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Activation of protein kinase C or cAMP-dependent protein kinase increases phosphorylation of the c-*erbA*-encoded thyroid hormone receptor and of the v-*erbA*-encoded protein

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The c-*erbA* proto-oncogene encodes a nuclear receptor for thyroid hormone (T_3), which is believed to stimulate transcription from specific target promoters upon binding to *cis*-acting DNA sequence elements. The v-*erbA* oncogene of avian erythroblastosis virus (AEV) encodes a ligand-independent version of this nuclear receptor. The v-*erbA* product inhibits terminal differentiation of avian erythroblasts, presumably by affecting the transcription of specific genes. We show here that the c-*erbA*-encoded nuclear receptor ($p46^{c\text{-}erbA}$) is phosphorylated on serine residues on two distinct sites. One of these sites, defined by the limit tryptic phosphopeptide ^{28}SSQCLVK, is retained on the v-*erbA*-encoded $P75^{gag-v\text{-}erbA}$ protein. This site is located in the amino-terminal domain of these molecules, 21 amino acids upstream of the DNA-binding region. Phosphorylation of this site in both $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$ is enhanced 10-fold following treatment of cells with activators of either protein kinase C or cAMP-dependent protein kinase. Since cAMP-dependent protein kinase phosphorylates both $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$ *in vitro* at the same site as that observed *in vivo*, at least part of the cAMP-dependent phosphorylation of *erbA* molecules in cells could result from direct phosphorylation by this enzyme. The possible role phosphorylation may play in the function of the *erbA*-encoded transcriptional factors is discussed.

Key words: erbA oncogene/thyroid hormone receptor/phosphorylation/protein kinase C/cAMP

Introduction

The v-*erbA* oncogene is found along with v-*erbB* in the genome of the ES4 strain of avian erythroblastosis virus (AEV) (Vennström and Bishop, 1982). AEV transforms avian fibroblasts and bone marrow-derived erythroblasts, both *in vivo* and *in vitro* (Graf and Stéhelin, 1982; Graf and Beug, 1983). Specific deletion analyses (Frykberg *et al.*, 1983; Sealy *et al.*, 1983) and characterization of other AEV field isolates (Yamamoto *et al.*, 1983) demonstrated that v-*erbB* alone is necessary and sufficient for initiation and maintenance of transformation, in both fibroblasts and erythroid cells. However, although it is by itself devoid of any detectable transforming activity, v-*erbA* potently contributes to AEV-induced oncogenesis. Expression of v-*erbA* in erythroblasts results in a complete blockade of differentiation of these cells at an immature stage (Frykberg *et al.*, 1983; Kahn *et al.*, 1986) and relieves the cells from the very specific pH and salt conditions they normally require for growth in tissue culture (Damm *et al.*, 1987). In addition, v-*erbA* can restore transformation of erythroblasts and fibroblasts expressing certain transformation-defective v-*erbB* mutants (Damm *et al.*, 1987; Jansson *et al.*, 1987). Expression of v-*erbA* stimulates growth of untransformed avian fibroblasts and enhances the tumorigenic potential of v-*erbB*-transformed fibroblasts (Gandrillon *et al.*, 1987).

