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Truncation of the human EGF receptor leads to differential transforming potentials in primary avian fibroblasts and erythroblasts

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The transforming capacity of the normal and mutant human EGF receptor (EGFR) was investigated in primary chicken cells. In fibroblasts, both N- and C-terminal truncations resulted in a weak, additive oncogenic activity. However, not even double truncations caused a v-erbB-like phenotype. Upon EGF-binding, on the other hand, both normal and C-terminally truncated EGFRs resembled v-erbB in their fibroblast transforming potential. In erythroblasts, N-terminal truncation was sufficient to induce constitutive self-renewal, which was enhanced by deletion of 32 C-terminal amino acids but abolished by a larger truncation of 202 amino acids. In contrast to the normal EGFR, the receptor lacking 32 C-terminal amino acids resembled v-erbB in conferring erythropoietin independence for spontaneous differentiation to the transformed erythroblasts. Our results indicate that the C-terminal domain of the EGFR is non-essential in fibroblast transformation, but seems to be crucial for both self renewal induction and specificity of receptor function in erythroblasts.

Key words: EGF receptor/fibroblasts/erythroblasts/transformation/oncogenes

Introduction

The v-*erb*B oncogene of the acutely transforming retroviruses AEV-ES4 and AEV-H is responsible for induction of erythroleukemia and fibrosarcoma in infected chicks, as well as for transformation of bone marrow erythroblasts and chicken embryo fibroblasts (CEFs) in culture (Graf *et al.*, 1976; Frykberg *et al.*, 1983; Fung *et al.*, 1983; Sealy *et al.*, 1983; Yamamoto *et al.*, 1983a,b; Beug *et al.*, 1985a). V-*erb*B represents an altered version of the gene encoding the avian epidermal growth factor receptor (EGFR), involving the deletion of most of the extracellular domain and of variable portions of the carboxy-terminal domain including one or two of the three C-terminal tyrosine autophosphorylation sites (Yamamoto *et al.*, 1983a,b; Nilson *et al.*, 1985; Downward *et al.*, 1984a,b; Ullrich *et al.*, 1984), as well as several amino acid changes in the tryosine kinase domain (Choi *et al.*, 1986; Scotting *et al.*, 1987).

The transformation induced by v-*erb*B in erythroblasts and fibroblasts is distinct. In bone marrow cells, v-*erb*B causes the outgrowth of erythroid progenitors (Graf, 1973; Graf *et al.*, 1976; Beug *et al.*, 1979, 1985a), although some can spontaneously enter terminal differentiation (Frykberg *et al.*, 1983). These transformed cells no longer require erythopoietin (EPO) for survival, proliferation and differentiation (Samarut and Bouabdelli, 1980; Samarut and Gazzolo, 1982; Beug *et al.*, 1982; Beug and Hayman, 1984). In CEFs, on the other hand, v-*erb*B induces characteristic changes in fibroblast phenotype referred to as 'transformation parameters' such as focus formation, anchorage independent growth, changes in cyto-architecture and hexose uptake but it does not affect their proliferation behavior or differentiation phenotype (Royer-Pokora *et al.*, 1978; Palmieri *et al.*, 1983). Whether v-*erb*B alters growth factor requirements of the fibroblasts is still unresolved.

Most of the early work has been performed with the AEV-ES4 strain, a virus that contains a second, non-transforming oncogene, v-*erb*A, which is a mutated version of a nuclear receptor for thyroid hormone (Sap *et al.*, 1986; Weinberger *et al.*, 1986). In hematopoietic cells, v-*erb*A enhances the transformed phenotype by completely arresting the differentiation of v-*erb*B transformed erythroblasts (Frykberg *et al.*, 1983; Beug *et al.*, 1985a; Kahn *et al.*, 1986; Damm *et al.*, 1987). It also alters some growth parameters of CEFs by enhancing the activity of v-*erb*B (Gandrillon *et al.*, 1987; Jansson *et al.*, 1987).

Despite several studies of v-*erb*B and its mutants (Sealy *et al.*, 1983; Bassiri and Privalski, 1986; Kawai *et al.*, 1987; Beug *et al.*, 1987; Jansson *et al.*, 1987), it is still not resolved how the various C- and N-terminal deletions and point mutations contribute to the oncogenic activation of the c-*erb*B/EGFR gene. Nor is it known whether overexpression of the normal EGFR is sufficient to cause transformation. The *erb*B genes transduced by retroviruses or activated by retroviral promoter insertion in erythroleukemic cells have identical 5'-endpoints, but the N termini of the corresponding proteins can vary depending on the location of the AUG used for translation. In addition, the 3' ends and the corresponding C termini of the proteins can vary considerably. For example, the v-*erb*B genes of the AEV strains ES4 and H exhibit deletions in their 3' ends corresponding to 73 and 34 amino acids, respectively, whereas the c-*erb*B derived genes activated by promoter insertion generally lack such deletions (Nilson *et al.*, 1985; Gamett *et al.*, 1986; B.Vennström and H.Beug, unpublished). Progressive artificial deletion of C-terminal sequences introduced into viral *erb*B genes of AEV-ES4 and AEV-H rendered the respective viruses incapable of transforming erythroblasts, but did not markedly affect their ability to transform fibroblasts, suggesting the existence of an erythroblast-transforming C-terminal domain (Yamamoto *et al.*, 1983b; Damm *et al.*, 1987; Jansson *et al.*, 1987). Analysis of the transforming capacity of mutant *erb*B genes, although generally supportive of this notion, do not permit the assignment of fibroblast- and erythroblast-transforming capacities to specific domains

of the molecule (Miles and Robinson, 1985; Beug *et al.*, 1986, 1987; H.Beug and B.Vennström, unpublished).

In this paper we have chosen a different approach to study the transforming potential of *erb*B, focusing on the question of whether a c-*erb*B/EGFR gene might function as an oncogene if introduced into primary chicken fibroblasts and erythroblasts and subsequently activated by EGF. For this, cDNAs encoding the complete human EGFR or various mutants carrying N- and C-terminal truncations were inserted into avian retrovirus vectors and analyzed for their ability to transform CEFs and erythroblasts in the presence or absence of EGF. These constructs also contained v-*erb*A to enhance the weak transforming activities of the respective EGFR genes.

Activation by EGF causes the non-transforming EGFR to induce phenotypically several parameters of transformation in both types of cells. In fibroblasts, deletion of the EGF binding domain conferred ligand independence and a constitutive transforming capacity whereas deletion of the C-terminal region further increased the effects seen after ligand activation or N-terminal truncation. However, the transformation was in most instances, regardless of the mutations, weaker than that observed with v-*erb*B, suggesting that the additional mutations in the viral protein contribute to its oncogenic effects. In erythroblasts, on the other hand, the EGFR was unable to confer independence of EPO for differentiation, in contrast to proteins lacking 32 C-terminal amino acids, whereas deletion of an additional 94 amino acids abolished transforming capacity. The results suggest that these 94 amino acids of the EGFR are responsible for its specificity for intracellular targets in erythroblasts.

Results

Chicken fibroblasts infected with EGFR constructs express functional receptors

To investigate the transforming effects of the complete human EGFR (HER-C) or its mutants in CEFs and erythroblasts, we introduced the cDNAs into the AEV-ES4 virus genome (Figures 1 and 9). We retained the v-*erb*A gene to enhance the transforming capacity of the constructs. One set of mutant EGFR constructs lacked 32 or 126 C-terminal amino acids, thereby deleting one (HER-6), or all three (HER-4) tryosine autophosphorylation sites (Downward *et al.*, 1984a). N-terminal mutants, lacking most of the extracellular domain necessary for EGF binding but containing either the complete C-terminus (HER-1) or carrying the same C-terminal deletions as described above (HER-1.6 and HER-1.4 respectively) were also studied. In

