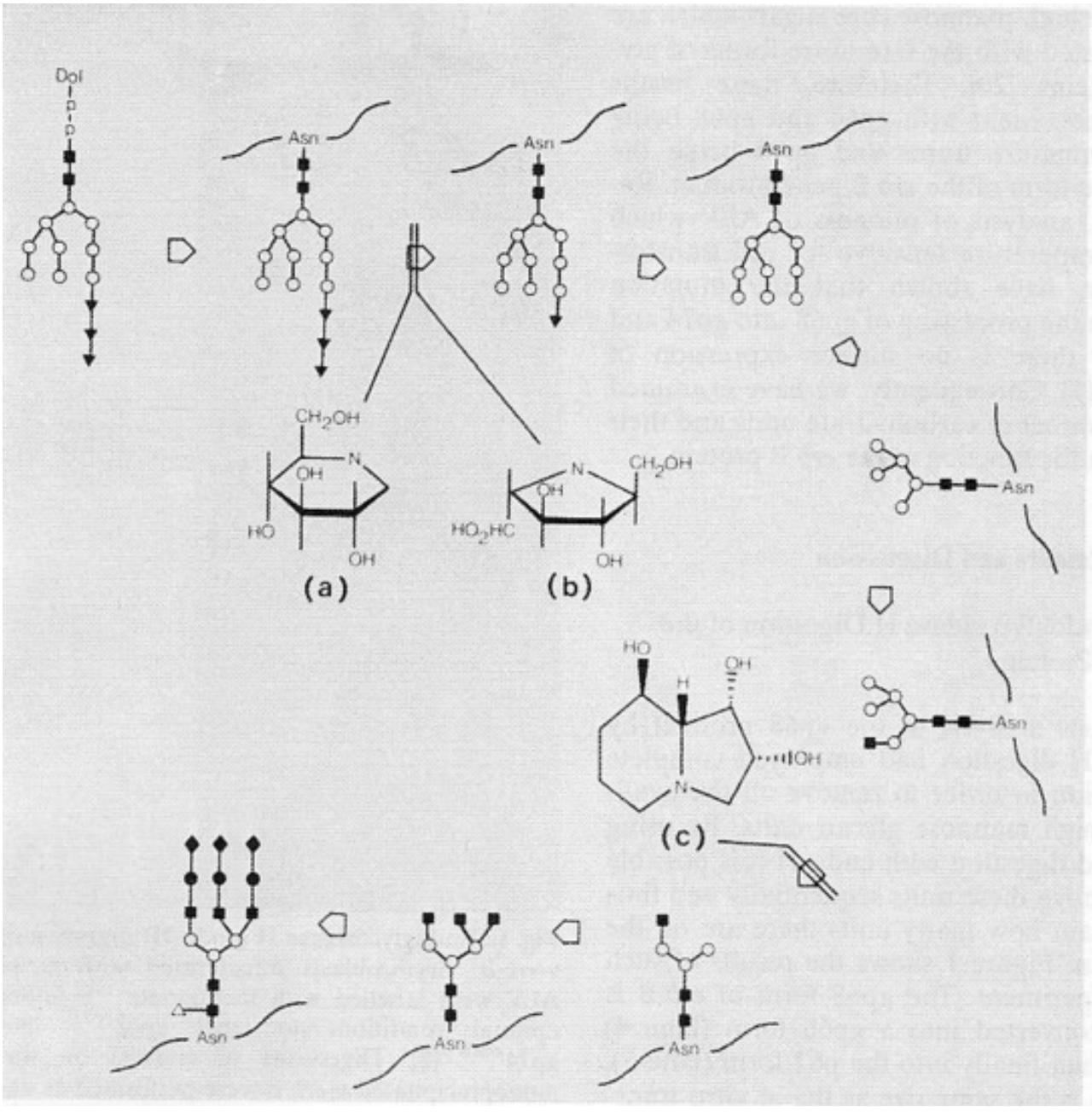

Fig. 2. Structures and the sites of action of the glycosylation inhibitors. ■ GlcNAc, ○ Man, ▲ Glc, ● Gal, ◆ NeuAc, △ Fuc; (a) 1-Deoxynojirimycin, (b) 2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine, (c) Swainsonine

useful in delineating the pathways of processing in addition to the functional role of carbohydrate side chains on a variety of proteins (see [17] for review). We have used three such inhibitors to study the processing of the *erb* B proteins. These are swainsonine, which inhibits the Golgi mannosidase II [21] leading to decreased expression of complex oligosaccharide chains on cell surface glycoproteins [5] and 1-deoxynojirimycin and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine which are both inhibitors of glucosidase I and interfere with the release of the outermost glucose ([15]; Elbein and Fellows, in press). These sites of action are shown diagrammatically in Fig. 2. AEV-transformed cells were grown in the presence of predetermined amounts of the three inhibitors to give maximum inhibition of the processing enzymes [Schmidt et al., in press]. Interestingly, there was no effect of these inhibitors on the transformed phenotype of the AEV-transformed cells, either erythroblasts or fibroblasts, by any criteria tested. Since these inhibitors had no effect on transformation it was obviously necessary to show that they were having the expected effect in chicken cells. Accordingly AEV-transformed cells grown in the presence of three different inhibitors were labelled with