The molecular bases of v-*erbA* and v-*erbB* actions were considerably clarified by elucidating the function of their respective cellular progenitors, c-*erbA* and c-*erbB*. The c-*erbB* proto-oncogene encodes the receptor for epidermal growth factor, which exhibits ligand-dependent mitogenic tyrosine kinase activity; the v-*erbB*-encoded protein is a truncated, constitutively active version of this receptor (Downward *et al.*, 1984; Gilmore *et al.*, 1985; Kris *et al.*, 1985). In contrast, the avian c-*erbA* proto-oncogene belongs to a superfamily that includes genes encoding steroid hormone receptors and other transcriptional regulators (Weinberg *et al.*, 1985; Green *et al.*, 1986). c-*erbA* is translated as two proteins with overlapping sequences, of respective mol. wts 46 kd ($p46^{c\text{-}erbA}$) and 40 kd ($p40^{c\text{-}erbA}$), both of which are nuclear, high-affinity receptors for the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) (Sap *et al.*, 1986). Mammalian genomes contain multiple c-*erbA*-related genes (Jansson *et al.*, 1983; Weinberger *et al.* 1986), some of which also encode high affinity T_3 receptors (Weinberger *et al.*, 1986; Thompson *et al.*, 1987; Benbrook and Pfahl, 1987). Like steroid receptors, T_3 receptors mediate hormone action by modulating transcription of specific genes; this process requires receptors to bind to regulatory ('hormone-responsive') DNA sequence elements located close to the target genes (Flug *et al.*, 1987; Ye and Samuels, 1987; Koenig *et al.*, 1987; Glass *et al.*, 1987). The v-*erbA* oncogene is expressed as the carboxy-terminal domain of a 75 kd fusion protein ($P75^{gag-v\text{-}erbA}$) which derives its amino-terminal domain from a partial retroviral *gag* gene (Hayman *et al.*, 1979; Beug *et al.*, 1980). $P75^{gag-v\text{-}erbA}$ is also a nuclear, DNA-binding protein, but it does not bind hormones (Sap *et al.*, 1986). $P75^{gag-v\text{-}erbA}$ is therefore believed to act as a ligand-independent transcriptional factor that perturbs the normal transcription of specific, differentiation-linked genes.

The activity of several transcriptional factors has been shown to depend on signals that stimulate cellular protein-kinases, suggesting that phosphorylation may be critical to the function of these nuclear proteins (Sen and Baltimore, 1986; Angel *et al.*, 1987).

We show here that the avian $p46^{c\text{-}erbA}/T_3$ receptor is phosphorylated on at least two distinct sites, defined by two limit tryptic phosphopeptides. Only one of these

phosphopeptides, which contains the serine residues located at positions 28 and 29, is retained in P75$^{gag-v\text{-}erbA}$. Phosphorylation of this common phosphopeptide is specifically increased following stimulation of either protein kinase C (PKC) or cAMP-dependent protein kinase.

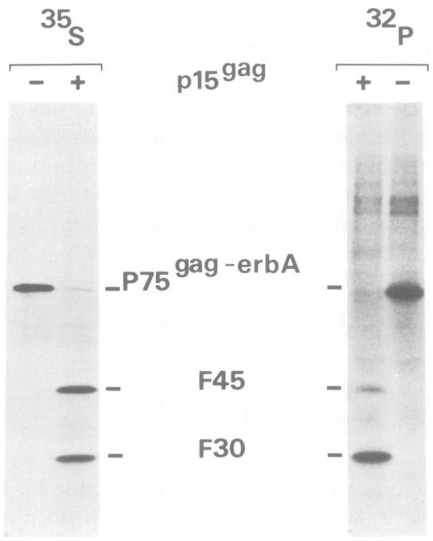

Fig. 1. Phosphorylation of P75$^{gag-v\text{-}erbA}$ in AEV-transformed erythroblasts. Cells were labelled either for 60 min with 250 μCi/ml L-[^{35}S]methionine or for 4 h with 1 mCi/ml [^{32}P]orthophosphate. The labelled cells were lysed and immunoprecipitation was carried out with serum B (described in Materials and methods) on two equal aliquots of each lysate (10^7 acid-precipitable radioactive counts per aliquot). For each lysate, one of the two immunoprecipitates was digested with p15^{gag}. Immunoprecipitates were analysed by electrophoresis on a 10% polyacrylamide slab gel. **Panel ^{35}S**: the gel was fluorographed with diphenyloxazole. **Panel ^{32}P**: the gel was autoradiographed with an intensifying screen.

