Phosphorylation of the *v-erbA* protein is required for its function as an oncogene

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The *v-erbA* oncogene of avian erythroblastosis virus (AEV) encodes a ligand-independent mutated version of the chicken *c-erbA α* -encoded thyroid hormone receptor. The *v-erbA* gene product, a 75-kD *gag/v-erbA* fusion protein, is phosphorylated on Ser-16/17 of its *v-erbA*-encoded domain, and phosphorylation at this site is increased in vivo after activation of either the PKA or PKC signal transduction pathways. To test the hypothesis that phosphorylation of Ser-16/17 regulates *gag/v-erbA* protein function, mutant proteins in which Ser-16/17 had been changed to alanine or threonine residues were analyzed for their ability to inhibit erythroid differentiation of *ts v-erbB* or *ts v-sea*-transformed erythroblasts at nonpermissive temperature. Conversion of Ser-16/17 into alanine, although not affecting nuclear localization or DNA binding of the *gag/erbA* protein, prevented phosphorylation of the *v-erbA*-encoded domain of the protein both in unstimulated cells or after stimulation by PKA and PKC activators. The nonphosphorylatable AA-*gag/v-erbA* protein proved unable to inhibit temperature-induced differentiation of *ts v-erbB* and *ts v-sea*-transformed erythroblasts and to block expression of the erythrocyte-specific genes band 3 and carbonic anhydrase II. Back mutation of these alanine residues to serine resulted in the recovery of both normal phosphorylation levels and wild-type biological activity. In contrast, substitution of Ser-16/17 for threonine, which preserved phosphorylation in unstimulated cells but not PKA- and PKC-enhanced phosphorylation, resulted in a partially active *gag/v-erbA* protein. These results, together with the fact that the protein kinase inhibitor H7 resulted in both a dose-dependent inhibition of *gag/v-erbA* protein phosphorylation and the induction of terminal differentiation of AEV-transformed erythroblasts show that phosphorylation of *gag/v-erbA* protein is required for full biological activity. These results support the hypothesis that phosphorylation of the *gag/v-erbA* protein is important for transcriptional repression of at least some of its target genes in erythroid cells.

[Key Words: *v-erbA*; oncogene; phosphorylation; erythroid differentiation]

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The mechanisms by which environmental factors essential to development and differentiation mediate transcriptional changes in the nucleus are still largely unknown. Binding of polypeptide hormones and growth factors to their plasma membrane receptor generates specific intracellular signaling events which, in turn, control the expression and/or activity of specific transcription factors (Greenberg and Ziff 1984; Sen and Baltimore 1986; Chiu et al. 1987; Montminy and Bilezikjian 1987; Lamph et al. 1988). On the other hand, a variety of hormones that exert profound effects on metabolic homeostasis, development, and differentiation (i.e., steroids, thyroid hormones, and retinoids) act through direct binding to specific nuclear receptors that are ligand-dependent transcription factors (for review, see Evans 1988).

The cellular progenitor (*c-erbA α*) of *v-erbA*, one of the oncogenes of avian erythroblastosis virus (AEV), encodes a nuclear receptor for the thyroid hormone 3,5,3'-triiodothyronine (T_3) (Sap et al. 1986). Upon hormone

binding, this receptor enhances transcription of specific genes by associating selectively with DNA sequences termed thyroid hormone-responsive elements (T_3RE ; Damm et al. 1989; Sap et al. 1989). The *c-erbA α* -encoded T_3 receptor shares a similar modular structure with receptors for steroid hormones and retinoids (Green and Chambon 1986; Weinberger et al. 1985, 1986; Sap et al. 1986; de The et al. 1987; Giguere et al. 1987; Petkovich et al. 1987). All members of this family contain a highly conserved central domain containing two zinc finger motifs responsible for specific binding to hormone-responsive elements. The DNA-binding domain is separated by a hinge region from a carboxy-terminal domain that is responsible for hormone binding. The function of the amino-terminal domain of the *c-erbA α* -encoded T_3 receptor has yet to be established.

The *v-erbA* gene product, a 75-kD *gag/v-erbA* fusion protein, binds to T_3RE s (Damm et al. 1989; Sap et al. 1989) but fails to bind hormone as the result of multiple mutations in its carboxy-terminal domain (Sap et al. 1986; Munoz et al. 1988). *v-erbA* has been shown to cooperate with *v-erbB*, the second oncogene of AEV, to in-

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duce transformation of chick erythroblasts in tissue culture and to cause acute erythroleukemia in chicks (Beug et al. 1982b, 1985a; Samarut and Gazzolo 1982; Frykberg et al. 1983; Sealy et al. 1983; Fung et al. 1983). More specifically, *v-erbB*, as well as other oncogenes encoding protein tyrosine kinases, are sufficient to induce extensive proliferation of erythroid progenitor cells but are unable to completely block the ability of these cells to differentiate terminally into erythrocytes, thus yielding a significant amount of mature cells in the transformed cultures (Beug et al. 1985a,b).

Expression of *gag/v-erbA* in these cells results in complete inhibition of their differentiation at an immature stage similar to that of CFU-E progenitor cells (Beug et al. 1979, 1985a; Samarut and Gazzolo 1982; Kahn et al. 1986b). In addition, expression of the *gag/v-erbA* protein results in the suppression of transcription of certain erythrocyte-specific genes such as carbonic anhydrase II (CA II) and erythrocyte anion transporter (band 3) (Zenke et al. 1988). Two sets of experimental evidence suggest that *gag/v-erbA* inhibits erythroid differentiation by acting as a constitutive dominant repressor of erythrocyte-specific gene transcription (or of regulators of these genes) that is normally regulated by T_3 or by retinoic acid (RA), because RA receptors have recently been shown to bind to and *trans*-activate at least some model T_3 REs (Graupner et al. 1989; Umesono et al. 1988). First, a *gag/c-erbA α* protein arrests erythroid differentiation and inhibits erythrocyte gene transcription when overexpressed in erythroblasts in the absence of hormone but promotes maturation and permits erythrocyte gene transcription in the presence of T_3 (Zenke et al. 1990). Second, the *gag/v-erbA* protein can function as a constitutive dominant repressor of model T_3 -responsive reporter plasmids in transient transfection assays (Damm et al. 1989; Sap et al. 1989).

We reported previously that the *c-erbA α* -encoded T_3 receptor is phosphorylated in its amino-terminal domain at two distinct sites (Goldberg et al. 1988). One of these sites (Ser-12) is phosphorylated in cells by casein kinase II or a kinase with similar specificity (Glineur et al. 1989), whereas phosphorylation of the second site (Ser-28/29) is mediated by an as yet uncharacterized protein kinase. Only this second phosphorylation site has been retained in the oncogenic *gag/v-erbA* protein (Ser-16/17 in *v-erbA* coordinates; Goldberg et al. 1988). Phosphorylation of both Ser-28/29 in the *c-erbA α* -encoded T_3 receptor and Ser-16/17 in the *gag/v-erbA* protein was found to be enhanced following treatment of cells with activators of either protein kinase C (PKC) or cAMP-dependent protein kinase (PKA; Goldberg et al. 1988), suggesting a role for these phosphorylation events in *c-erbA α* and *v-erbA* protein function.