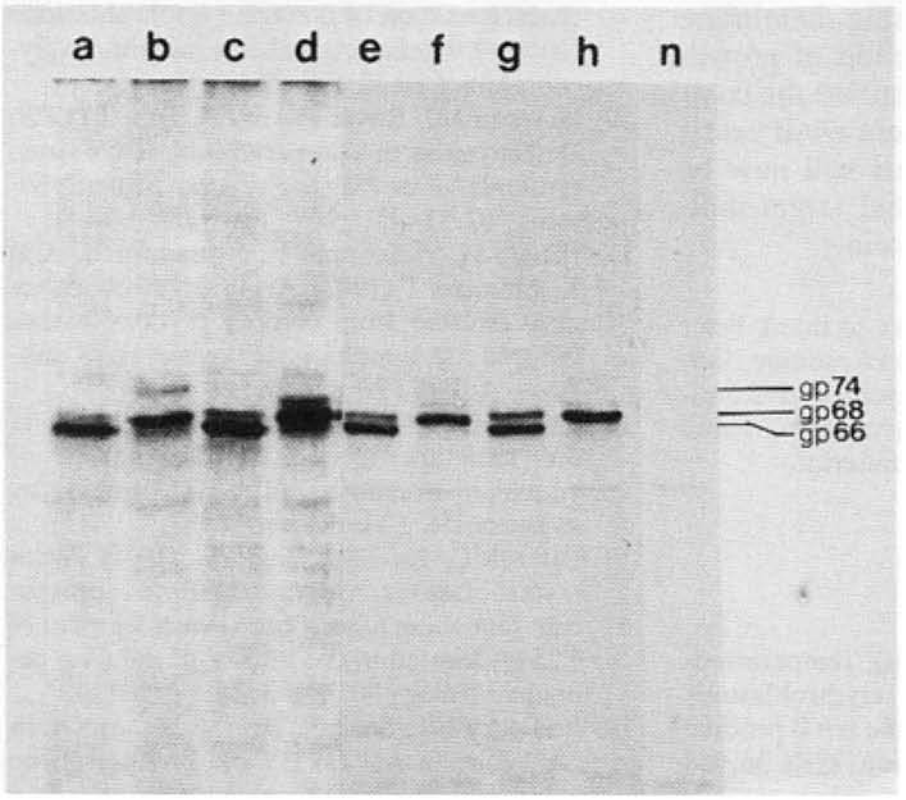

Fig. 3. Pulse-chase analysis of the synthesis of v-*erb* B in the presence or absence of glycoprotein processing inhibitors. Erythroblasts transformed with *ts* 34 AEV were pulse labelled with methionine ^{35}S for 1 h, samples taken (*lanes a, c, e, g*) and then chased in excess cold methionine for a further 4 h (*lanes b, d, f, h*). Glycoprotein processing inhibitors were added to a final concentration of either 10 μ*M* (swainsonine, *lanes c* and *d*) or 2 m*M* (1-deoxynojirimycin, *lanes g* and *h* and 2,5-dihydroxymethyl- 3,4- dihydroxypyrrolidine, *lanes e* and *f*) at least 2 h before addition of label and were present throughout the labelling period. Cell extracts were immunoprecipitated with *erb* B-specific serum and analysed by polyacrylamide gel electrophoresis. *Lanes a* and *b* are control AEV cells grown in the absence of any inhibitors, *lane n* is a control cell extract immunoprecipitated with normal rat serum

methionine ^{35}S and then chased to allow the processing to take place. Detergent extracts were prepared and immunoprecipitated with anti-*erb* B-specific serum [8]. Figure 3 shows the results of such an experiment. In control cells grown in the absence of inhibitors, methionine ^{35}S can be chased from $gp66^{erb\ B}$ through gp68 and into gp74 as shown previously (Fig. 3 lanes a and b). Similar pulse-chase analysis of v-*erb* B in cells grown in the presence of swainsonine contained a 70 000 molecular weight protein instead of gp74 (Fig. 3 lanes c and d) as would be predicted for a protein containing a "hybrid" oligosaccharide structure often formed on glycoproteins synthesized in the presence of swainsonine [12]. Cells grown in the presence of 2 m*M* 1-deoxynojirimycin or 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine had arrested the processing of *erb* B at the gp68 form (Fig. 3 lanes e–h); again this is the result predicted (see Fig. 2). Therefore the inhibitors are having their predicted effects on the processing of *erb* B, but this inhibition does not affect the ability of this protein to maintain the transformed phenotype.

Preliminary results indicate that these abnormally processed forms of *erb* B synthesized in the presence of the inhibitors are nevertheless transported to the plasma membrane (unpublished observations). These data, together with those on the *ts* mutants of AEV [1] would indicate that it is the expression of the *erb* B product in the plasma membrane that is crucial for it to exert its oncogenic effect. Given the recently described homology of this protein with EGF-R [4, 22] this result is perhaps

not surprising since the plasma membrane is most likely the site of action of growth factor receptors. Having identified the compartment within the cell where *erb* B exerts its effect, further experiments will now be necessary to identify potential target molecules for this oncogenic protein.