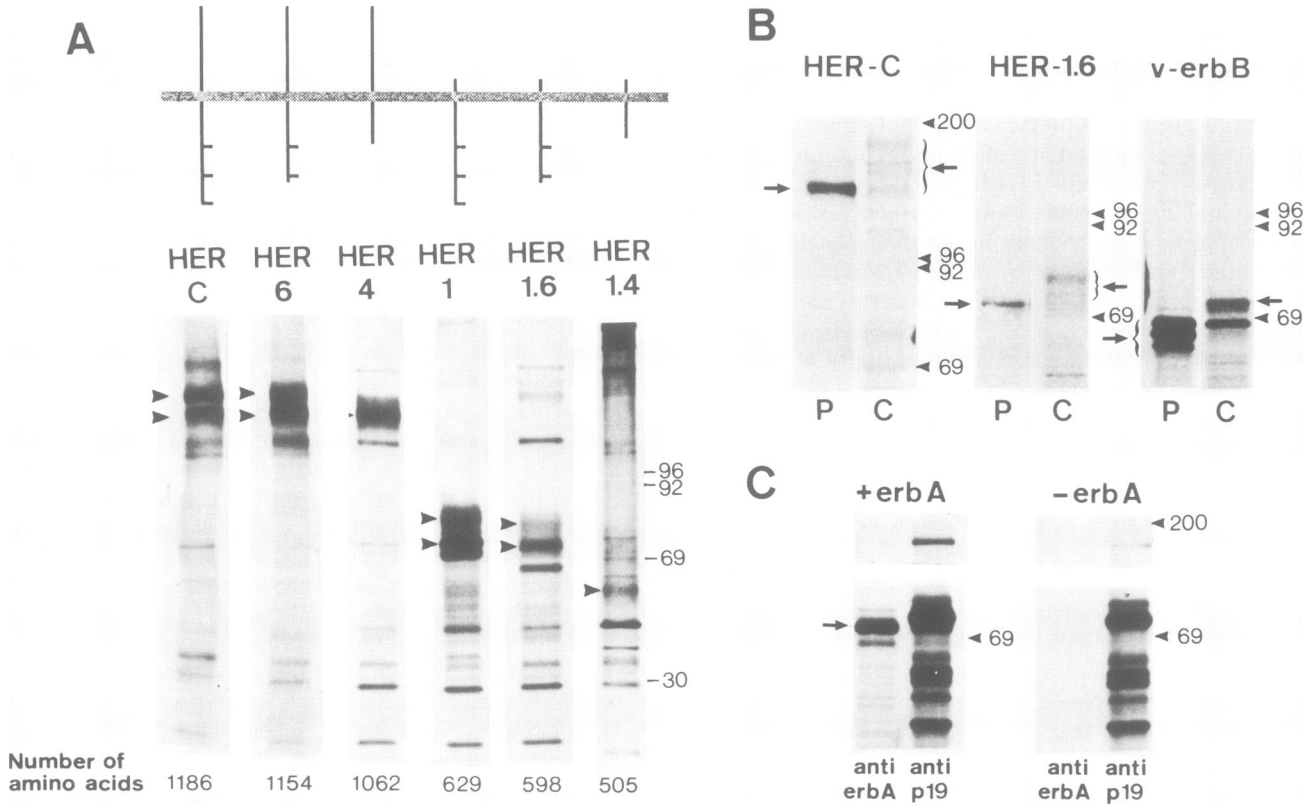

Fig. 1. Expression of normal and mutated human EGF-receptor proteins in chick embryo fibroblasts after infection with the respective recombinant retroviruses. **A.** Immunoprecipitation of human EGFR proteins expressed in chick embryo fibroblasts infected with recombinant retroviruses containing normal and truncated human EGFR (HER) cDNAs. Their nomenclature and structure is schematically depicted on top of the panel (hatched horizontal bar; plasma membrane; short horizontal 'hooks': position of the tyrosine autophosphorylation sites in the C-terminal intracellular domain). Proteins specifically immunoprecipitated by *erb*B antiserum (as determined by comparison with control immunoprecipitation using preimmune serum) are indicated by large arrowheads. Small numerals (right lane) indicate the position of mol. wt markers. **B.** Pulse-chase analysis of chick fibroblasts expressing the complete EGFR (HER-C) a double-truncated receptor (HER-1.6), or the AEV v-erbB protein (v-erbB). Cells were pulse labeled with [^{35}S]methionine (P) or chased for 4 h (C) as described in Materials and methods. The pulse–chase labeling of the HER-C cells was done in presence of EGF (20 ng/ml). Note the efficient processing of one precursor polypeptide into higher molecular weight forms in case of HER-C and HER 1.6 (arrows) and the persistence of gp68^{erbB} during abnormal processing of the v-erbB protein (Hayman *et al.*, 1983). **C** Expression of p75$^{gag\text{-}erb\text{-}A}$ in HER-C (+erbA) and HER-C A2$^-$ (−erbA) transformed fibroblasts. Immunoprecipitation of labeled lysates with antisera to the v-erbA protein and to viral structural proteins (anti p19) reveals that p75$^{gag\text{-}erbA}$ is expressed in the HER-C cells but not in the HER-C-A^{2-} cells, although both types express similar levels of viral structural proteins.

a second set of constructs, the v-*erb*A gene of AEV-ES4 was exchanged for an inactive v-*erb*A allele, v-*erb*A2$^-$ (Frykberg *et al.*, 1983). All constructs were designed to express the inserted EGFR cDNAs from spliced, subgenomic mRNAs, using the AUG of the EGFR signal peptide sequence for translation initiation. The N- and C-terminal mutations in the EGFR genes mimicked the deletions found in the v-*erb*B gene of AEV strain H (HER-1.6) and its deletion mutant *td*130 (HER-1.4) (Yamamoto *et al.*, 1983a),

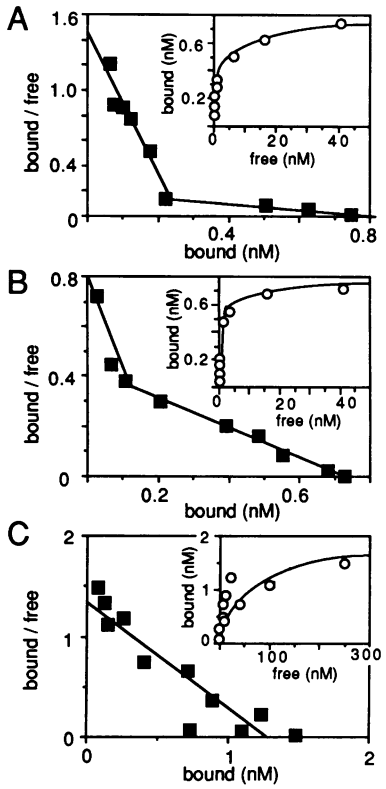

Fig. 2. Analysis of [^{125}I]EGF binding to chick fibroblasts expressing normal and mutated human EGFR. Scatchard plots are shown for binding of EGF to HER-C cells (**A**) HER-6 cells (**B**) and HER-4 cells (**C**). The saturation curves obtained are shown in the inserts. Each value represents the mean of triplicate determinations.

Table I. Characteristics of human EGF receptor constructs expressed by CEFs

Construct	No. of receptors per cell	K_d, high affinity (nM)	K_d, low affinity (nM)
HER-C	4.1 ± 1.2×10^5 (9)[a]	0.19 ± 0.09 (9)	2.7 ± 0.8 (9)
HER-6	13 ± 3.6×10^5 (3)	0.27 ± 0.02 (3)	1.9 ± 0.7 (3)
HER-4	22 ± 15×10^5 (3)	undetectable (3)	1.7 ± 0.6 (3)
HER-1, 1.6, 1.4	<10^3[c]	NR[d]	NR
Control (NR-6)[e]	14 × 10^5 (1)	0.12 (1)	1.1 (1)

[a]Number of determinations.
[b]No evidence for high affinity receptors was revealed by Scatchard analysis (Figure 3).
[c]No binding of [^{125}I]EGF above background was seen with these cells.
[d]NR not relevant.
[e]NR-6 cells, which normally lack EGF receptors, were transfected with a plasmid construct expressing the human EGF receptor from an HSV thymidine kinase promoter.

except for the signal peptide sequence added to the human genes.

To test whether the recombinant viruses replicated and correctly expressed their EGFR and v-*erb*A genes, CEFs were transfected with virus construct DNA together with RAV-2 helper virus DNA. Transfected cells were selected for resistance to G418, encoded in the *neo* gene of the plasmid vector, or for anchorage-independent growth in soft agar. After selection and expansion, cells were labeled with [^{35}S]methionine and extracts immunoprecipitated with anti-erbB antisera. Figure 1A shows that proteins of expected sizes were expressed at high levels in the infected fibroblasts, the different bands probably representing glycosylated precursors at different stages of processing. Possibly due to their intact signal peptide, EGFR proteins lacking the ligand binding domain were normally processed into mature forms like the unmutated EGFR protein, thus differing from v-erbB protein which is processed abnormally (Figure 1B). Virus constructs containing a *wt* v-*erb*A gene expressed p75$^{gag\text{-}v\text{-}erbA}$ at levels similar to those of the virus structural proteins pr180$^{gag\text{-}pol}$ and pr76^{gag}, while those carrying the defective v-*erb*A2$^-$ allele expressed no stable v-erb-A-related polypeptides (Figure 1C).

To test ligand-binding properties of the various EGFR proteins, Scatchard analyses using ^{125}I-labeled human and mouse EGF were performed with the fibroblasts described above. Figure 2 and Table I demonstrate that cells expressing the HER-C and HER-6 proteins exhibited both high (k_d 0.1−0.3 nM) and low affinity receptors (k_d 1−4 nM), 17−25% of the receptors being of the high-affinity type. In contrast, fibroblasts expressing the extensively truncated HER-4 protein lacking all three autophosphorylation sites exhibited exclusively low affinity receptors (k_d 1−2 nM).

Determination of the receptor number per cell (Table I) revealed that all ligand-binding EGFR-proteins were highly expressed. HER-C cells contained 2−4 × 10^5 recep-

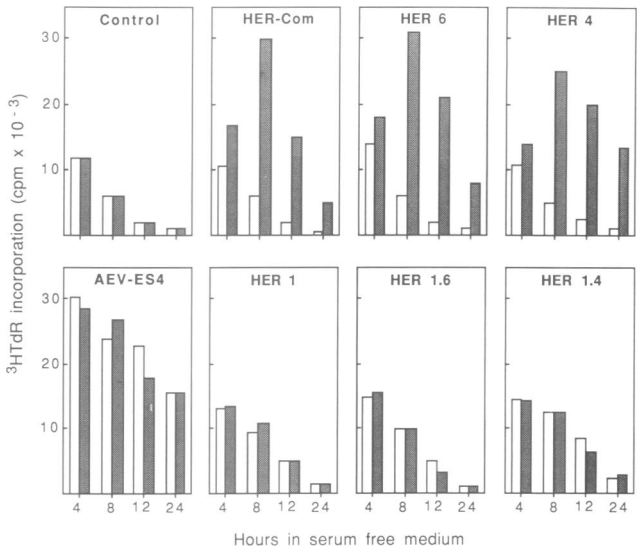

Fig. 3. Proliferation of chick fibroblasts expressing normal and mutated EGF receptors in serum-free medium. Fibroblasts infected the different HER constructs, RAV-1 (control) and AEV-ES4 virus were pregrown in standard medium and then washed and incubated in serum-free medium in the presence (hatched bars) and absence (open bars) of 10 μg/ml EGF for the times indicated, and analyzed for [^{3}H]TdR incorporation as described in Materials and methods. Note the induction of serum-independent growth by EGF with the constructs containing a ligand-binding domain, and the constitutive serum-independent growth with v-*erb*B.