To study the functional significance of *gag/v-erbA* protein phosphorylation in oncogenesis, we analyzed the biochemical and biological properties of *gag/v-erbA* proteins in which Ser-16 and Ser-17 have been altered by site-directed mutagenesis into either unphosphorylatable alanine residues or into threonine. These studies, together with the effects of a protein kinase inhibitor on

both *gag/v-erbA* protein phosphorylation and differentiation of AEV-transformed erythroblasts show that phosphorylation of the *gag/v-erbA* protein on Ser-16/17 is required for the protein to exert its full biological activity. Therefore, our results suggest that the *gag/v-erbA* protein has to be phosphorylated to repress at least a subset of the genes normally regulated by T_3 and/or other hormones (e.g., RA) during erythroid differentiation.

Results

Construction of *v-erbA* phosphorylation mutants by site-directed mutagenesis

To analyze the importance of phosphorylation of Ser-16/17 for the biological activity of the *gag/v-erbA* protein, the codons for both Ser-16 and Ser-17 (Fig. 1A) were replaced with codons for either alanine (AA) or threonine (TT) by oligonucleotide site-directed mutagenesis. We also prepared a revertant form of the *gag/v-erbA* protein in which the codons for Ala-16/17 in the AAgag/*v-erbA* protein were converted back to serine codons (SS*; Fig. 1B).

The altered *gag/v-erbA* proteins were introduced into retroviral vectors containing temperature-sensitive versions of either *v-erbB* or *v-sea* (Fig. 1C; Scotting et al. 1987; Knight et al. 1987; Zenke et al. 1988). Virus stocks were prepared by cotransfection of these constructs together with RAV-1 DNA in chicken embryo fibroblasts.

Figure 1. Structure of wild-type and mutant *v-erbA* proteins and recombinant retroviruses. (A) Structure of the *v-erbA* oncogene, indicating the positions of the phosphorylated serine residues at positions 16 and 17 (○). The amino-terminal, DNA-, and hormone-binding regions are indicated. (●) Point mutations. (B) Nucleotide and amino acid sequences of the amino-terminal regions (nucleotides 28–69, corresponding to amino acids 10–23) of the wild-type *gag/v-erbA* protein (SS), the mutant proteins alanine (AA) and threonine (TT) *gag/v-erbA*, and the serine (SS*) *gag/v-erbA* back mutant, as constructed by site-directed mutagenesis (see Materials and methods). (C) Structure of the *ts v-erbB*- or *ts v-sea*-containing retroviruses used to express wild-type and mutant *v-erbA* proteins in cells (Zenke et al. 1988).

These viruses, as well as viruses containing a wild-type *v-erbA* oncogene (*ts* 167 AEV and *ts v-sea/v-erbA*; Zenke et al. 1988) were used to generate transformed erythroblast clones from infected chicken bone marrow cells (see Materials and methods).

Both fibroblasts and erythroblasts transformed by viruses containing either wild-type or the various mutant *gag/v-erbA* oncogenes expressed comparable levels of the respective *gag/v-erbA* proteins, as evidenced by both immune precipitation of L-[³⁵S]methionine pulse-labeled cells and by Western blotting analyses, using an *erbA*-specific antiserum (data not shown). In addition, both wild-type and AAgag/*v-erbA* proteins were found to be localized in the nucleus of transformed cells by immunofluorescence analysis (Fig. 2A) and were found to bind with similar efficiency to a synthetic palindromic T₃RE oligonucleotide (Fig. 2B). Similar results were obtained for the TTgag/*v-erbA* protein (not shown). This indicates that substitution of Ser-16/17 for either alanine or threonine residues does not detectably alter the stability, subcellular localization, or DNA-binding ability of *gag/v-erbA* protein in transformed cells.

In vivo phosphorylation of mutant *gag/v-erbA* proteins

We then analyzed the effect of the serine substitutions both on basal *gag/v-erbA* protein phosphorylation and on the enhanced phosphorylation observed following treatment of cells with either PKC activators or pharmacological agents that increase the intracellular concentration of cAMP. Transformed erythroblasts were labeled with [³²P]orthophosphate for 4 hr and either left untreated or further incubated for 20 min with either phorbol myristate acetate (PMA) or forskolin. The *gag/v-erbA* proteins were immunoprecipitated with an *erbA* antiserum and analyzed by SDS-PAGE before or after proteolytic digestion with the p15^{gag} protease of avian retroviruses. This protease cleaves the *gag/v-erbA* protein into a *gag*-encoded 30-kD fragment (F30^{gag}) and a *v-erbA*-encoded 45-kD fragment (F45^{v-erbA}). Because Ser-16 and Ser-17 are the only residues phosphorylated in the *v-erbA*-encoded domain of the *gag/v-erbA* protein, this procedure allows a simple visualization of the phosphorylation status of these residues (Goldberg et al. 1988). As shown in Figure 2C, digestion of wild-type *gag/v-erbA* protein with p15^{gag} generated both phosphorylated F30^{gag} and F45^{v-erbA} fragments. The level of phosphate incorporation into F45 was enhanced ~10-fold upon pulse treatment of labeled cells for 20 min with either forskolin or PMA (Fig. 2C). As demonstrated previously, the enhanced phosphorylation of F45^{v-erbA} occurred exclusively at Ser-16/17 and therefore represents an increase in the population of *gag/v-erbA* proteins phosphorylated at these residues (Goldberg et al. 1988). Similar results were obtained for erythroblasts expressing the revertant SS* *gag/v-erbA* protein (data not shown). In contrast, conversion of Ser-16/17 into alanine residues completely abolished both basal and forskolin- or PMA-enhanced phosphorylation of the *gag/v-erbA* protein in its F45^{v-erbA} fragment while leaving that of the

gag-encoded domain unaffected (Fig. 2C, AA). These results confirm our prior biochemical identification of the

Figure 2. Properties of wild-type and mutant *v-erbA* proteins. (A) Immunofluorescence analyses. Fibroblasts transformed by either wild-type AEV (wt-*erbA*) or a mutant AEV encoding the AA-*gag/v-erbA* (AA-*erbA*) were grown on glass coverslips and fixed in paraformaldehyde, permeabilized, and stained with *erbA*-specific antiserum (anti-*erbA*) or nonimmune serum (N.I.) plus FITC swine anti-rabbit IgG antibodies, as described in Materials and methods. Note prominent nuclear fluorescence in both wt-*erbA* and AA-*erbA* cells. (B) Electrophoretic mobility shift assays. Mobility shift assays were performed, using a palindromic T₃RE oligonucleotide (Glass et al. 1988) as probe, and either whole-cell extracts of COS cells transfected with the pKCR3 expression plasmid (control) or pKCR3-based plasmids expressing the wild-type *gag/v-erbA* protein (*v-erbA*-SS) or the mutant AA *gag/v-erbA* protein (*v-erbA*-AA). Free probe was electrophoresed in the first lane (free). The positions of free probe (free) and DNA-protein complex (complex) are indicated. (C) Phosphorylation of wild-type and mutant *v-erbA* proteins. Erythroblast cells containing either the wild-type *gag/v-erbA* protein (SS) or the mutant AA- and TT-*gag/v-erbA* proteins (AA) and (TT) were labeled for 4 hr with 1 mCi/ml [³²P]orthophosphate. Cells were incubated further for 20 min with no agent (UNT.), 10 μ M forskolin (FOR), or 40 nM PMA. Immunoprecipitation was carried out with an *erbA*-specific antiserum on equal aliquots of each lysate (10⁷ acid-precipitable radioactive counts per aliquot). For each lysate, one of the two immunoprecipitates was digested with p15^{gag}. Immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide slab gels.

phosphorylation site of the wild-type *gag/v-erbA* protein of AEV as Ser-16/17 [Goldberg et al. 1988].