Results

Phosphorylation of P75$^{gag-v\text{-}erbA}$ protein

To investigate the phosphorylation state of P75$^{gag-v\text{-}erbA}$, AEV-transformed erythroblasts were labelled with [^{32}P]-orthophosphate, or, as a control experiment, with [^{35}S]-methionine. P75$^{gag-v\text{-}erbA}$ was immunoprecipitated with a specific anti-*erbA* serum raised against a bacterially expressed *erbA* polypeptide (see Materials and methods). This serum efficiently precipitated P75$^{gag-v\text{-}erbA}$ from lysates of [^{35}S]methionine-labelled AEV-transformed erythroblasts (Figure 1). Immunoprecipitation of the ^{32}P-labelled cell proteins showed ^{32}P incorporation into P75$^{gag-v\text{-}erbA}$ (Figure 1). This result was not unexpected since P75$^{gag-v\text{-}erbA}$ contains all of the sequence of p19^{gag}, which is the major structural phosphoprotein of avian retroviruses (Lai, 1976). To analyse whether specific labelling of the v-*erbA*-encoded domain of P75$^{gag-v\text{-}erbA}$ also occurred, we took advantage of the fact that P75$^{gag-v\text{-}erbA}$ can be cleaved by the p15^{gag} protease of avian retroviruses into a *gag*-encoded 30 kd fragment (F30^{gag}), and v-*erbA*-encoded 45 kd fragment (F45$^{v\text{-}erbA}$) (Vogt *et al.*, 1979; Beug *et al.*, 1980). Digestion of phosphorylated P75$^{gag-v\text{-}erbA}$ with p15^{gag} indicates that, although most of the phosphate incorporated into P75$^{gag-v\text{-}erbA}$ is found in the *gag* domain, a low level of incorporation is also observed in F45$^{v\text{-}erbA}$ (Figure 1). Phosphoamino acid analysis of the gel-purified F45$^{v\text{-}erbA}$ fragment yielded exclusively phosphoserine (data not shown).

To analyse whether the activation of known cellular protein-kinases might modulate the phosphorylation of F45$^{v\text{-}erbA}$, [^{32}P]orthophosphate-labelled AEV-transformed erythroblasts were incubated for 30–45 min with various protein kinase activators. After p15^{gag} cleavage of the immunoprecipitated P75$^{gag-v\text{-}erbA}$, the [^{32}P]phosphate incorporated into F30^{gag} and F45$^{v\text{-}erbA}$ was analysed by SDS–PAGE. Two groups of agents were found to specifically

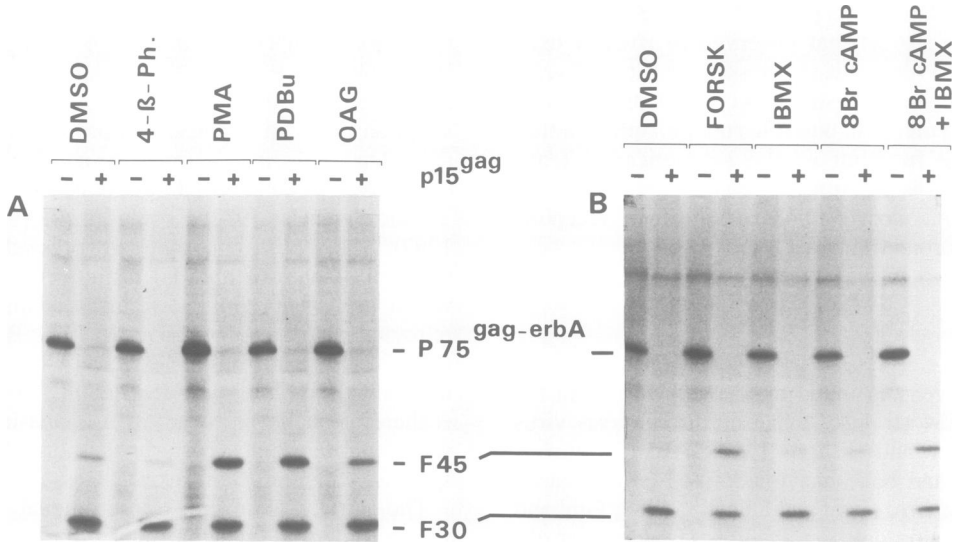

Fig. 2. Stimulation of P75$^{gag-v\text{-}erbA}$ in AEV-transformed erythroblasts. **Panel A**. Cells were labelled for 4 h with 1 mCi/ml [^{32}P]orthophosphate, then divided into five equal fractions. To each fraction was added, respectively: DMSO (vehicle), 0.3% (v/v) final concentration; 4β-phorbol, 100 nM; PMA, 40 nM; PdBu, 100 nM; OAG, 150 μg/ml. Cells were incubated for 30 min in the presence of the agents, then lysed. Immunoprecipitation was carried out with serum B on two equal aliquots of each lysate (10^7 acid-precipitable radioactive counts per aliquot). For each lysate, one of the two immunoprecipitates was digested with p15^{gag}. Immunoprecipitates were analysed by electrophoresis on 10% polyacrylamide slab gels. **Panel B**. As for panel A except that the agents were DMSO, 0.3% (v/v); forskolin, 10 μM; IBMX, 125 μM; 8-bromo-cAMP, 1 mM.