Table II. Colony formation in soft agar of chick fibroblasts transfected with EGFR encoding viruses

Construct	Number of agar colonies[a]			
	−v-*erb*A[(b)]		+v-*erb*A	
	−EGF	+EGF[(c)]	−EGF	+EGF
HER-C	1.5	90	34	372
HER-6	26	225	126	224
HER-4	62	520	200	600
HER-1	256	160	260	256
HER-1.6	483	486	602	548
HER-1.4	510	560	689	852
AEV ES4	ND[d]	ND	688	609

[a]Average of colonies from one to two parallell dishes counted after 8−10 days as described in Materials and methods. Values shown were calculated by subtracting the number of colonies obtained (2−10) in control cultures transfected with RAV-2 DNA.
[b]Constructs containing a deleted, non-functional v-*erb*A gene was used.
[c]10 ng/ml mouse EGF.
[d]ND, not done.

Table III. Transformation parameters and focus forming ability of EGFR virus constructs in fibroblasts

Infecting virus	EGF addition	Cells with actin cables (%)	Hexose transport (fold stimulation)	Focus formation (FFU/ml)
HER-C	−	89	1.2	6.0×10^1
	+	14	3.0	3.0×10^3
HER-6	−	ND	1.6 ± 0.5[a]	6.0×10^1
HER-4	−	73	1.2	$<10^1$
	+	7	3.1	7.5×10^3
HER-1	−	75	2.4 ± 0.9[a]	4.0×10^1
HER-1.6	−	74	2.0 ± 0.2[a]	1.6×10^2
HER-1.4	−	76	2.3 ± 0.6[a]	5.0×10^1
AEV ES4	−	35	3.3 ± 0.4[a]	6.0×10^4
RAV-2	−	90	1.0	$<10^1$
	+	85	1.0	ND

[a]Pooled data from several experiments employing different fibroblast cultures.

Table IV. Erythroblast transformation by mutant EGF receptor viruses

Construct	− *erb*A		+ *erb*A	
	Co-cultivation[a]		Co-cultivation[c]	Colony assay[b,c] (No. of colonies)
	−EGF	+EGF[c]		
HER-C	−[d]	+	+	0
HER-6	−	+	+	0
HER-4	−	−	−	0
HER-1	+	+	+	5
HER-1.6	+	+	+	19
HER-1.4	ND	ND	−	0

[a]Normal bone marrow cells were co-cultivated with high-titer virus-producing fibroblast clones as described in experimental procedures.
[b]Bone marrow cells infected by co-cultivation were seeded into semisolid medium as described in experimental procedures.
[c]The co-cultivation was done in the presence of 20 ng/ml EGF (added daily).
[d]Cocultivations were done at least twice, in case of a negative (−) result three times.
[c]Assays were done in the absence of EGF.

tors/cell, while the HER-6 and HER-4 cells expressed even 5- to 10-fold higher receptor numbers ($1-4 \times 10^6$ receptors/cell). Neither the receptor number nor their affinity to ligand was affected by the presence of v-erbA protein (data not shown). As expected, fibroblasts expressing EGFR-proteins without ligand binding domain as well as uninfected or RAV-2 helper virus infected fibroblasts did not bind iodinated mouse or human EGF.

Mitogenic response of the EGFR and its mutants in fibroblasts

To investigate mitogenic effects of the various EGF-receptor mutants in chick fibroblasts is difficult since CEFs are inefficiently arrested in G_0 upon cultivation in serum-free or low-serum medium and respond poorly to growth factors after starvation (A.Johnsson and H.Beug, unpublished data). However, serum withdrawal from CEF cultures leads to an almost complete cessation of growth within 24 h as measured by thymidine incorporation ([^{3}H]TdR). We therefore asked whether EGFR-expressing CEFs, when submitted to the latter protocol and treated with EGF, could maintain growth. Figure 3 demonstrates that in the absence of EGF none of the constructs completely reversed the growth retardation induced by serum withdrawal (as seen with v-*erb*B-transformed controls CEFs). The decrease in thymidine incorporation occurred, however, more slowly in cells expressing HER-1, HER-1.6 and was even more pronounced with HER-1.4 proteins. In contrast, addition of EGF at high concentrations (10 ng/ml) to cells expressing the EGFR proteins with an intact ligand binding domain delayed or even prevented their arrest. This was true even for HER-4-expressing cells, suggesting that neither the C-terminal autophosphorylation sites nor high affinity receptors are required for EGF dependent stimulation of DNA synthesis in fibroblasts.

Phenotypic transformation of fibroblasts by EGFR activated by ligand

To study whether these proteins also induce a transformed phenotype in fibroblasts, the cells were seeded into soft agar in the presence or absence of EGF. Table II shows that in the absence of ligand HER-C was essentially inactive, the HER-6 and HER-4 genes induced colonies poorly whereas co-expression with v-*erb*A increased the number of colonies 3- to 10-fold. The addition of EGF stimulated soft agar colony formation 5- to 40-fold, although no additive effect of v-*erb*A was seen. In contrast, constructs lacking an intact ligand-binding domain induced agar colonies even in the absence of EGF and no further increase was observed after addition of EGF. Again, the C-terminally truncated versions (HER-1.6, HER-1.4) were more active than HER-1 which has an intact C-terminus. In these experiments the presence of v-*erb*A yielded larger colonies without affecting their number (Table II).

None of the constructs, regardless of whether or not the construct tested contained v-*erb*A, induced the formation of well defined foci in the absence of ligand. In the presence of ligand, however, both HER-C and HER-4 efficiently induced foci although they were still fewer and more diffuse than those obtained with v-*erb*B (Table III).

The data thus show that N- and C-terminal truncations of the EGFR generate partially transforming proteins, and that EGF greatly increases the transforming ability of the pro-

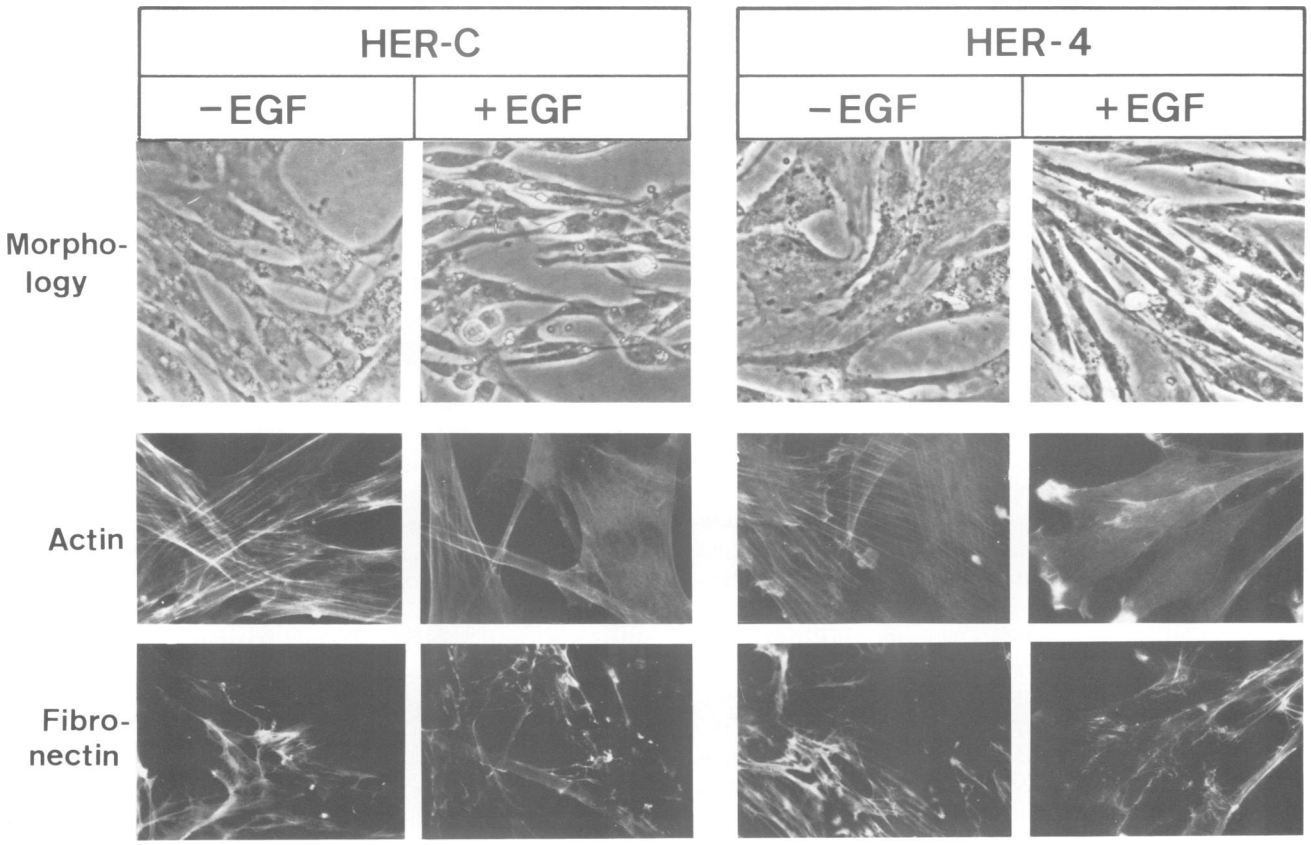

Fig. 4. EGF-dependent, phenotypic transformation of chick fibroblasts expressing a normal (HER-C) or C-terminally truncated EGFR (HER-4). Cells were incubated overnight in presence or absence of 20 ng/ml EGF. Morphologies are shown in the phase micrographs (**upper panels**). The same field of cells stained by double immunofluorescence for actin cables (**middle panels**) and fibronectin network (**bottom panels**) are shown.

teins containing the ligand-binding domain. This notion was confirmed by analyzing additional fibroblast transformation parameters. Figure 4 and Table III show that in the absence of ligand all HER proteins caused minor changes in cell morphology, cytoskeleton architecture or hexose transport. Those lacking the EGF-binding domains were more active than those retaining it. A 20 h incubation with EGF, however, caused both HER-C and HER-4 expressing fibroblasts to assume a transformed, spindle-like morphology, to lose organized actin filament bundles, and to express elevated levels of hexose transport similar to that observed with v-erbB-transformed cells (Figure 4, Table III).