Conversion of Ser-16/17 into threonine residues resulted in phosphorylation of the *gag/v-erbA* protein in both the F30^{gag} fragment on serine and the F45^{v-erbA} fragment on threonine residues (Fig. 2C, TT, and data not shown). We reproducibly observed that the ratio of phosphate incorporation in F45^{v-erbA} versus that of F30^{gag} was lower in the TT *gag/v-erbA* protein than in the wild-type *gag/v-erbA* protein. This suggests that this variant *erbA* protein is a less efficient substrate for the protein kinase(s) involved in the phosphorylation of the wild-type protein in erythroblasts. In striking contrast to wild-type *gag/v-erbA* protein, however, treatment of erythroblasts and fibroblasts expressing TT *gag/v-erbA* with either forskolin or PMA failed to result in enhanced phosphorylation of the F45^{v-erbA} fragment (Fig. 2C, TT, and data not shown).

Biological activity of mutant *gag/v-erbA* proteins

The rationale for testing the effects of the serine substitutions on the biological activity of *gag/v-erbA* proteins in erythroblasts was to combine them with a conditional transforming oncogene [e.g., temperature-sensitive mutants of *v-erbB* and *v-sea*; Fig. 1C]. Previous analyses have shown that *ts v-erbB*- or *ts v-sea*-transformed erythroblast clones grown at the permissive temperature (37°C) differentiated terminally into erythrocyte-like cells after "switching off" the temperature-sensitive kinase oncogene at the nonpermissive temperature (42°C). In contrast, expression of the wild-type *gag/v-erbA* protein in these cells resulted in a dominant inhibition of differentiation, as evidenced by cell morphology, cell-surface antigen expression, and transcription of erythrocyte-specific genes [Kahn et al. 1986a,b; Zenke et al. 1988].

Differentiation phenotype As an initial screen for possible alterations in biological activity, a series of erythroblast clones expressing the SS-, AA-, and TT-*gag/v-erbA* proteins were tested for hemoglobin expression by acid benzidine staining after cultivation at 37 and 42°C for two days [Graf et al. 1978; Damm et al. 1987]. As a negative control, erythroblast clones transformed by *ts v-sea* were used in this assay (no *erbA* in Fig. 3). Whereas the SS *gag/v-erbA*-expressing clones and, to a lesser extent, the TT-*gag/v-erbA* clones, exhibited the expected low hemoglobinization at 37°C and often contained <95% hemoglobin-positive cells even at 42°C (Fig. 3B,D), the AA-*gag/v-erbA*-expressing erythroblast clones closely resembled erythroblasts without any active *erbA* protein by this assay (Fig. 3A,C). These results suggest that the AA-*gag/v-erbA* protein might be devoid of biological activity, whereas the TT-*gag/v-erbA* protein displays a (perhaps weaker) activity of a *v-erbA* oncoprotein.

To determine in more detail the phenotypes induced by the mutant *gag/v-erbA* proteins in differentiating erythroblasts, representative erythroblast clones (se-

Figure 3. Hemoglobin expression in erythroblast clones expressing wild-type or mutant *v-erbA* proteins. Individual erythroblast clones transformed by *ts v-sea* (A; no *erbA*), SS *gag/v-erbA*/*ts erbB* (B, SS-*erbA*), AA *gag/v-erbA*/*ts erbB* (C, AA *erbA*), and TT *gag/v-erbA*/*ts sea* (D, TT *erbA*) were grown at 37 or 42°C for 2 days and analyzed for hemoglobin expression by acid benzidine staining (see Materials and methods). The histograms shown depict the clonal variation of acid benzidine staining within the various sets of clones. The data in B are pooled from several experiments involving both *ts erbB/v-erbA* and *ts sea/v-erbA* retroviruses.

lected for high in vitro lifespan together with typical differentiation behavior; see Materials and methods) were cultivated at 42°C for 3 days and analyzed for other parameters of erythroid differentiation. The results indicate that the AA-*gag/v-erbA* was severely affected in its ability to arrest erythroid differentiation (Fig. 4; Table 1). Erythroblasts expressing this mutant protein formed CFU-E-like red colonies in semisolid medium (Fig. 4A). In mass culture, they differentiated into erythrocyte-like oval cells that were strongly hemoglobin-positive as evidenced by neutral benzidine staining (Fig. 4B) and expressed erythrocyte antigens at their cell surface (Table 1). Thus, these cells exhibited a phenotype very similar to that of *ts v-sea*-transformed erythroblasts devoid of exogenous *gag/v-erbA* protein (Fig. 4A,B; Table 1).

The inability of the AA-*gag/v-erbA* protein to inhibit

Figure 4. The nonphosphorylated AA-*v-erbA* mutant protein fails to arrest erythroid differentiation. Erythroblast clones transformed by retroviruses expressing wild-type or mutant *erbA* proteins (see legend to Table 1) were seeded in pH 7.2 semisolid methocel medium (Zenke et al. 1988; see Materials and methods). Photographs of typical colonies obtained after 3–4 days at 42°C are shown in A. (Insets) Some colonies at higher magnification reveal morphological details. [B] Mass cultures of erythroblasts from the same clones as in A were incubated in pH 7.2 medium at 42°C and 5% CO₂ for 3 days. Cytospin preparations of these cells were stained with neutral benzidine plus histological dyes (see Materials and methods) and were photographed under blue light to reveal histochemical staining for hemoglobin (Beug et al. 1982).

erythroid differentiation is directly attributable to the substitution of Ser-16/17 for alanine because a revertant SS*-*gag/v-erbA* protein was found to recover a biological activity indistinguishable from that of the wild-type SS-*gag/v-erbA* protein (Table 1; data not shown).

In contrast, erythroblasts containing the TT-*gag/v-erbA* protein exhibited a clearly differentiation-arrested phenotype which, however, appeared to be less penetrant than the phenotype caused by either SS- or SS*-*gag/v-erbA*. The TT-*gag/v-erbA* erythroblasts formed pale, relatively large colonies in semisolid medium that seemed to consist of partially differentiated, sometimes oval cells (Fig. 4A), corresponding to the mixture of erythroblasts and early reticulocytes observed in mass

cultures (Fig. 4B). These cells were found to express both immature and mature differentiation antigens (Table 1). Thus, the phenotype induced by TT-*gag/v-erbA* was similar to, but also distinct from, the typical, essentially immature phenotype displayed by both SS-*gag/v-erbA*- and SS*-*gag/v-erbA*-expressing erythroblasts in these assays (Fig. 4; Table 1).