enhance ~10-fold the phosphorylation of $F45^{v\text{-}erbA}$ as judged by densitometric scanning of the autoradiograms, while leaving that of $F30^{gag}$ unaffected (Figure 2). The first group includes direct activators of PKC, i.e. two different phorbol esters, phorbol myristate acetate (PMA) and phorbol dibutyrate (PdBu), and the synthetic diacylglycerol, octyl-acetyl-glycerol (OAG) (Figure 2, panel A). The second group includes agents that cause or mimic an increase in intracellular cAMP concentration, i.e. the adenylate cyclase activator, forskolin, or a combination of 8-bromo-cAMP, and the phosphodiesterase inhibitor, isobutyl-methyl-xanthine (IBMX) (Figure 2, panel B). Control agents, such as the inactive unesterified 4β-phorbol, or dimethylsulfoxide (DMSO), the solvent vehicle used for all hydrophobic inducers, did not affect $F45^{v\text{-}erbA}$ phosphorylation (Figure 2, panels A and B). Treatment of cells with either the calcium ionophore A23187 or dibutyryl cGMP failed to evoke any modification in the phosphorylation of $P75^{gag-v\text{-}erbA}$ (data not shown). These observations were not restricted to erythroblasts, since similar results were obtained in AEV-transformed chicken embryo fibroblasts (data not shown). Since fibroblasts could be labelled long enough (18–20 h) to reach isotopic equilibrium, these results indicate that the increased ^{32}P incorporation in the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$ is due to authentic *de novo* phosphorylation, rather than to an increased phosphate turnover.

Analysis of the v-erbA phosphorylation sites

^{32}P-Labelled AEV-transformed erythroblasts were incubated with either vehicle or PMA or forskolin, and the phosphorylation sites in $P75^{gag-v\text{-}erbA}$ were analysed in each case by two-dimensional tryptic mapping analysis of $P75^{gag-v\text{-}erbA}$, $F45^{v\text{-}erbA}$ and $F30^{gag}$ (Figure 3). Most of the phosphopeptides of $P75^{gag-v\text{-}erbA}$ isolated from untreated AEV-transformed erythroblasts are derived from the *gag*-encoded domain of the molecule, since these phosphopeptides were also detected in the tryptic peptide map of $F30^{gag}$ (Figure 3, compare panels A and C). Only three phosphopeptides were specific for $F45^{v\text{-}erbA}$ (Figure 3, compare panels A and B); these are referred to as peptides 1, 2 and 3 (Figure 3, panel E). Peptides 1, 2 and 3 became the major phosphopeptides in $P75^{gag-v\text{-}erbA}$ isolated from cells treated either with forskolin (Figure 3, compare panels A and D) or with PMA (results not shown). Beside phosphopeptides 1, 2 and 3, no other phosphopeptides were found in $F45^{v\text{-}erbA}$ isolated from either untreated or forskolin- or PMA-treated cells (Figure 3, panels A and D; schematized in panel E) and in all cases phosphate was found exclusively in the form of phosphoserine (data not shown). We conclude from these experiments that the same tryptic peptides are phosphorylated in the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$ in either untreated or forskolin- or PMA-treated cells.

Since *in vivo* stimulation of both PKC and PKA resulted in an increase in the population of $P75^{gag-v\text{-}erbA}$ molecules phosphorylated in the v-*erbA*-encoded domain, we next analysed whether either of these enzymes could directly catalyse $P75^{gag-v\text{-}erbA}$ phosphorylation *in vitro*. Immuno-