Stimulation of EGF as well as N-terminal truncation render the EGFR transforming in avian erythroblasts

To study the effects of the EGFR retrovirus constructs on self-renewal and differentiation of erythroid progenitors, infected fibroblast clones were co-cultivated with fresh bone marrow cells in the presence of anemic serum as a source of EPO and, in some experiments, with EGF.

Table IV shows that in the absence of EGF the HER-C and HER-6 viruses caused erythroblast transformation provided that they also expressed v-*erb*A, while the N-terminally truncated constructs HER-1 and HER-1.6 caused transformation of erythroblasts even without v-*erb*A. The double-truncated construct HER-1.6 was more active than HER-1, since it transformed bone marrow erythroblasts more rapidly and reproducibly. Futhermore, HER-4 and HER-1.4, the constructs with the most extensive C-terminal truncation, lacked the ability to transform erythroblasts even in the presence of v-*erb*A. Viruses harvested from transformed erythroblasts were indistinguishable from the original fibroblast-grown viruses with respect to their ability to induce EGFR protein expression and transformation in fibroblasts (not shown), suggesting that mutated viruses had not been selected.

In co-cultivations performed in the presence of EGF, the HER-C and the HER-6, but not the HER-4 viruses, induced the stable outgrowth of erythroblasts regardless of v-*erb*A expression. The HER-C and HER-6 cells were dependent of EGF for proliferation and survival (see below), suggesting that the normal EGFR is indeed able to induce self-renewal in erythroid progenitors. Surprisingly, the HER-4 + v-*erb*A construct was able to induce a transient outgrowth of erythroblasts, suggesting that extensive truncation of the EGFR C terminus reduces but does not completely abolish its capacity to induce self-renewal in erythroblasts. Immunoprecipitation revealed that the transformed erythroblasts expressed proteins of the expected sizes (not shown). The HER-C cells contained 2.2×10^4 receptors/cell, which bound iodinated EGF both with high (0.1 nM) and low (2 nM) affinity.

Proliferation and differentiation capacity of erythroblasts expressing normal and mutant EGFR

Next we determined how the differentiation program of erythroid progenitors was altered by the various forms of EGFR proteins. Erythroblasts transformed by the HER-C,

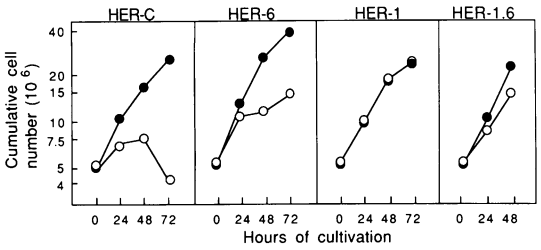

Fig. 5. Proliferation of erythroblasts transformed by normal and mutant EGFR proteins in the presence or absence of EGF. Proliferation of transformed erythroblasts was measured by cell counting in the presence (closed symbols) or absence of 20 ng/ml EGF (open circles).

HER-6, HER-1 and HER-1.6 viruses (containing the v-*erb*A2$^-$ genes) and cultivated in presence of EGF were reseeded in EPO-containing differentiation medium in presence or absence of EGF, and analyzed for proliferation by cell counting. Figure 5 demonstrates that the proliferation of HER-C and HER-6 erythroblasts was strictly dependent on EGF. Withdrawal of EGF caused a rapid proliferation arrest in the HER-C erythroblasts while the HER-6 erythroblasts continued to proliferate for 24−48 h before they withdrew from the cell cycle. In contrast, growth of the HER-1 and the HER-1.6 cells was not affected by EGF. Finally, cells containing the ligand-activated receptors grew faster than cells expressing the proteins without a ligand binding domain. Determination of [^{3}H]TdR incorporation throughout these experiments corroborated the above findings (not shown).

Analysis of the ability of the cells to differentiate in the presence of both EPO and EGF showed that both the HER-C and the HER-6 cultures contained ≥90% erythroblasts (data not shown). The HER-C and HER-6 cells resembled v-*erb*B$^-$ and v-*sea*-transformed cells with respect to morphology, cytochemical staining, volume (not shown) and antigen expression (Figure 6; Knight *et al.*, 1988). Withdrawal of EGF for 3 days caused the cells to differentiate into early reticulocytes (ER, 25−50%), late reticulocytes (LR, 45−60%) or even erythrocytes (Ery, 1-6%), as determined by cytochemical staining. Analysis of the same cells for differentiation antigens revealed the appearance of erythrocyte antigens (EryAg, MC5J2A antigen) after 2 days while erythroblasts markers had almost disappeared. After 3 days, no blast-like cells were observed and the cells exclusively expressed erythrocyte markers (not shown). HER-6 erythroblasts required 5 days of EGF withdrawal to differentiate terminally (Figure 6).

In contrast, HER-1 cultures grown in presence or absence of EGF contained ~65% erythroblasts, the remaining cells being more mature (25% ER, 20% LR + Ery). The spontaneous differentiation exhibited by the HER-1 cells was similar to that observed with RAV-1 induced erythroleukemia cells containing N-terminally truncated c-*erb*B proteins (Nilson *et al.*, 1985; Beug *et al.*, 1986), while v-*erb*B-transformed erythroblasts contain lower proportions of mature cells (Damm *et al.*, 1987).

Ligand-activated HER-C protein induces self-renewal, but not EPO-independent differentiation

The v-*erb*B oncogene may induce self-renewal through intracellular signal(s) similar to those generated by the

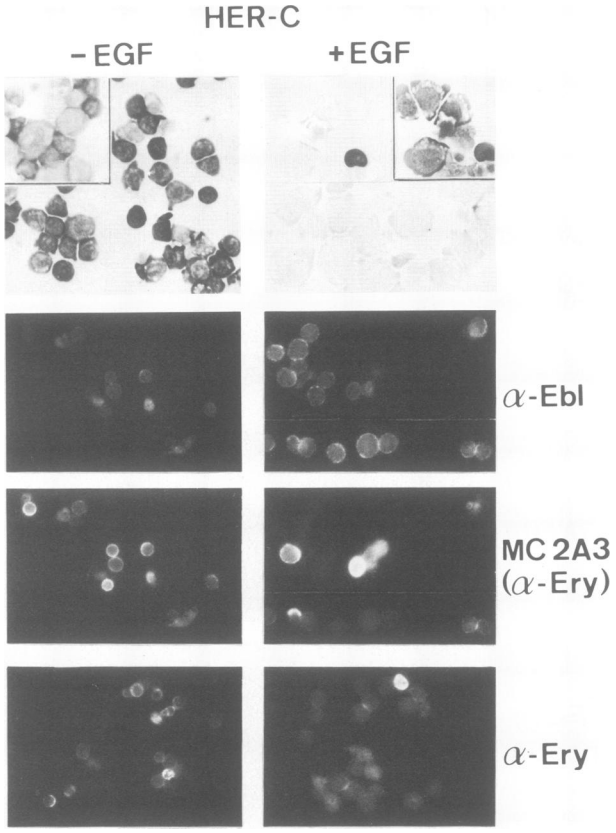

Fig. 6. Dependence on EGF for differentiation arrest of HER-C transformed erythroblasts. Cells grown in the presence or absence for 2−3 days were cytocentrifuged, stained with benzidine and photographed under blue light to reveal hemoglobin-positive cells. Alternatively, cells were stained by indirect immunofluorescence with various differentiation-specific antibodies [rabbit anti-erythroblast serum; α-Ebl; erythrocyte-specific monoclonal antibody MC2A3 or rabbit anti-erythrocyte serum, α-Ery (**lower panels**)]. The aEbl and MC2A3 panels show the same fields photographed for FITC and TRITC fluorescence, respectively. Note that a small proportion of erythrocyte-like cells are stained by the α-Ery serum in the immature cell population transformed by HER-C and grown in the presence of EGF (arrows). Inserts in the **top panels** show benzidine-stained cells grown in the absence of EGF for 2 days (left) and the morphology of the immature HER-C erythroblasts obtained in the presence of EGF, as photographed under green light to reveal their morphology.

putative receptor(s) for erythropoietin and/or erythroid-specific growth factors (Beug *et al.*, 1985b). Since the ligand-activated HER-C protein induced self-renewal in erythroblasts, we tested whether the normal and the mutated receptor proteins would, like v-*erb*B, promote hormone-independent, spontaneous differentiation of transformed erythroblasts (Kahn *et al.*, 1984; Beug *et al.*, 1985b). HER-C and HER-6 erythroblasts cultivated in differentiation medium containing EGF were replated in medium without EPO and lacking or containing EGF for 3 days. Cells were assayed daily for their state of differentiation by cell size determination and cytochemical staining. The results show that both the HER-C and the HER-6 cultures contained erythroblasts to >95% in media with 20 ng/ml EGF in the presence and absence of EPO. As shown in three different experiments, withdrawal of EGF from these cultures caused the HER-6 cells to differentiate equally well in the presence or absence of EPO, whereas the HER-C cells dif-

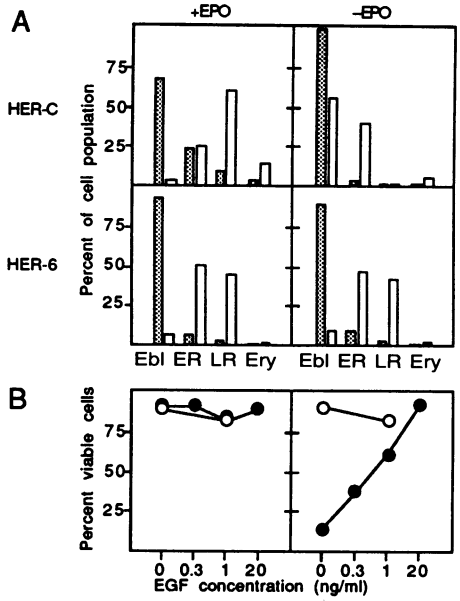

Fig. 7. Dependence on EGF and EPO for differentiation and survival of transformed erythroblasts cultures. **A.** Aliquots of HER-C and HER-6 erythroblasts grown for 3 days in the presence (hatched bars) or absence (open bars) of 1 ng/ml EGF were analyzed for their content of erythroblasts (Ebl), early reticulocytes (ER), late reticulocytes (LR) and erythrocytes (Ery). The presence or absence of EPO is indicated in the figure. **B.** The percentage of viable cells (HER-C: closed circles; HER-6: open circles) was determined after incubation for 2 days in media containing the indicated concentrations of EGF, +/− EPO.