Medium requirements Besides causing a differentiation arrest, *gag/v-erbA* protein enables protein tyrosine kinase oncogene-transformed erythroblasts to proliferate in standard growth media, whereas erythroblasts devoid of a functional *erbA* protein require more complex growth conditions (CFU-E medium) mainly characterized by a narrow tolerance of pH, NaCl, and NaHCO₃ concentrations (Kahn et al. 1986a,b; Damm et al. 1987). Accordingly, we compared the ability of erythroblasts expressing wild-type or mutant *gag/v-erbA* proteins to proliferate in these two media. Whereas erythroblasts expressing wild-type *erbA* proteins (SS-*gag/v-erbA*; SS*-*gag/v-erbA*) grew in standard growth medium, cells containing the mutant proteins AA-*gag/v-erbA* or TT-*gag/v-erbA* did not, resembling *ts v-sea* erythroblasts in their medium requirements (Table 1). This inability to proliferate in standard medium was accompanied by development of large vacuoles in the AA- and TT-*gag/v-erbA*-expressing erythroblasts, eventually leading to cell disintegration. These results confirm that the TT-*gag/v-erbA* protein is only partially functional, perhaps corresponding to its weaker phosphorylation that cannot be enhanced by PKA or PKC activation.

Erythrocyte gene expression Because the AA-*gag/v-erbA* protein neither arrested erythroid differentiation nor altered the growth requirements of erythroblasts, it was of interest to analyze whether expression of this mutant protein is also accompanied by a loss in the ability to suppress erythrocyte-specific gene expression. Mass cultures of AA-*gag/v-erbA*-expressing erythroblasts were cultivated for 2 days at 42°C, and aliquots were analyzed at either protein or RNA levels for the expression of band 3 and CA II. As controls, cultures of wild-type-*gag/v-erbA* (SS-*gag/v-erbA*)-expressing erythroblasts and *ts v-sea*-transformed erythroblasts lacking *erbA* were treated similarly. Immunoprecipitation analyses of labeled lysates from these cells showed that expression of the AA-*gag/v-erbA* protein only reduced band 3 protein synthesis slightly, whereas expression of the SS-*gag/v-erbA* protein resulted in essentially complete suppression of band 3 protein levels under the same conditions (Fig. 5A). Similarly, expression of the CA II and band 3 RNAs was not or only partially reduced in AA-*gag/v-erbA*-expressing clones, whereas, as expected, their expression was almost completely abolished in erythroblasts transformed by the wild-type protein (Fig. 5B). These results suggest that the inability of the nonphosphorylated AA-*gag/v-erbA* mutant protein to block erythroid differentiation is mediated, at least in part, by its inability to completely shut down the expression of specific erythrocyte genes.

Table 1. Differentiation phenotype and growth characteristics of wild-type and mutant *erbA* protein-expressing erythroblast clones

Construct ^a	Differentiation parameter ^{b,c}					Growth ^d	
	LR + E (%)	ER (%)	Ebl (%)	anti- α Ery (%)	anti- α Ebl (%)	doubling time in EBM (hr)	doubling time in S13M (hr)
no <i>erbA</i>	88	10	2	93	>5	>120	20
v-(SS)- <i>erbA</i>	5 ^e	16	78	15	84	33	19
AA- <i>erbA</i>	83	17	3	91	12	>120	16
TT- <i>erbA</i>	20 ^e	68	12	56	48	>120	17
SS*- <i>erbA</i>	4 ^e	13	83	ND	ND	22	15

^aThe following erythroblast clones were used: (no *erbA*) *ts sea* clone H₂; [v-(SS)-*erbA*] *ts sea/v-erbA* clone F₁; (AA-*erbA*) *ts erbB/AA-erbA* clone C8; (TT-*erbA*) *ts sea/TT-erbA* clone B3; (SS*-*erbA*) *ts sea/SS*-erbA* clone E₁.

^bPercentage of late reticulocytes (LR) plus erythrocytes (E), early reticulocytes (ER), and erythroblasts (Ebl) was determined by using cytopins stained with neutral benzidine plus histological dyes (Beug et al. 1982) after incubating the cells for 3 days at 42°C (see Materials and methods).

^cPercent cells distinctly positive with anti-erythrocyte serum (α Ery; Beug et al. 1979) or monoclonal antibody MC 4.5.A.5 (α Ebl; Hayman et al. 1982). Cells were antibody-stained after incubation for 3 days at 42°C. (ND) Not determined.

^dCells were grown in standard growth medium (EBM) or CFU-E-medium (S13M) at 37°C, as described in Materials and methods. Doubling times were calculated from semilogarithmic plots of cumulative cell number vs. time, using the time interval between 96 and 148 hr.

^ePercentage of LR + E after 3 days at 42°C in pH 8.1 medium (Zenke et al. 1988) was >95%.

Figure 5. The AA *gag/v-erbA* protein is unable to suppress erythrocyte gene expression. (A) *ts v-sea* erythroblasts (clone A4; no *erbA*), *ts v-erbB/v-erbA* erythroblasts (clone E3; SS-*erbA*) and *ts erbB/AA-erbA* erythroblasts (clone C8; AA-*v-erbA*) were grown at 42°C in pH 7.2 medium for 3 days and labeled with [³⁵S]methionine, and extracts were immunoprecipitated with antibodies against band 3 protein (lanes 1), *v-erbA* (lanes 2), and globin (lanes 3; see Materials and methods; Zenke et al. 1988). The positions of molecular weight markers (right) are indicated by arrows. (B) Cells from the same clones as in A were cultivated at 42°C for 2 days and analyzed for expression of CA II-, band 3-, and globin-specific mRNA by slot-blot hybridization (5 mg cytoplasmic RNA per slot, exposure times CA II, 16 hr; band 3, 16 hr; β -globin, 2 hr). It remains unclear whether the two- or threefold lower expression of band 3 RNA in the AA-*erbA* erythroblasts is due to clonal variation or to a partial arrest of band 3 gene expression by the AA-*erbA* protein.

Inhibition of *gag/v-erbA* protein phosphorylation by the protein kinase inhibitor H7 abolishes biological activity of the oncoprotein

The results described thus far demonstrate that integrity of the phosphorylation site of the *gag/v-erbA* oncogene protein is essential for its biological activity, suggesting an important role of phosphorylation in *gag/v-erbA* protein function. However, these experiments do not rule out the alternative possibility that mutation at Ser-16/17 leads to phosphorylation-independent conformational changes that impair protein function. If the former notion is correct, inhibition of *gag/v-erbA* protein phosphorylation by appropriate protein kinase inhibitors should result in a phenocopy of the AA-*gag/v-erbA* mutant.