Acknowledgments. We would like to thank Peter Dorling for providing purified swainsonine, Linda Fellows for 1-deoxynojirimycin and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine, and Irene Esmond for typing the manuscript.

References

1. Beug H, Hayman MJ (1984) Temperature-sensitive mutants of avian erythroblastosis virus: surface expression of the *erb* B product correlates with transformation. Cell 36:963–972
2. Bister K, Duesberg PH (1979) Structure and specific sequences of avian erythroblastosis virus RNA; evidence for multiple classes of transforming genes among avian tumor virus. Proc Natl Acad Sci USA 76:5023–5027
3. Debuire B, Henry C, Benaissa M, Biserte G, Claverie JM, Saule S, Martin P, Stehelin D (1984) Sequencing the *erb* A gene of avian erythroblastosis virus reveals a new type of oncogene. Science 224:1456–1459
4. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD (1984) Close similarity of epidermal growth factor receptor and *v-erb* B oncogene protein sequences. Nature 307:521–527
5. Elbein AD, Pan YT, Solf R, Vosbeck K (1983) Effect of swainsonine, an inhibitor of glycoprotein processing, on cultured mammalian cells. J Cell Physiol 115:265–275
6. Frykberg L, Palmieri S, Beug H, Graf T, Hayman MJ, Vennstrom B (1983) Transforming capacities of avian erythroblastosis virus mutants deleted in the *erb* A or *erb* B oncogenes. Cell 32:227–238
7. Fung YKT, Lewis WG, Kung HJ, Crittenden LB (1983) Activation of the cellular oncogene C-*erb* B by LTR insertion: molecular basis for induction of erythroblastosis by avian leukosis virus. Cell 33:357–368
8. Hayman MJ, Beug H (1984) Identification of a form of the avian erythroblastosis virus *erb*-B gene product at the cell surface. Nature 309:460–462
9. Hayman MJ, Ramsay G, Savin K, Kitchener G, Graf T, Beug H (1983) Identification and characterisation of the avian erythroblastosis virus *erb* B gene product as a membrane glycoprotein. Cell 32:579–588
10. Hayman MJ, Royer-Pokora B, Graf T (1979) Defectiveness of avian erythroblastosis virus: synthesis of a 75k *gag*-related protein. Virology 92:31–45
11. Hihara H, Yamamoto H, Shimohira H, Arai K, Shimizu T (1983) Avian erythroblastosis virus isolated from chicken erythroblastosis induced by lymphatic leukemia virus subgroup A. JNCI 70:891–898
12. Kang MS, Elbein AD (1983) Alterations in the structure of the oligosaccharide of vesicular stomatitis virus G protein by swainsonine. J Virol 46:60–69
13. Lai MMC, Hu SSF, Vogt PK (1979) Avian erythroblastosis virus: transformation-specific sequences form a contiguous segment of 3.25 kb located in the middle of the 6 kb genome. Virology 97:366–377
14. Privalsky ML, Sealy L, Bishop JM, McGrath JP, Levinson AD (1983) The product of the avian erythroblastosis virus *erb* B locus is a glycoprotein. Cell 32:1257–1267
15. Romero PA, Datema R, Schwartz RT (1983) N-methyl-1-deoxynojirimycin, a novel inhibitor of glycoprotein processing and its effect on fowl plague virus maturation. Virology 130:238–242
16. Roussel M, Saule S, Lagron C, Rommens C, Beug H, Graf T, Stehelin D (1979) Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. Nature 281:452–455
17. Schwartz RT, Datema R (1984) Inhibitors of trimming: new tools in glycoprotein research. Trends Biol Sci 9:31–34
18. Sealy L, Privalsky ML, Moscovici G, Moscovici C, Bishop JM (1983) Site-specific mutagenesis of avian erythroblastosis virus: *erb* B is required for oncogenicity. Virology 130:155–178
19. Stehelin D, Saule S, Roussel M, Sergeant A, Lagrou C, Rommeno C, Raes MB (1980) Three new types of viral oncogenes in defective avian leukemia viruses. 1. specific nucleotide sequences of cellular origin correlate with specific transformation. Cold Spring Harbor Symp Quant Biol 44:1215–1223
20. Tarentino AL, Maley F (1974) Purification and properties of an endo-β-N-acetylglucosaminidase from Streptomyces griseus. J Biol Chem 249:811–817
21. Tulsiani DPR, Harris TM, Touster O (1982) Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of golgi α-mannosidase II. J Biol Chem 257:7936–7939

22. Ullrich A, Cousseno L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH (1984) Human epidermal growth factor receptor; cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418–425

23. Yamamoto T, Hihara H, Nishida T, Kawai S, Toyashima K (1983) A new avian erythroblastosis virus, AEV-H, carries *erb* B gene responsible for the induction of both erythroblastosis and sarcomas. Cell 34:225–232