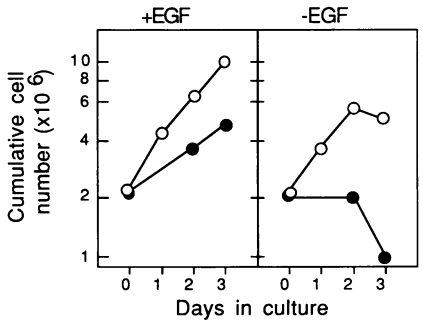

Fig. 8. Dependence on EGF in the absence of EPO for proliferation of transformed erythroblasts. HER-C (closed circles) and HER-6 (open circles) cells were grown in the absence or presence (20 ng/ml) of EGF in media lacking EPO. Cumulative cell number was determined daily, and corrected for cell losses during removal of dead cells.

ferentiated normally in the presence of EPO, but failed to mature beyond the early reticulocyte stage in the absence of EPO (Figure 7A). A similar result was obtained with cells cultivated with 1 ng/ml EGF, again showing that spontaneous differentiation was EPO dependent with HER-C cells (12% LR + Ery with EPO, 1% without) whereas it was EPO independent with HER-6 cells (Figure 7A).

Determination of the percentage of viable cells during growth in different concentrations of EGF by cytochemical staining (Figure 7B) or determination of cell numbers (Figure 8) revealed that the HER-6 erythroblasts (open symbols) survived and continued to grow in the absence of EPO for up to 2 days after EGF withdrawal, whereas the HER-C cells (closed symbols) quickly ceased to grow and disintegrated. Finally [^{3}H]TdR incorporation by the HER-C cells in the absence of EPO was strictly dependent on EGF (not shown).

Table V. Leukemogenicity of the retroviruses containing v-*erb*A and human EGF receptor genes

*erb*B gene	Incidence of erythroblastosis[a]		Mean latency (days)	
	L15	Spafas	L15	Spafas
HER-C	2/4	0/8	14	NR[b]
HER-6	1/4	0/8	14	NR
HER-1	3/4	4/9	14	15
HER-1.6	4/4	4/9	14	16

[a]Leukemia was determined by staining bloodsmears and determining the appearance of erythroblasts.
[b]NR not relevant.

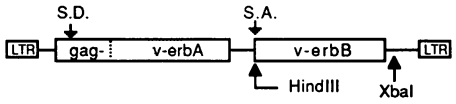

Fig. 9. Structure of the AEV derived vector used for expression of the human EGFR cDNAs. V-*erb*B was excised with *Hin*dIII and *Xba*I, and the respective cDNAs inserted (see Figure 1A). The hatched area in v-*erb*A shows the 500 nt *Pst*I−*Pst*II fragment deleted in the inactive A2-variant. S.A.: splice acceptor site; S.D.: splice donor site.

Thus the HER-C but not the HER-6 erythroblasts are dependent on EPO for survival and differentiation, unless induced to self-renew by high concentrations of EGF.

Leukemogenic potential of normal and mutated human EGF receptors

Since the human EGF-containing retrovirus constructs HER-C, HER-6, HER-1 and HER-1.6 were all able to transform erythroblasts *in vitro* when co-expressed with v-*erb*A, we assessed their leukemogenicity in chicks. The viruses were injected intravenously into 1-week-old F1 chicks of a cross between the K28 and the $L15_1$ strains, in which erythroleukemia is readily induced by RAV-1 activated *erb*B genes encoding complete C termini (Fung *et al.*, 1983; Nilson *et al.*, 1985; L.Crittenden, personal communication). Injections were also done into Spafas chicks, which are resistant to RAV-1 induced erythroleukemia (Robinson *et al.*, 1985).

Erythroblastosis was observed with all constructs in K28 × $L15_1$ chicks, whereas only those viruses encoding erbB proteins without the ligand binding domain (HER-1, HER-1.6) were leukemogenic in the Spafas strain (Table V). *In vitro* cultivated leukemic cells from HER-6 and HER-1 infected K28 × $L15_1$ birds expressed HER proteins of the expected sizes as shown by immunoprecipitation, whereas the cells from the single, leukemic HER-C chick synthesized the normal 170 kd receptor as well as a smaller protein of ~95 kd. Although fibroblasts infected with HER-C virus from these leukemic cells formed no foci and exhibited unchanged high and low affinity binding of the iodinated EGF (data not shown), we cannot rule out that the HER-C leukemia was caused by a mutated version of the EGFR molecule. Nevertheless, our results suggest that the N-terminally truncated EGFR proteins HER-1 and HER-1.6 (and perhaps also the ligand binding constructs HER-C and HER-6) are leukemogenic in chicks.

Discussion

Is overexpression and ligand activation sufficient to convert the normal EGF-receptor into a transforming protein?

An unexpected result of our studies was that primary CEFs overexpressing the normal human EGFR (HER-C) could be induced by ligand (EGF) to exhibit most phenotypic changes characteristic of v-*erb*B transformation. Only focus formation and colony growth in soft agar were somewhat less efficiently induced by HER-C than by v-*erb*B. Likewise, overexpression of ligand-activated EGFR led to induction of self renewal in erythroblasts to an extent similar to v-*erb*B. However, HER-C could not, unlike HER-6 and v-*erb*B (Beug *et al.*, 1985a,b), promote EPO independent differentiation.

Our fibroblast data differ from those of DiFiore *et al.* (1987) and Velu *et al.* (1987) who demonstrated induction of foci and agar colonies by EGF in 3T3 cells overexpressing the EGFR with an efficiency similar to that seen with v-erbB. Since these authors did not analyze the transformed phenotype of their EGFR expressing cells, the results are difficult to compare. The difference may be due to the hypersensitivity of the immortalized 3T3 cell lines to the action of oncogenes (Land *et al.*, 1983), a problem we have tried to avoid by using primary CEFs that cannot be immortalized.

Our findings suggest that the normal signal(s) generated by the ligand-activated EGF receptor are sufficient to induce most of the morphological and physiological changes attributed to a 'transformed' fibroblast phenotype. This concept agrees with the observation, that normal human fibroblasts assume a transformed morphology and grow in a focus-like fashion when treated with high doses of PDGF, an effect that can be completely reversed by stripping off the PDGF from its receptor with suramin (Johnsson *et al.*, 1985, 1986; Betsholtz and Westermark, 1984; R.Klein and J.Thiel, personal communication). Likewise, normal chicken fibroblasts overexpressing the complete avian c-*erb*B gene assume a transformed phenotype when treated with TGF-α, an effective agonist of the chicken c-erbB protein (Lax *et al.*, 1988). The transformed phenotype induced by CEFs by the ligand-activated EGF receptor might therefore reflect the normal behavior of fibroblasts under physiological conditions, such as during PDGF stimulation of skin fibroblast outgrowth in a healing wound.

In erythroblasts, which normally do not express any detectable EGFR and do not self-renew under normal circumstances, the effects of ligand-activated EGFR must be due to a different mechanism. Since very early, pluripotent hematopoietic stem cells may respond to non-hematopoietic growth factors like PDGF and FGF (N.N.Iscove, personal communication), committed progenitors like erythroblasts, although no longer expressing the receptors, may still contain the signal transduction machinery required for these early responses. If the ligand-activated EGFR would utilize such stem cell pathways different from those employed by late-acting growth factors such as erythropoietin, our finding that the activated EGFR was not able to replace erythropoietin during terminal differentiation becomes understandable.

After completion of these studies, Pierce *et al.* (1988) reported that an IL-3-dependent myeloid cell line showed IL-3-independent growth and partial differentiation, when overexpressing the ligand-activated EGF receptor. These studies are difficult to compare with ours, since the established cell line used by these authors had already acquired the ability to self renew by unknown transformation events. Furthermore, the cells were able to grow continuously in EGF only after an 'adaptation' period suggesting selection of aberrant cell types. Recent results of von Rüden *et al.* (T.von Rüden and E.Wagner personal communication) indeed indicate that primary myeloid mouse bone marrow cells cannot be induced to proliferate in the absence of IL-3 by an overexpressed, ligand-activated EGFR.