H7 is one of several isoquinoline sulfonamide derivatives that act as direct inhibitors of several protein kinases at micromolar concentrations, including cyclic nucleotide-dependent protein kinases and PKC (Hidaka et al. 1984; Kawamoto et al. 1984). To analyze whether this inhibitor would affect growth and/or differentiation of cells lacking the *v-erbA* oncogene, the following experiments were performed. First, *ts v-erbB*- or *ts v-sea*-transformed erythroblasts were cultivated at either 37°C (for proliferation assays) or 42°C (to test differentiation) in the appropriate media (see Materials and methods) containing different amounts of H7. At concentrations of 10–15 μ M, H7 had no detectable effects on the proliferation and spontaneous differentiation of these cells at 37°C [they grew with comparable doubling times (18 vs. 19 hr), incorporated similar amounts of tritiated thymidine, and expressed similar proportions of partially and completely differentiated cells (23.5 vs. 21%) in the absence or presence of H7, respectively]. At 42°C, H7 did not detectably affect temperature-sensitive maturation of *ts v-sea*-transformed erythroblasts (Table 2). In addition, no reduction of proliferation rate or alterations in differentiation capacity were seen in myeloid cells in-

Table 2. Abrogation of the *v-erbA*-induced arrest of erythroid differentiation by the protein kinase inhibitor H7

Construct	H7 ^a	Differentiation parameter ^b			
		LR + E (%)	ER (%)	Ebl (%)	anti-Ery3 (%)
no <i>erbA</i>	–	90	10	<0.5	90
(<i>ts sea/ts erbB</i>)	+	88	11	<0.5	95
SS- <i>erbA</i>	–	10	41	49	19
	+	78	20	2	80
AA- <i>erbA</i>	–	78	16	2	85
	+	80	15	1	85

For abbreviations, see Table 1 footnote.

^aThe protein kinase inhibitor H7 (Seikagaku) was used at 15 μ M (see Materials and methods).

^bDifferentiation parameters (see Table 1) were determined after incubating the cells at 42°C for 3 days.

fects with an E26 virus temperature-sensitive mutant and grown at 37 or 42°C in the presence of 15 μ M H7 (H. Beug, unpubl.).

Higher concentrations (25–100 μ M) of H7 were increasingly toxic on erythroblast clones. Cells from the *ts* 34 AEV-transformed cell-line HD3 (Beug et al. 1982a) when tested for proliferation at 37°C tolerated much higher doses of the drug, showing a significant growth inhibition only at 30–40 μ M (data not shown).

The effects of H7 on *gag/v-erbA* protein phosphorylation were analyzed next. HD3 erythroblasts were incubated for 2 days in either S13 medium at 37°C or differentiation medium at 42°C in the continuous presence of 20 μ M H7. Thereafter, cells were labeled with [³²P]orthophosphate for 3 hr in the presence of 20 μ M H7 and either were left untreated or were further treated for 20 min with forskolin or PMA before lysis and immune precipitation analyses. Treatment of AEV-transformed erythroblasts at either 37 or 42°C resulted in an almost complete inhibition of phosphorylation of the *gag/v-erbA* protein in both its *gag*- and *v-erbA* encoded domains (Fig. 6; data not shown). This inhibition of phosphorylation was dose-dependent with a maximal inhibition occurring at 20 μ M H7 for the HD3 cell line (data not shown). Similar results were obtained by using an erythroblast clone freshly transformed with a *ts v-erbB/v-erbA* virus (*ts*167 AEV) in the presence of 10 μ M H7 (data not shown). In these experiments, H7 proved equally efficient at inhibiting both basal and forskolin-, or PMA-enhanced phosphorylation of the *v-erbA*-encoded domain of the *gag/v-erbA* protein at all concentrations tested (Fig. 6). We therefore conclude that H7 effectively inhibits *gag/v-erbA* phosphorylation at concentrations that have no detectable toxic effects on erythroblasts lacking a *v-erbA* oncogene.

The above results encouraged us to test whether H7 under the conditions described above would impair the biological activity of the *gag/v-erbA* protein. Erythroblasts expressing either wild-type SS-*gag/v-erbA* or mutant *erbA* protein (AA- and TT-*gag/v-erbA*) were cultivated at 42°C in the presence and absence of 10 μ M H7 and analyzed for their differentiation phenotype and ex-

pression of erythrocyte genes as described above. H7 was found to release the differentiation arrest in both SS- and TT-*gag/v-erbA* erythroblasts, as shown by the induction of red, compact colonies and reticulocyte/erythrocyte-like cells strongly expressing hemoglobin and erythrocyte antigens (Fig. 7; Table 2). As expected, differentiation of erythroblasts expressing the nonphosphorylated AA-*gag/v-erbA* proteins was not grossly affected by H7 (Fig. 7; Table 2).

These results, suggesting that H7 strongly inhibits the biological activity of *v-erbA*, were corroborated by the finding that the same inhibitor, to a large extent, was also able to relieve the repression of erythrocyte genes in transformed erythroblast clones expressing the *gag/v-erbA* protein. H7 was found to restore both CA II and band 3 mRNA expression in SS-*gag/v-erbA*-containing erythroblasts to levels only slightly lower than that in cells without *v-erbA* but did not affect the expression of control genes (e.g., globin, *c-myc*) in these cells (Fig. 8; data not shown). The H7-induced up-regulation of band 3 mRNA was matched by a proportional increase in band 3 protein synthesis (data not shown). Again, H7 did not grossly alter CA II and band 3 mRNA and protein expression in erythroblasts expressing either no *erbA* or the AA-*gag/v-erbA* protein (Fig. 8; data not shown). In conclusion, the results described here indicate that the arrest of both erythroid differentiation and erythrocyte

Figure 6. The protein kinase inhibitor H7 inhibits phosphorylation of *v-erbA* protein. Erythroblasts from the *ts* AEV-transformed cell line HD3 were cultivated for 2 days in the absence (–H7) or the continuous presence of 20 μ M H7 (+H7). Cells were labeled with [³²P]orthophosphate for 3 hr in the presence of 20 μ M of H7 (+H7) or in the absence of H7 (–H7) and were treated further for 20 min with no agent (unt), 10 μ M forskolin (FOR), or 40 nM PMA. Immunoprecipitation was carried out with an *erbA*-specific antiserum on equal aliquots of each lysate (10⁷ acid-precipitable radioactive counts per aliquot). For each lysate, one of the two immunoprecipitates was digested with p15⁸⁸. Immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide slab gels.

Figure 7. H7 relieves the differentiation arrest caused by the phosphorylated SS *gag/v-erbA* and TT *gag/v-erbA* oncogene proteins. Erythroblasts containing no *erbA*, wild-type *erbA* (SS-*erbA*) or mutant *erbA* (AA- and TT-*erbA*; same clones used as in Fig. 4 and Table 1) were either seeded in semisolid medium or grown in mass cultures at 42°C for 3 days in the presence (+H7) or absence (–H7) of 15 mM H7. Photographs of typical colonies obtained in semisolid medium are shown in the upper portions (colonies), whereas cytospin preparations of the respective mass cultures photographed under blue light after staining with neutral benzidine plus histological dyes (see legend to Fig. 3) are shown in the lower portions (hemoglobin).

gene transcription by the *gag/v-erbA* oncogene protein is effectively modulated by phosphorylation of Ser-16/17.