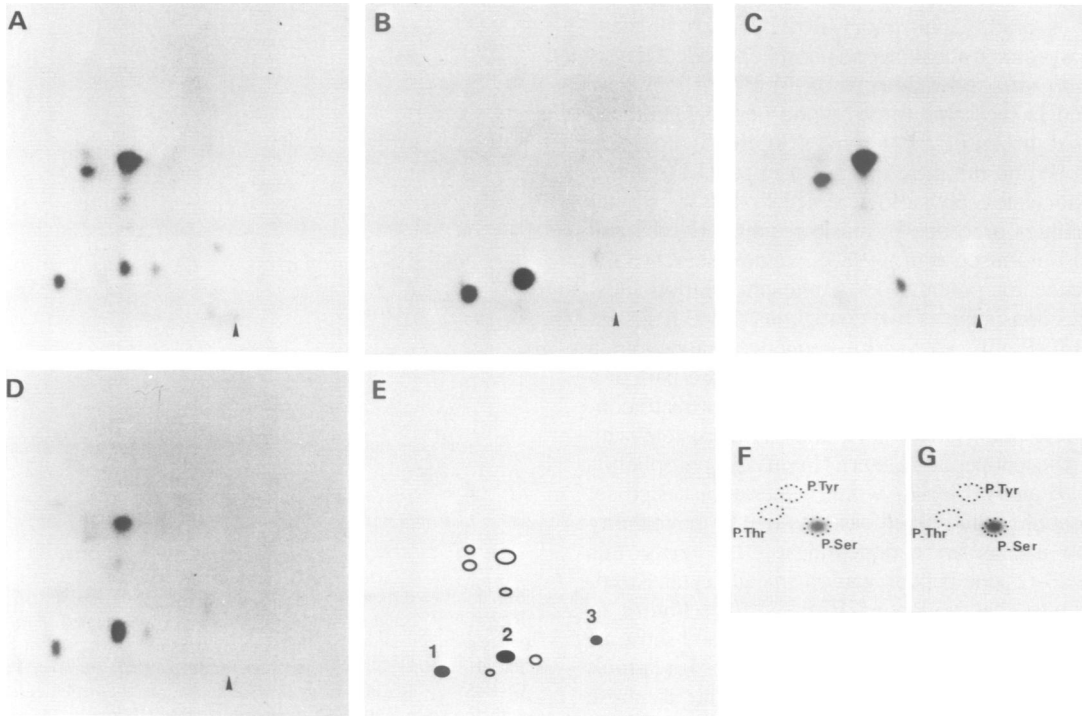

Fig. 3. Two-dimensional tryptic phosphopeptide analysis of $P75^{gag-v\text{-}erbA}$ in unstimulated and stimulated conditions. [^{32}P]Orthophosphate-labelled $P75^{gag-v\text{-}erbA}$ or $F45^{v\text{-}erbA}$, or $F30^{gag}$, were excised from gels similar to those of Figure 2, eluted, oxidized with performic acid and digested with TPCK-treated trypsin. The resulting peptides were separated by electrophoresis in the first dimension (origin: arrow; cathode at the left) and chromatography in the second dimension. Exposure was at −70°C with an intensifying screen using Kodak X-AR5 film. **Panel A**: $P75^{gag-v\text{-}erbA}$ from untreated AEV-transformed erythroblasts. **Panel B**: $F45^{v\text{-}erbA}$ from untreated cells. **Panel C**: $F30^{gag}$ from untreated cells. **Panel D**: $P75^{gag-v\text{-}erbA}$ from forskolin-treated cells. **Panel E**: schematic drawing of panel A; solid spots (peptides 1, 2, 3) represent peptides derived from $F45^{v\text{-}erbA}$ and open spots represent peptides derived from $F30^{gag}$. **Panel F**: phosphoamino acid analysis of $p46^{c\text{-}erbA}$ isolated from untreated COS-1 cells transfected with the pKCR3-based c-*erbA* expression vector. **Panel G**: as in panel F but cells were treated with forskolin.