Oncogenic activation of the EGFR c-erbB gene

The mutations introduced into the EGFR cDNA were chosen to mimic the N- and C-terminal truncations found in the v-*erb*B gene of AEV-H (HER-6, HER-1.6) and its derivative *td*130 which is transformation-deficient in erythroblasts (HER-4, HER-1.4, Yamamoto *et al.*, 1983a). It was therefore surprising that none of the mutated EGFR, not even the doubly truncated HER-1.6 and HER-1.4 proteins, were able to induce a fully transformed phenotype in chick fibroblasts similar to that induced by v-*erb*B. Possible explanations include the presence of several point mutations in the v-erbB proteins of strains H and ES4 (Nilsen *et al.*, 1985; Yamamoto *et al.*, 1983a; Choi *et al.*, 1986; Scotting *et al.*, 1987), and the lack of a signal peptide in the v-*erb*B glycoprotein, leading to its aberrant glycoprotein processing and inefficient incorporation into the plasma membrane (Beug and Hayman, 1984; Privalski and Bishop, 1984). Another explanation for the low activity of our HER mutants could be that the human EGFR is inherently less active in chicken cells. We consider this unlikely since chicken cells overexpressing a full length, normal avian c-*erb*B cDNA were indistinguishable from HER-C expressing cells both in the absence and presence of ligand (A.Johnsson and B.Vennström, unpublished). On the other hand, the ligand-binding domain containing HER-6 and HER-4 proteins, when stimulated by EGF, were even more transforming than HER-C, giving rise to a v-erbB-like transformation with the exception of a weaker focus formation.

Our data also show that removal of the ligand-binding domain results in a weak, but constitutive oncogenic activation of the EGF receptor, while binding of ligand leads to a much stronger, but EGF-dependent activation. This is in accord with the finding that the v-erbB protein displays a much weaker tyrosine kinase activity than the ligand-activated EGF receptor, both in autophosphorylation and on exogenous substrates (Lax *et al.*, 1985; Hayman *et al.*, 1986; Gilmore *et al.*, 1985). On the other hand, truncation of the C-terminal domain enhanced the oncogenic activation conferred by EGF binding or removal of the ligand-binding domain, but did not efficiently activate the EGF receptor on its own. In this context it is noteworthy, that neither high affinity binding of EGF nor the presence of any of the three C-terminal autophosphorylation sites seems to be required for mitogenic stimulation and transformation by the EGFR in fibroblasts.

Involvement of the C-terminal EGFR domain in signal transduction

Our results show that although the normal ligand-activated EGF receptor was mitogenic in both fibroblasts and erythroblasts, the effects of N- and C-terminal truncations on its transforming ability were different in the two cell

types. In erythroblasts, truncation of the N-terminal domain was sufficient to induce self renewal, the HER-1 cells being very similar to the erythroleukemic cells obtained after activation of the c-*erb*B by RAV-1 integration (Beug *et al.*, 1986). Moreover, truncation of 32 C-terminal amino acids enhanced erythroid transformation regardless of whether the ligand-binding domain was present or absent. A more extensive C-terminal truncation (126 amino acids) essentially abolished the erythroblast transforming capacity, while the same truncation caused enhanced transforming activity in fibroblasts. In combination with earlier data obtained with various v-erbB mutants (Yamamoto *et al.*, 1983a; Beug *et al.*, 1986; Damm *et al.*, 1987; Jansson *et al.*, 1987), the results suggest that mitogenic signalling by the EGFR in erythroblasts might proceed via different pathways than in fibroblasts, ruling out simple mechanisms such as different threshold levels of tyrosine kinase activity being required for transformation in the two cell types.

Although the C-terminal domain clearly plays a role in activating signal transduction in erythroblasts, the presence of the C-terminal domain is not itself a prerequisite for self renewal since a v-erbB protein that has lost ~200 C-terminal amino acids is still capable of transforming erythroblasts (H.Beug and B.Vennström, unpublished). We nevertheless propose that the C-terminal domain is instrumental in defining the substrate specificity of the EGFR tyrosine kinase, and that changes in this domain could lead to an EGFR kinase with altered specificity. This idea is corroborated by our finding that the HER-6 protein but not the complete EGFR altered the requirement of erythroblasts for erythroid growth factors during differentiation, suggesting that the structure of the mutated EGFR allowed it to interact with target proteins that are specific for hematopoietic growth factor receptors and different from the targets normally recognized by the unmutated EGFR.

Outlook

The studies presented here have shown that overexpression and ligand activation of the normal EGFR is sufficient to cause transformation in primary, non-established avian fibroblasts and hematopoietic cells, and that the mutations accumulated during conversion of c-*erb*B to v-*erb*B all seem to contribute to the induction of constitutive ligand-independent transformation. It is tempting to speculate that the different *erb*B mutations might in fact cooperate to bring about two types of functional changes. On the one hand, N-terminal truncations, C-terminal truncations and point mutations might all cause ligand-independent activation of the EGFR kinase activity, with the additive effects of all changes being required to obtain a ligand-independent receptor activity strong enough to transform cells. On the other hand the altered intracellular processing of the v-erbB protein, perhaps leading to recycling and thus to an increased half-life (I.Killisch, G.Griffiths and H.Beug, unpublished), might be important for its transforming ability, since even the combined effects of all mutations in v-*erb*B might still result in a lower kinase activity than ligand binding to the normal receptor. Finally and perhaps most likely, mutations in the C-terminal domain might also change the specificity of the receptor for sets of target proteins, thus rendering the mutated receptor capable of transforming cell types which do not express the EGFR and normally would be unresponsive to it.

Materials and methods

Construction of avian retroviruses expressing the human EGF receptor

The genome of AEV strain ES4 (clone AEV 11, Vennström *et al.*, 1980) was used for all constructions. Plasmid pCG-1, which harbors a permuted form of the AEV genome (Jansson *et al.*, 1987) in pSV2-*neo* (Southern and Berg, 1982) was first modified by removing the *Hin*dIII site between the SV40 promoter and the *neo* gene by cleavage and blunt-end ligation. The plasmid was then cleaved with *Apa*1, which cleaves at the splice acceptor site for v-*erb*B, blunted with T4 polymerase, and an 8-mer *Hin*dIII linker inserted. This plasmid was further modified by cleavage with *Eco*RI close to the 3′ end of the v-*erb*B, treated with *Bal*31 exonuclease to remove 113 nt from the downstream sequences (determined by sequence analysis), and an *Xba*I linker added. This basic vector was denoted pCR-X, encodes the complete v-*erb*A gene, contains a *Hin*dIII site at the splice acceptor site for v-*erb*B, and an *Xba* site after v-*erb*B. A variant of pCR-X was also made by replacing *wtv-erb*A with an inactive gene (v-*erb*A2$^-$, Frykberg *et al.*, 1983).

The EGF receptor cDNA (Ullrich *et al.*, 1984) was modified to give five different mutant cDNAs as described in a separate paper. The EGFR cDNAs, having *Hin*dIII sites in the 5′ and *Xba*I sites in the 3′ ends were then inserted into the pCR-X vector after excision of the v-*erb*B sequences with *Hin*dIII and *Xba*I. A corresponding set of vectors with v-*erb*A2$^-$ was also made. The AUG in the leader sequence of the subgenomic viral mRNAs encoding the EGFR genes is in another reading frame as compared to those of the cDNA inserts, thus allowing translation to occur from the authentic EGFR AUGs.

RAV-2 DNA was obtained from Dr G.Payne and Dr M.Bishop in a circularly permuted form. A proviral genome was reconstructed by cloning the circularized genome, recleaved with *Sac*I, into the *Sac*I site of an AEV LTR-containing fragment (Frykberg *et al.*, 1987). Secondary CEFs were transfected with 10 μg recombinant virus DNA together with 1–2 μg RAV-2 helper virus DNA to allow for virus spread. In some experiments the cells were selected for resistance to G418, as the plasmid vector encodes the *neo* gene transcribed from the SV40 promoter. Supernatants from the cells were used as source of virus for infection of fresh CEFs. Infected cells were placed in agar suspension and isolated clones were checked by dot blot analysis to identify high producer clones. These were expanded, mixed with fresh fibroblasts, and used as the source of infectious virus for further experiments (for further details, see below).

Immunoprecipitation analysis

7.5–15 × 10^6 transformed fibroblasts were labeled for 2 h with [^{35}S]methionine as described (Hayman *et al.*, 1979, 1983; Beug and Hayman, 1984). Cell lysates were immunoprecipitated with a rabbit antiserum to bacterially expressed v-*erb*B protein (Hayman *et al.*, 1986). A monoclonal antibody R1 obtained from Drs M.Waterfield and P.Goodfellow, was used to immunoprecipitate EGFR proteins with ligand-binding domain. Rabbit anti-p19 antibody (a kind gift from K.Moelling, Berlin, FRG) and rabbit anti-erbA antibodies (#21, a kind gift from J.Ghysdael, Lille, France) were used to immunoprecipitate virus structural proteins and p75$^{gag\text{-}erbA}$, respectively. For pulse–chase analysis, 15 × 10^6 cells were labelled for 30 min with 500 μCi [^{35}S]methionine and an aliquot chased for 4 h with CFU-E medium without isotope, essentially as described earlier (Hayman and Beug, 1984). Immunoprecipitated proteins were analyzed on 6–12% SDS polyacrylamide gradient gels and fluorographed as described (Beug and Hayman, 1984).

Virus dot blot

To analyze infected cells for virus production, 1 ml cell supernatant growth medium containing virus was filtered, and made up to 10 mM EDTA, 100 μg/ml proteinase K and 0.5% SDS. This was incubated at 37°C for 30 min. Five μg carrier tRNA was added, followed by phenol extraction and ethanol precipitation. The precipitate was resuspended in 50 μl Tris–HCl (10 mM, pH 7.5) and 1 mM EDTA, and mixed with 75 μl de-ionised formamide and 25 μl formaldehyde. After heating for 10 min at 60°C, 150 μl 20× SSC was added and the samples were subjected to serial 3× dilutions with 10× SSC. Samples were then loaded onto a dot blot apparatus. Transferred nucleic acids were fixed by baking the nitrocellulose sheet at 80°C for 1 or 2 h, and hybridized to an appropriate, labeled probe by standard techniques.