Discussion

Possible pathways involved in regulating phosphorylation and biological activity of the *gag/v-erbA* protein

In this study three lines of evidence have been presented that suggest phosphorylation at Ser-16/17 is involved in the regulation of *v-erbA* oncoprotein function. First, mutation of Ser-16/17 to alanine residues, which prevents phosphorylation at this site, resulted in an essentially inactive *gag/v-erbA* protein. Second, an analogous substitution to threonine residues, which preserves a basal phosphorylation level but fails to maintain the ability of the *gag/v-erbA* protein to respond to either PKA or PKC activators by enhanced phosphorylation, yielded a partially active *gag/v-erbA* protein. Third, the function of the *gag/v-erbA* protein can be severely affected in vivo by preventing its phosphorylation, using a rather nonspecific inhibitor of serine protein kinases (H7) at concentrations low enough to prevent toxic effects. This latter result, together with the fact that an antibody raised against a synthetic peptide containing amino acid residues 5–28 of wild-type *gag/v-erbA* protein bound to both AA-*gag/v-erbA* and SS-*gag/v-erbA* proteins with the same affinity (C. Glineur et al., un-

publ.) make it unlikely that conformational changes unrelated to phosphorylation are responsible for the loss of function of the AA-*gag/v-erbA* protein.

The identity of the proximal protein kinase responsible to basal phosphorylation of *gag/v-erbA* protein in erythroblasts remains unknown. Two lines of evidence suggest that it is unlikely to be PKC. First, immunoaffinity-purified *gag/v-erbA* protein appears to be a poor substrate for purified PKC in vitro (Goldberg et al. 1988). Second, down-regulation of intracellular PKC in AEV-transformed erythroblasts by long-term treatment with PMA failed to affect the basal phosphorylation of the *gag/v-erbA* protein, although it completely suppressed the enhanced phosphorylation by PKC activators (Y. Goldberg and J. Ghysdael, unpubl.). In contrast, the catalytic subunit of PKA efficiently phosphorylated *gag/v-erbA* protein at Ser-16/17 in vitro (or a synthetic peptide encompassing Ser-16/17; Goldberg et al. 1988). However, in parallel assays, PKA proved unable to phosphorylate the TT-*gag/v-erbA* protein in vitro (C. Glineur et al., unpubl.), yet this protein is phosphorylated in both erythroblasts and fibroblasts, although at a lower level in its *v-erbA*-encoded domain than the wild-type protein (Fig. 2). If the same kinase is responsible for the basal phosphorylation of both wild-type and TT-*gag/v-erbA* proteins in vivo, it would therefore appear that PKA is also not involved in the basal phosphorylation of *gag/v-erbA* protein in cells. Because mutation of Ser-16/17 to threonine suppresses the enhanced phosphorylation of the *gag/v-erbA* protein following treatment with forskolin, our data are consistent with the idea that PKA is directly responsible for the enhanced phosphorylation of wild-type *gag/v-erbA* protein observed in vivo following treatments that result in an increase in the intracellular concentration of cAMP.

The TT-*gag/v-erbA* protein shows a weaker biological activity in *v-erbB* or *v-sea*-transformed erythroblasts as compared to the wild-type SS-*gag/v-erbA* protein, both in terms of its effects on the differentiation phenotype of transformed cells and on their growth medium require-

Figure 8. H7 relieves suppression of erythrocyte gene expression. Erythroblasts containing no *erbA*, SS *gag/v-erbA*, or AA *gag/v-erbA* (AA-*erbA*; same clones as in Fig. 5) were grown in pH 7.2 medium at 42°C for 2 days in the presence (+H7) or absence (–H7) of 15 μM H7, and expression of CA II-, band 3-, or β-globin-specific mRNA was analyzed by slot blot hybridization, as described in the legend to Fig. 5.

ments (Fig. 3; Table 1). Whether these properties are a result of its lower stoichiometric phosphorylation under basal conditions or to its inability to respond to external signals by enhanced phosphorylation involving the PKA or PKC pathways cannot be rigorously determined on the basis of available experimental data. Trials to directly demonstrate an effect of PKA and PKC activators (e.g., forskolin and phorbol esters, respectively) on differentiation of *gag/v-erbA*-containing erythroblasts were hampered by toxic side effects of these drugs (as evidenced using *ts v-sea* erythroblasts) although the observed effects—a somewhat stronger differentiation arrest caused by forskolin and an induction of differentiation by phorbordibutyrate (which, when applied for 48 hr, probably caused down-regulation of PKC)—are in agreement with the idea that the PKA and/or PKC pathways might be involved in some way.

How does phosphorylation affect erbA oncoprotein function?

Several lines of evidence indicate that lack of phosphorylation of the *gag/v-erbA* protein does not render the protein completely nonfunctional. First, the AA-*gag/v-erbA* protein retains its nuclear localization in transformed cells and its ability to bind in vitro either non-specific DNA (C. Glineur et al., unpubl.) or a palindromic T₃RE (Fig. 2B). Second, the AA *gag/v-erbA* protein, like the wild-type protein (Damm et al. 1989), is able to *trans*-repress a construct containing a T₃RE linked to the herpes simplex virus thymidine kinase promoter in transient transfection assays (C. Glineur et al., unpubl.). Further support for the idea that regulation of *v-erbA* protein function by phosphorylation may modulate its activity rather than simply turn it on or off stems from observations using *c-erbA*α-expressing retroviruses. We have described elsewhere (Zenke et al. 1990) that the *c-erbA*α protein fused to viral *gag* sequences as in *v-erbA* arrests differentiation, as well as erythrocyte gene expression in the absence of T₃, but induces (abnormal) differentiation and up-regulation of erythrocyte gene transcription in the presence of hormone. Preliminary experiments testing the effect of H7 on cells expressing this *gag/c-erbA*α protein suggested that phosphorylation regulates its repressor function in the absence of hormone but not its function as an activator of gene expression in response to T₃. We are currently testing this hypothesis by constructing the homologous phosphorylation mutant in *gag/c-erbA*α and *c-erbA*α.

The *gag/v-erbA* protein has recently been shown to act as a constitutive repressor of model T₃RE in transient transfection assays (Damm et al. 1989; Sap et al. 1989). Because this property requires integrity of the DNA-binding domain of *gag/v-erbA* protein, it has been proposed that competition occurs at the level of the response element by interfering with the binding of thyroid hormone receptors (Damm et al. 1989). The fact that phosphorylation of *gag/v-erbA* protein at Ser-16/17 is required for full biological activity in transformed erythroblasts and yet does not affect its DNA-binding

ability in vitro indicates that the mode of action of *v-erbA* might be more complex than anticipated previously. Phosphorylation of Ser-16/17 would appear to be required for the activity of the *gag/v-erbA* protein on a subset of its responsive elements, some of which would regulate the activity of genes essential to erythroid differentiation. T₃RE-like elements are present upstream of the CA II gene promoter region (C. Disela, H. Beug, and M. Zenke, in prep.). If these elements proved able to mediate T₃-dependent transcriptional regulation by the *c-erbA* protein, the CA II gene would represent a likely direct target for repression by the *gag/v-erbA* protein.

In this context, phosphorylation of Ser-16/17 could contribute to the interaction of *gag/v-erbA* protein either with other factors to stabilize its binding to these elements or with specific transcriptional activators to neutralize their *trans*-activating function.