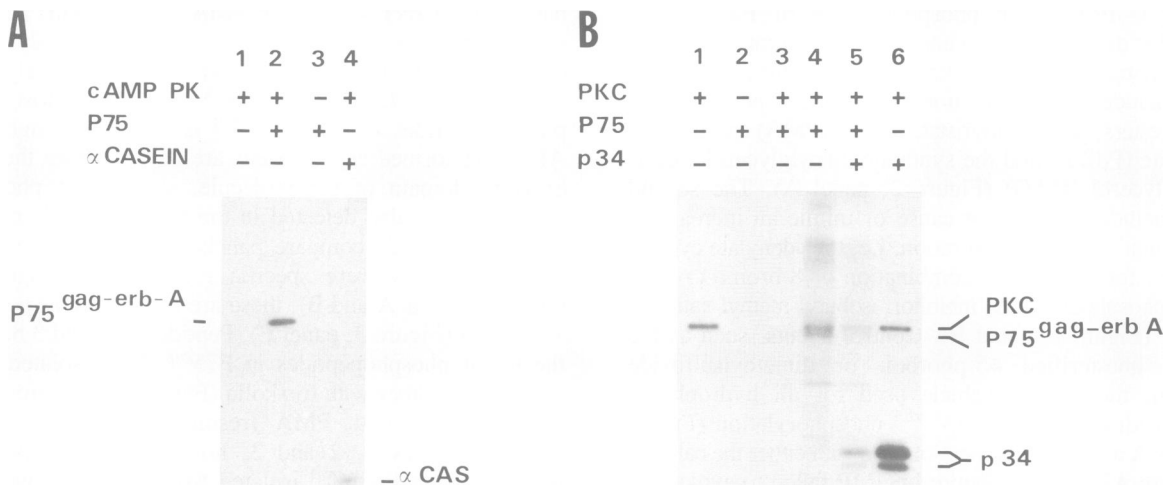

Fig. 4. *In vitro* phosphorylation of $P75^{gag-v\text{-}erbA}$. Immunoprecipitated $P75^{gag-v\text{-}erbA}$ (~500 ng) was used as a substrate for the catalytic subunit of cAMP-dependent protein kinase or purified PKC; reactions were carried out for 10 min at 30°C in the presence of [γ-^{32}P]ATP (see Materials and methods). Reactions were stopped by boiling in gel loading buffer and analysed on a 10% polyacrylamide slab gel followed by autoradiography. **Panel A. Lane 1**: purified PKA catalytic subunit incubated with no substrate. **Lane 2**: $P75^{gag-v\text{-}erbA}$ incubated with purified PKA catalytic subunit. **Lane 3**: a $P75^{gag-v\text{-}erbA}$ immunoprecipitate identical to that of lane 2 was incubated with no added enzyme. **Lane 4**: same as lane 2, except that $P75^{gag-v\text{-}erbA}$ was replaced with 1 μg of α-casein. Exposure time of the autoradiogram was 20 min. **Panel B. Lane 1**: purified PKC incubated with no substrate; **lane 2**: $P75^{gag-v\text{-}erbA}$ immunoprecipitate incubated with no added enzyme; **lane 3**: $P75^{gag-v\text{-}erbA}$ incubated with purified PKC in the absence of phosphatidylserine and Ca^{2+}; **lane 4**: $P75^{gag-v\text{-}erbA}$ incubated with purified PKC in the presence of phosphatidylserine and Ca^{2+}; **lane 5**, same as lane 4 except that 0.5 μg of chicken p34 calpactin I/lipocortin II was included as a PKC standard substrate (Gould *et al.*, 1986); **lane 6**: same as lane 5 except for the absence of $P75^{gag-v\text{-}erbA}$. Exposure time of the autoradiogram was 18 h.

precipitated $P75^{gag-v\text{-}erbA}$ was incubated with either the catalytic subunit of PKA or purified PKC, in assay conditions proper for each enzyme. As shown in Figure 4, panel A, $P75^{gag-v\text{-}erbA}$ is readily phosphorylated by PKA in these conditions. Two-dimensional tryptic phosphopeptide analysis showed that *in vitro* phosphorylation of $P75^{gag-v\text{-}erbA}$ by PKA occurred in the same three tryptic peptides as those phosphorylated in $F45^{v\text{-}erbA}$ *in vivo* (Figure 5, compare panels A and B, and mixing experiment in panel D). Since PKA phosphorylates physiological substrates at serine/threonine residues preceded by basic residues (Krebs and Beavo, 1979; Feramisco *et al.*, 1980), we next searched the v-*erbA* sequence for potential PKA phosphorylation sites. A unique locus consisting of two contiguous serine residues, Ser-16 Ser-17 (Figure 6, v-*erbA* sequence), appeared a plausible candidate because (i) these residues are part of a sequence (Arg-Lys-Arg-Lys-Ser-Ser) that conforms to consensus PKA phosphorylation sites, and (ii) consistent with the observed phosphopeptide pattern (Figure 5), phosphorylation of Ser-16 and/or Ser-17 would generate a nested set of phosphopeptides with graded basicity, due to the inability of trypsin to act as an endopeptidase. To verify this hypothesis, a 23-residue peptide was chemically synthesized, corresponding to that region of $P75^{gag-v\text{-}erbA}$ (Figure 6, v-*erbA* sequence). This peptide proved to be a good substrate for PKA *in vitro* (K_m = 25 μM; V_{max} = 1.1 μmol/min/mg). These kinetic parameters are similar to those of optimalized peptide substrates of PKA (Feramisco *et al.*, 1980). Two-dimensional analysis of a tryptic digest of this phosphorylated synthetic peptide yielded three peptides indistinguishable from those seen in *in vitro* phosphorylated $P75^{gag-v\text{-}erbA}$ and *in vivo* phosphorylated $F45^{v\text{-}erbA}$ (Figure 5, compare panels A, B and C, and mixing experiment in panel D). We conclude from these experiments that phosphorylation of the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$,