Cells and cell culture

Primary chick embryo fibroblasts were prepared from 11 day old embryos of the Spafas flock maintained in Heidelberg (Graf, 1973). They were grown

in standard growth medium, which consisted of modified Dulbecco's minimal essential medium (DMEM) plus 8% fetal calf serum, 2% chicken serum (both from GIBCO) and 10 mM HEPES (pH 7.3). Transformed erythroblasts were cultivated in CFU-E medium without anemic serum, unless stated otherwise (Radke *et al.*, 1982).

In vitro transformation of bone marrow cells

To infect erythroid cells in liquid culture, 20×10^6 bone marrow cells prepared from 2–8 day old Spafas chicks as described earlier (Graf *et al.*, 1973) were suspended in 5 ml of CFU-E medium plus 100 μl pretested anemic serum and plated onto 60 mm Falcon plates which had been seeded the day before with 7×10^5 virus producing CEFs. The following day, non-adherent cells were flushed from the adherent cell population and transferred to a new plate of virus producing fibroblasts as before. On the third day, non-adherent cells were transferred to a new plate without fibroblasts and grown in CFU-E medium lacking anemic serum. Cultured cells were concentrated and/or depleted of differentiated erythrocytes when necessary by centrifugation and purification on Ficoll (Beug *et al.*, 1981). In some experiments the entire co-cultivation was also performed in the presence of 20 μg/ml EGF (Collaborative Research) which was added daily.

Scatchard analysis for EGF binding

Chicken embryo fibroblasts were plated (2×10^5 cells per well) into 12-well Nunc dishes, the day before analysis. The next day, the confluent cultures were placed on ice and washed once with wash-buffer (PBS containing Mg^{2+}, Ca^{2+} and 1 mg/ml BSA), precooled to 4°C. To triplicate dishes were added 0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0, 80.0, 200 and 500 ng/ml cold EGF (in 1% PBS), in 0.5 ml of the above wash-buffer. To each well was added 0.5 ng/ml [^{125}I]EGF (8.0×10^4 c.p.m./ng), diluted in 10 μl wash buffer.

Culture dishes were rocked gently in cold room on ice for 2 h, then washed five times with wash buffer, lysed with RRA lysis buffer (HEPES pH 7.3, 20 mM + glycerol 10% + Triton X-100 1%), and counted in gamma counter. Maximum bound radioactivity was in the range of 20 000 c.p.m.

Scatchard analysis of HER-C-expressing erythroblasts was done similarly, except that 2×10^6 cells were used per well and that the cells were washed in ice-cold wash-buffer (supplemented with 1% detoxified BSA) (Radke *et al.*, 1982) by centrifugation.

Focus and agar colony assays

Focus assay with chicken embryo fibroblasts were performed as described by Frykberg *et al.* (1983). Colony assays were performed in 35 mm Nunc dishes, pre-layered with 1 ml bottom agar (in Dulbecco's medium containing 8% FCS, 2% heat inactivated chick serum, antibiotics, 10 mM HEPES pH 7.2, 0.6% Noble agar, Difco). CEFs to be assayed were trypsinised, mixed at 3×10^5 cells per plate with 2 ml top agar (0.25% agar containing the same ingredients as bottom agar) and poured onto the pre-layered plates. Plates were layered with 1 ml top agar and checked for growth of colonies at 4 day intervals.

Growth in serum-free medium

Virus infected fibroblasts, pretested for completeness of infection by immunofluorescence (see below, Hayman and Beug, 1984), were seeded (2×10^5 cells) into 35 mm dishes with 2 ml standard growth medium and incubated overnight at 37°C. The plates were then washed three times in serum-free DMEM and incubated further in serum-free DMEM containing transferrin (50 ng/ml) and insulin (1 μg/ml). EGF (10 ng/ml) was added to the dishes and the cells were further incubated for 4–24 h at 37°C. They were then labeled for 2 h with [^{3}H]thymidine (Amersham-Buckler 15 Ci/mmol, 4 μCi/ml), and radioactivity in DNA determined as described by Betsholz and Westermark, 1984.

Morphology, actin cables, fibronectin, hexose transport

Cells were seeded subconfluently in standard growth medium, incubated overnight in the presence or absence of 20 μg/ml EGF and photographed using phase optics. Assays for actin cables, fibronectin network expression and uptake of [^{3}H]deoxyglucose were performed as described earlier (Royer-Pokora *et al.*, 1978; Palmieri *et al.*, 1983) except that the appropriately seeded cells were incubated overnight in the presence or absence of 20 ng/ml EGF.

Immunofluorescence

The virus-transformed fibroblasts were analyzed for their expression of normal and mutant EGFR proteins at the cell surface by live cell immunofluorescence as described earlier (Hayman *et al.*, 1983; Beug *et al.*, 1986) using adsorbed rat anti-erbB antiserum for EGFR proteins without ligand-binding domain (Hayman *et al.*, 1983), and the R1 anti-EGFR monoclonal antibody for those with a ligand-binding domain. To determine the differentiation phenotype of transformed erythroblasts, they were stained with rabbit anti-erythroblast antibody (Beug *et al.*,1979) anti-erythrocyte monoclonal antibody (5J2A3, Schmidt *et al.*, 1986) and rabbit anti-erythrocyte antibody (Beug *et al.*, 1979) in double indirect immunofluorescence, using FITC-labeled goat anti-mouse IgG and TRITC-labeled goat anti-rabbit IgG as second antibodies (Beug *et al.*, 1982; Beug and Hayman, 1984).

Analysis of transformed erythroblasts for morphology, hemoglobin content and size

Cytospin preparations of the various transformed erythroblasts were stained with neutral benzidine plus histological dyes as described earlier (Beug *et al.*, 1982). Size distributions were determined as described in Knight *et al.*, 1988.

Assays for proliferation of transformed erythroblasts

Erythroblasts transformed by the various HER constructs and pregrown in CFU-E medium (containing 20 ng/ml EGF in case of HER-C and HER-6 erythroblasts) were seeded in differentiation medium (Zenke *et al.*, 1988) supplemented with 2% anemic chicken serum and 1 μg/ml insulin at 1×10^6 cells/ml and cultivated at 41°C and 5% CO_2 in presence or absence of EGF (20 ng/ml). In some experiments an erythroid growth factor from reticuloendotheliosis virus (REV) transformed lymphoid cells was added as well. This however did not change the results. At daily intervals, cells were counted using a Coulter counter, aliquots were removed for cytospin preparations and measurement of [^{3}H]TdR incorporation, and the cells were resuspended in a mixture of old (1/3) and fresh medium (2/3) at $\sim 1-1.5 \times 10^6$ cells/ml. [^{3}H]TdR incorporation was determined by incubating 100 000 cells suspended in 100 μl of their own supernatant plus 8 μCi of [^{3}H]TdR for 2 h and harvesting in a cell harvester (Skatron) as described earlier (Leutz *et al.*, 1984).

Purification of erythropoietin and avian erythroid growth factor

Avian EPO was purified ~2000-fold from anemic serum as described in Kowenz *et al.*, 1987. Avian erythroid growth factor produced by REV_T-transformed lymphoblasts was purified by hydroxylapatite and reverse phase HPLC (A.Leutz and H.Beug, unpublished) yielding a highly purified (>10 000-fold) preparation (Zenke *et al.*, 1988).

Proliferation and differentiation assay of HER-C and HER-6 erythroblasts

Differentiation medium (Zenke *et al.*, 1988) was supplemented with 1 μg/ml insulin, various concentrations of EGF (0.1–20 ng/ml) and either 5% of high-titer anemic serum or ~10 μg/ml of reverse-phase HPLC purified chick EPO or 1 μg/ml of the same EPO plus avian erythroid growth factor at optimal concentration (determined as in Zenke *et al.*, 1988). Cells (HER-C v-*erb*A2$^-$ and HER-6 v-*erb*A2$^-$ erythroblasts) were seeded at 1×10^6/ml in 5 ml of the different media and incubated at 41°C and 5% CO_2. Twenty-four and 48 h later, the cells were counted in the Coulter counter (which did not count disintegrated cells at the setting used), dead cells were removed by centrifugation through Percoll, the cells re-counted (to account for cell losses during Ficoll purification), aliquots removed for [^{3}H]TdR incorporation and cytospin preparation, and the cells reseeded in fresh aliquots (kept frozen until used) of the same media. Cumulative cell numbers were corrected for losses during Ficoll purification and removal of cells for the different assays. [^{3}H]TdR incorporation was measured as described above, using again 10^5 viable cells resuspended in 100 μl of their own supernatant.

Animal experiments

White Leghorn chickens of the Heidelberg flock (originally derived from the Spafas flock) or K28 × $L15_1$ chicks (hatched in Heidelberg from fertilized eggs kindly provided by L.Crittenden) were used in the experiments. Virus harvested from pretested, transformed fibroblasts were injected i.v. into 7 day old chicks of both strains. Beginning 1 week after infection, the animals were monitored twice weekly for the onset of leukemia by examining blood smears stained with Diff Quick (Harleco) plus neutral benzidine.

Peripheral blood was obtained by heart puncture from moribund animals in 0.37% sodium citrate to prevent clotting. Buffy coat cells were prepared as described earlier (Radke *et al.*, 1982; Beug *et al.*, 1981). Single cell suspensions from bone marrow and spleen from diseased animals were prepared as described earlier (Radke *et al.*, 1982).