Implications for transcriptional regulation in other systems

Several other members of the steroid/thyroid hormone receptor superfamily have been shown to be phosphoproteins (Dougherty et al. 1984; Logeat et al. 1985; Housley et al. 1985; Migliaccio et al. 1986; Sullivan et al. 1988), but the significance of these phosphorylation events on receptor function has not been established. The results presented here provide definitive evidence that phosphorylation can modulate the function of the oncogenic version of at least one member of the steroid/thyroid hormone receptor superfamily. Because the phosphorylation site homologous to Ser-16/17 of *gag/v-erbA* protein is conserved and phosphorylated in the thyroid hormone receptor encoded by the chicken *c-erbA*α proto-oncogene (Ser-28/29; Goldberg et al. 1988), phosphorylation at this site is likely to play a role in regulating the function of the *c-erbA*α-encoded thyroid receptor as well. Because phosphorylation sites homologous to Ser-28/29 do not exist in the various mammalian thyroid hormone receptors sequenced to date (Weinberger et al. 1986; Thompson et al. 1987; Benbrook and Pfahl 1987; Izumo and Madhavi 1988; Lazar et al. 1988; Mitsuhashi et al. 1988; Nakai et al. 1988) nor in the avian *c-erbA*β receptor (Forrest et al. 1990), it is unlikely that the function of these receptors is regulated in a similar fashion. This is not surprising, given the lack of identity between the amino-terminal domains of the different members of the T₃ receptor family. The amino-terminal domain of the estrogen and progesterone receptors appears to be involved in cell-specific regulation of a subset of responsive genes (Kumar et al. 1987; Tora et al. 1988a,b). The heterogeneity of the amino-terminal domains of the various members of the thyroid hormone receptor family may be one of the components contributing to target gene specificity.

There is increasing evidence that gene expression is regulated by phosphorylation of transcription factors. Activation of PKC and cAMP-mediated pathways is known to regulate transcriptional events (Hashimoto et

al. 1984; Comb et al. 1986; Montminy et al. 1986; Chiu et al. 1987], and several transcription factors involved in these responses have been shown to be phosphoproteins (Barber and Verma 1987; Müller et al. 1987; Gonzalez and Montminy 1987). In the case of cAMP-responsive element nuclear binding protein (CREB)—the 43-kD protein that mediates the transcriptional response of at least some genes to cAMP (Montminy and Bilezikjian 1987)—phosphorylation by PKA has recently been shown to be required for transcriptional activity (Gonzalez and Montminy 1989). Our results suggest that phosphorylation plays a critical role in the activity of the *gag/v-erbA* protein as a transcriptional repressor of some of its target genes in erythroid cells. Because these results cannot easily be obtained in transient expression assays due to our limited understanding of direct target sequences for the *gag/v-erbA* protein in these cells, our alternative approach of using stably integrated normal and mutated transcriptional regulators that act on target genes in their native configuration should be considered as a complementary way in helping to understand the functional regulation of transcription factors.

Materials and methods

Oligonucleotide site-directed mutagenesis of v-erbA phosphorylation sites and construction of recombinant retroviruses

A *Bam*HI fragment of pCG1 (Jansson et al. 1987), encompassing all of *v-erbA* and a portion of *v-erbB*, was subcloned into the *Bam*HI site of M13mp18 RF DNA. Substitution of Ser-16/17 to threonine or alanine was performed by using the oligonucleotide 5'-C CAA ACA TTG GGC GGT CTT TCT TTT G-3' and 5'-CAA ACA TTG GGC GGC CTT TCT TTT-3', respectively. Back mutation of the alanine residues at positions 16 and 17 to serine made use of the oligonucleotide 5'-CTT CAC CAA ACA TTG GGC GGC CTT TGT TTT GTG-3'.

Mutagenesis was performed by using the Amersham system, based on the method of Taylor et al. (1985). Single-stranded DNA from individual plaques was analyzed for the presence of the expected mutations by the dideoxy sequencing method. The mutagenized *v-erbA* genes were recovered from M13mp18 RF by digestion with *Xho*I and *Ap*aI and subcloned at the *Xho*I-*Ap*aI of cloned *ts* 167 AEV or *erbA/ts sea* proviruses (Knight et al. 1988).

Plasmids corresponding to these constructs were cotransfected together with a plasmid containing a biologically active RAV-1 provirus in chicken embryo fibroblasts by calcium phosphate coprecipitation, followed by selection with 250 µg/ml neomycin (G418; GIBCO).

Electrophoretic mobility-shift assays

COS cells were transfected with the SV40 promoter-based expression plasmid pKCR3 (Breathnach and Harris 1983), encoding either SS-*gag/v-erbA* or AA-*gag/v-erbA* or pKCR3 alone as control. Two days after transfection, cells were washed three times in phosphatase-buffered saline (PBS) and 10^7 cells were lysed in 200 µl of 10 mM HEPES (pH 7.4), 1 mM dithiothreitol (DTT), 0.1% Triton X-100 (wt/vol), 1 mM EDTA, 400 mM NaCl, 1% aprotinin, 100 µg/ml phenyl methylsulfonylfluoride (PMSF), and 10 µg/ml leupeptin (all from Sigma). Lysates were centrifuged at 100,000g for 20 min, and supernatants were col-

lected. For DNA-binding reactions, 4 µl of whole-cell lysates was incubated at 0°C in a final volume of 16 µl containing 10 mM HEPES (pH 7.4), 100 mM NaCl, 1.25 mM Na-phosphate, 0.175 mM EDTA, 0.075 mM EGTA, 1 mM DTT, 5 mM MgCl₂, 2 µg of poly[d(I-C)], and 0.6 µg of salmon sperm DNA. After 20 min, 50 fmol of an end-labeled T₃RE palindromic oligonucleotide (Glass et al. 1989) were added, and incubation continued for 10 min on ice. Samples were loaded on a 5% polyacrylamide gel (acrylamide/bisacrylamide ratio 30 : 1) in 0.25 × TBE [$1 \times TBE = 0.089$ M Tris, 0.089 M boric acid, 2 mM EDTA (pH 8.3)], and electrophoresed at 140 V for 90 min at room temperature. Gels were fixed, dried, and exposed to Kodak XAR films for 60 min.

Cells and cell culture

Chicken embryo fibroblasts were prepared from 11-day-old embryos of SPAFAS flocks maintained in Vienna or Lille. They were grown in standard growth medium [consisting of modified Dulbecco's modified Eagle medium (DMEM) plus 8% fetal calf serum, 2% chicken serum, and 10 mM HEPES (pH 7.3)]. Transformed erythroblasts were grown in CFU-E medium without anemic serum (Radke et al. 1982), unless otherwise stated.

Generation of transformed erythroblast clones

Erythroblast clones transformed by the recombinant viruses illustrated in Figure 1 were generated by infecting bone marrow cells with the respective viruses (by cocultivation of bone marrow cells with virus-producing fibroblasts for 2 days; Beug et al. 1986) and seeding them in either standard methocel (Beug and Hayman 1984) supplemented with 5% chicken serum and 1% dimethyl sulfoxide (DMSO) or, in later experiments, CFU-E-methocel (Fuhrmann et al. 1989), which contained the medium additions and erythroid hormones of differentiation medium (Zenke et al. 1988). This was necessary because some of the constructs yielded insufficient numbers of transformed colonies in standard methocel (Beug and Hayman 1984). Erythroblast clones exhibiting a long in vitro lifespan, as well as a high capacity to differentiate terminally under suitable conditions (i.e., at pH 8.1 in case of active *v-erbA*-containing constructs; Zenke et al. 1988), were selected as described (Knight et al. 1988; Zenke et al. 1988).