Buffy coat cells from the peripheral blood of leukemia chicks as well as bone marrow and spleen cells were seeded at a density of 2.5×10^6 cells/ml CFU-E medium. Cells were flushed from the adherent cell population at 2–3 day intervals and concentrated by centrifugation whenever necessary to maintain a density $>10^6$ cells. Fully differentiated erythrocytes

and dead cells were removed when required by Ficoll centrifugation as described above.

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References

Bassiri,M. and Privalsky,M.L. (1986) *J. Virol.*, **59**, 525–530.
Betsholz,C. and Westermark,B. (1984) *J. Cell. Physiol.*, **118**, 203–210.
Beug,H. and Hayman,M.J. (1984) *Cell*, **36**, 963–972.
Beug,H., v.Kirchbach,A., Döderlein,G., Conscience,J.F. and Graf,T. (1979) *Cell*, **18**, 375–390.
Beug,H., Müller,H., Grieser,S., Döderlein,G. and Graf,T. (1981) *Virology*, **115**, 295–309.
Beug,H., Palmieri,S., Freudenstein,C., Zentgraf,H. and Graf,T. (1982) *Cell*, **28**, 907–919.
Beug,H., Kahn,P., Vennström,B., Hayman,M.J. and Graf,T. (1985a) *Proc. R. Soc. Lond.*, **B226**, 121–126.
Beug,H., Kahn,P., Döderlein,G., Hayman,M.J. and Graf,T. (1985b) In Neth,R., Gallo,R., Greaves,M. and Janka,K. (eds), *Modern Trends in Human Leukemia VI.* Springer Verlag, Berlin, Vol. 29, pp. 290–297.
Beug,H., Hayman,M.J., Raines,M.B., Kung,H.J. and Vennström,B. (1986) *J. Virol.*, **57**, 1127–1138.
Beug,H., Hayman,M.J. and Vennström,B. (1987) In Kahn,P. and Graf,T. (eds), *Oncogenes and Growth Control.* Springer Verlag, Berlin Heidelberg, pp. 85–89.
Choi,H.R., Trainor,C., Graf,T., Beug,H. and Engel,D. (1986) *Mol. Cell. Biol.*, **6**, 1751–1759.
Damm,K., Beug,H., Graf,T. and Vennström,B. (1987) *EMBO J.*, **6**, 375–382.
DiFiore,P.P., Pierce,J.H., Fleming,T.P., Hazan,R., Ullrich,A., Richter-King,C., Schlessinger,J. and Aaronson,S.A. (1987) *Cell*, **51**, 1063–1070.
Downward,J., Parker,P. and Waterfield,M.D. (1984a) *Nature*, **311**, 483–485.
Downward,J., Yarden,Y., Mayes,E., Scrace,G., Totty,N., Stockwell,P., Ullrich,A., Schlessinger,J. and Waterfield,M.D. (1984b) *Nature*, **307**, 521–527.
Frykberg,L., Palmieri,S., Beug,H., Graf,T., Hayman,M.J. and Vennström,B. (1983) *Cell*, **32**, 227–238.
Frykberg,L., Graf,T. and Vennström,B. (1987) *Oncogene*, **1**, 415–421.
Fung,T., Lewis,W.G., Kung,H.-J. and Crittenden,L.B. (1983) *Cell*, **33**, 357–368.
Gamett,D., Tracy,S. and Robinson,H. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 6053–6057.
Gandrillon,O., Jurdic,P., Benchaibi,M., Xiao,J.H. Ghysdael,J. and Samarut,S. (1987) *Cell*, **49**, 687–697.
Gilmore,T., DeClue,J.E. and Martin,G.S. (1985) *Cell*, **40**, 609–618.
Graf,T. (1973) *Virology*, **54**, 398–413.
Graf,T., Royer-Pokora,B., Schubert,G.E. and Beug,H. (1976) *Virology*, **71**, 423–433.
Hayman,M.J. and Beug,H. (1984) *Nature*, **309**, 460–462.
Hayman,M., Royer-Pokora,B. and Graf,T. (1979) *Virology*, **92**, 31–45.
Hayman,M.J., Ramsay,G.M. Savin,K., Kitchener,G., Graf,T. and Beug,H. (1983) *Cell*, **32**, 579–588.
Hayman,M.J., Kitchener,G., Knight,J., Mc Mahon,J., Watson,R. and Beug,H. (1986) *Virology*, **150**, 270–275.
Jansson,M., Beug,H., Gray,C., Graf,T. and Vennström,B. (1987) *Oncogene*, **1**, 167–173.
Johnsson,A., Bestholz,C., Heldin,C.H. and Westermark,B. (1985) *Nature*, **317**, 438–440.
Johnsson,A., Bestholz,C., Heldin,C.H. and Westermark,B. (1986) *EMBO J.*, **5**, 1535–1541.
Kahn,P., Adkins,B., Beug,H. and Graf,T. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 7122–7126.
Kahn,P., Frykberg,L., Brady,C., Stanley,I.J., Beug,H., Vennström,B. and Graf,T. (1986) *Cell*, **100**, 349–356.
Kawai,S., Nishizino,M., Yamamoto,T. and Toyoshima,K. (1987) *J. Virol.*, **61**, 1665–1669.
Knight,J., Zenke,M., Diseal,Ch., Kowenz,E., Vogt,P., Engel,D., Hayman,M.J. and Beug,H. (1988) *Genes Dev.*, in press.
Kowenz,E., Leutz,A., Döderlein,G., Graf,T. and Beug,H. (1987) In Neth,R. Gallo,R., Greaves,M. and Kabish,H. (eds), *Modern Trends in Human Leukemia VII.* Springer Verlag, Berlin, vol. 31, pp. 199–209.
Land,H., Parada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.
Lax,I., Kris,R., Sasson,I., Ullrich,A., Hayman,M.J., Beug,H. and Schlessinger,J. (1985) *EMBO J.*, **4**, 3179–3182.
Lax,I., Johnsson,A., Sap,J., Vennström,B., Howk,R., Bellot,F., Ullrich,A., Schlessinger,J. and Givol,D. (1988) *Mol. Cell. Biol.*, in press.
Leutz,A., Beug,H. and Graf,T. (1984) *EMBO J.*, **3**, 3191–3197.
Miles,B.D. and Robinson,H.L. (1985) *J. Virol.*, **54**, 295–303.
Nilsen,T.W., Maroney,P.A., Goodwin,R.E., Rottman,F.M. Crittenden,L.B., Raines,M.A. and Kung,H.-J. (1985) *Cell*, **41**, 719–726.
Palmieri,S., Kahn,P. and Graf,T. (1983) *EMBO J.*, **2**, 2385–2389.
Pierce,J.H., Ruggiero,M., Fleming,T.P., Di Fiore,P.P., Greenberger,J., Varticovski,L., Schlessinger,J., Rovera,G. and Aaronson,S.A. (1988) *Science*, **239**, 628–631.
Privalski,M.L. and Bishop,J.M. (1984) *Virology*, **135**, 356–368.
Radke,K., Beug,H., Kornfeld,S. and Graf,T. (1982) *Cell*, **31**, 643–653.
Robinson,H.L., Miles,B.D., Catalano,D.E., Briles,W.E. and Crittenden,L.B. (1985) *J. Virol.*, **55**, 617–622.
Royer-Pokora,B., Beug,H., Claviez,M., Winkhardt,H.-J., Friis,R.R. and Graf,T. (1978) *Cell*, **13**, 751–750.
Samarut,J. and Bouabdelli,M. (1980) *J. Cell Physiol.*, **105**, 269–280.
Samarut,J. and Gazzolo,L. (1982) *Cell*, **28**, 921–929.
Sap,J., Muñoz,A., Damm,K., Goldberg,Y., Ghysdael,J., Leutz,A., Beug,H. and Vennström,B. (1986) *Nature*, **324**, 635–640.
Schmidt,J.A., Marshall,J., Hayman,M.J., Döderlein,G. and Beug,H. (1986) *Leukemia Res.*, **10**, 257.
Scotting,P., Vennström,B., Jansson,M., Graf,T., Beug,H. and Hayman,M.J. (1987) *Oncogene Res.*, **1**, 265–278.
Sealy,L., Privalsky,M.L., Moscovici,G., Moscovici,C. and Bishop,J.M. (1983) *Virology*, **130**, 155–178.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Gen.*, **1**, 327–341.
Ullrich,A., Coussens,L., Hayflick,J.S., Dull,T.J., Gray,A., Tam,A.W., Lee,J., Yarden,Y., Libermann,T.A., Schlessinger,J., Downward,J., Mayes,E.L.V., Whittle,N., Waterfield,M.D. and Seeburg,P.H. (1984) *Nature*, **309**, 418–425.
Velu,T.J., Beguinot,L., Vass,W.C., Willingham,M.C., Merlino,G.T. Pastan,I. and Lowy,D.R. (1987) *Science*, **238**, 1408–1410.
Vennström,B., Fanshier,L., Moscovici,C. and Bishop,J.M. (1980) *J. Virol.*, **36**, 575–585.
Weinberger,C., Thompson,C., Ong,E., Lebo,R., Gruol,D. and Evans,R. (1986) *Nature*, **324**, 641–646.
Yamamoto,T., Hihara,T., Nishida,T., Kawai,S. and Toyoshima,K. (1983a) *Cell*, **34**, 225–232.
Yamamoto,T., Nishida,T., Mitajimi, N., Kawai,S., Ooi,T. and Toyoshima,K. (1983b) *Cell*, **35**, 71–78.
Zenke,M., Kahn,P., Disela,Ch., Vennström,B. Leutz,A., Keegan,K., Hayman,M., Choi,H.R., Yew,N., Engel,J.D., Beug,H. (1988) *Cell*, **52**, 107–119.

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