Induction of differentiation transformed erythroblast clones

For induction of differentiation in semisolid medium, pH 7.2 methocel was supplemented with erythropoietin (2% high titer anemic serum) and REV factor at optimum concentration (Kowenz et al. 1987; Zenke et al. 1988). Then, 3×10^4 to 5×10^4 cells from transformed erythroblast clones were mixed with 0.6 ml of the above methocel mixture. After 3–4 days at 42°C and 5% CO₂, colonies were either photographed or isolated with fine capillaries and spread on slides by using a stream of CO₂ gas emerging from a Pasteur pipette at 0.5–1 atm.

For differentiation induction in mass cultures, cells were purified by centrifugation through Percoll (density 1.070 g/cm³; Schmidt et al. 1986), and seeded at 1×10^6 to 1.5×10^6 cells/ml into either differentiation medium (AA-*erbA*) or pH 7.2 medium and pH 8.1 medium (SS-*erbA*, SS*-*erbA*), or both (TT-*erbA*, AA-*erbA*), depending on the respective cell behavior elucidated in pilot experiments (Zenke et al. 1988). Media were supplemented with 1–2% high-titer anemic sera, REV factor at optimum concentration (Kowenz et al. 1987), and insulin (Zenke et al. 1988). For treatment with the protein kinase inhibitor H7 (Seikagaku, Gainesville, FL), the drug was dissolved

in sterile PBS (10 mM) and added to a final concentration of 15 μ M. To account for possible uptake or decay of H7, the addition of 10 μ M H7 was repeated daily. After 2 days (RNA) or 3–4 days (proteins, differentiation markers) at 42°C, cells were harvested by centrifugation and centrifugation through Percoll (1.070 g/cm³) and Ficoll (always combining the Percoll pellet with the Ficoll interphase; Zenke et al. 1988) and were used for differentiation marker analysis, as well as immunoprecipitation and RNA extraction (see below).

Analysis of erythroid differentiation markers

Hemoglobin content of individual clones was analyzed by acid benzidine staining as described earlier (Damm et al. 1987). Stages of erythroid differentiation (erythroblasts, early reticulocytes, late reticulocytes, and erythrocytes) were analyzed by cytocentrifugation and staining with neutral benzidine plus histological dyes (Beug et al. 1982; Zenke et al. 1988). Note that the acid benzidine technique is much more sensitive than the neutral benzidine staining method, because cells heavily positive with the former method (TT-*erbA*; Fig. 3D) are still essentially negative with the other method (Beug et al. 1982; Zenke et al. 1990).

Cell-surface antigens were analyzed by indirect immunofluorescence as described earlier (Beug and Hayman 1984), using antisera to immature (antierythroblast serum; Beug et al. 1979) and mature erythroid cells (antierythrocyte serum; Beug et al. 1979).

Cell proliferation analysis

Erythroblasts from exponentially growing cultures of freshly thawed cells (cells approaching senescence are unsuitable for this assay) were seeded at 1×10^6 cells/ml into either standard growth medium or CFU-E medium. After daily counting in a Coulter Counter, cells were readjusted to 1×10^6 cells/ml by using the respective media. Cumulative cell numbers were calculated, accounting for feeding with fresh medium and cell losses by counting and plotted semilogarithmically against time in culture to allow determination of doubling times.

Cell labeling and immunoprecipitation procedures

Detection of erythrocyte proteins (hemoglobin, band 3) by [³⁵S]methionine labeling and immunoprecipitation analysis was performed as described earlier (Beug et al. 1981; Zenke et al. 1988; Knight et al. 1989).

For [³²P]orthophosphate labeling, cells were incubated for 2 hr in phosphate-free MEM (GIBCO), supplemented with 3% dialyzed fetal calf serum and incubated in the same medium with 1 mCi/ml of [³²P]orthophosphate for 4 hr. At the end of the labeling period, cells were rinsed in ice-cold PBS and lysed in 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 50 mM NaCl, 10 mM NaF, 40 mM *p*-nitrophenylphosphate, 1% aprotinin, 0.1 mg/ml PMSF, and 1 μ g/ml leupeptin (all from Sigma). For L-[³⁵S]methionine labeling in controls, cells were rinsed with methionine-free MEM (GIBCO), supplemented with 5% dialyzed fetal calf serum, and incubated in the same medium with L-[³⁵S]methionine at 250 μ Ci/ml for 60 min and lysed as above. The lysates were centrifuged at 100,000g for 60 min and immune precipitations were carried out using either normal rabbit sera or an *erbA*-specific antisera followed by cleavage with protease p15, as described previously (Goldberg et al. 1988). Immune precipitates were analyzed on 10% polyacrylamide slab gels in the presence of 0.1% SDS (SDS-PAGE). Gels were fixed in meth-

anol/water/acetic acid (5 : 5 : 1), dried, and processed for either autoradiography or fluorography.

RNA preparation and blot hybridization

Total RNA was prepared by using the guanidinium thiocyanate CsCl step gradient procedure (Glisin et al. 1974), with a modification as described before (Zenke et al. 1988). To obtain cytoplasmic RNA, cells were lysed with 0.5% NP-40 in 25 mM Tris-HCl (pH 7.5), 5 mM NaCl, and 1 mM MgCl₂ in the presence of 5 mM Vanadyl-ribonucleoside-complex (BRL) for 10 min on ice. Following centrifugation (10 sec, 15,600g, 4°C), the cytoplasmic supernatant was digested extensively with proteinase K (Merck, 5 mg/ml, 2 hr, 37°C) in the presence of 40 mM EDTA, 1% SDS, and 150 mM NaCl. The RNA obtained after phenol-chloroform extraction and ethanol precipitation was transferred to GeneScreen membranes (NEN) by slot blotting. For this, 5 μ g of RNA was denatured in 50% (vol/vol) formamide and 0.2 M formaldehyde (10 min at 60°C), chilled on ice, diluted 10-fold with 20 $\times$ SCC, and transferred onto the membrane in a Millipore slot blotting apparatus. Hybridization with radiolabeled probes was performed as described previously (Zenke et al. 1988).

Immunofluorescence analysis

Chicken embryo fibroblasts infected with wild-type or mutant AEVs were fixed in 3.8% formaldehyde in PBS (freshly prepared from paraformaldehyde) for 1 hr at room temperature. Cells were permeabilized with 1% Triton X-100 followed by a treatment with methanol at -20°C for 5 min. Fixed cells were preincubated with a 5% solution (wt/vol) of powdered milk in PBS for 30 min at room temperature and then incubated with a 1 : 200 dilution of an *erbA*-specific rabbit antiserum (Goldberg et al. 1988) or a nonimmune rabbit control serum for 60 min at room temperature. After four washes in PBS, cells were incubated with a 1 : 400 dilution of fluorescein isothiocyanate-conjugated swine anti-rabbit IgG antiserum (Bioss). After washing and mounting in Elvanol, immunofluorescence was examined under a Zeiss epifluorescence microscope.

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