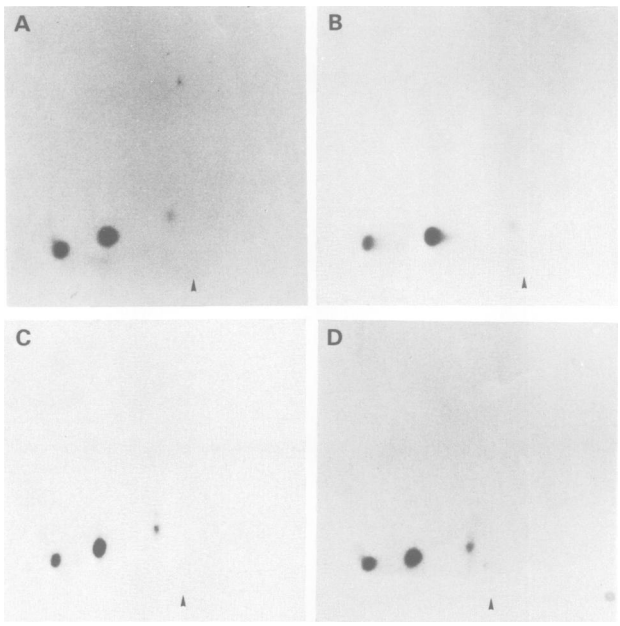

Fig. 5 .Two-dimensional tryptic phosphopeptide analysis of $P75^{gag-v\text{-}erbA}$ and of synthetic peptide phosphorylated *in vitro*. Trypsin digestion and two-dimensional peptide analysis were as in Figure 3. **Panel A**: $P75^{gag-v\text{-}erbA}$ phosphorylated by PKA *in vitro*. **Panel B**: $F45^{v\text{-}erbA}$ isolated from [^{32}P]orthophosphate-labelled cells treated with forskolin. **Panel C**: synthetic peptide (shown in Figure 6 v-*erbA* sequence) phosphorylated by PKA *in vitro*. **Panel D**: mixture of the samples run in panels B and C.

both in basal and stimulated conditions, occurs in a unique region of the molecule, defined by the limit tryptic peptide ^{16}SSQCLVK. Whether phosphorylation in basal or stimulated conditions involves Ser-16 and/or Ser-17 cannot be distinguished by these analyses.

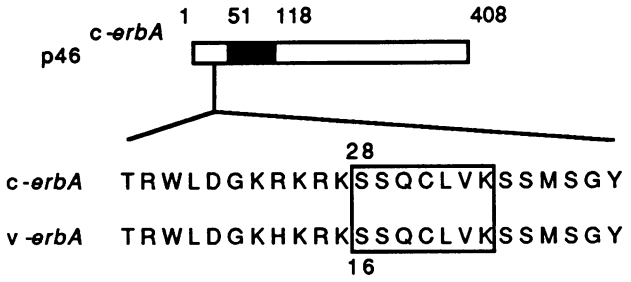

Fig. 6. Location of the limit tryptic phosphopeptide common to $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$. Shown are the portion of the v-*erbA*-encoded sequence that was chemically synthesized, and the homologous portion of the c-*erbA*-encoded sequence (Sap *et al.*, 1986). Boxed sequence: limit tryptic phosphopeptide common to $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$. The position of the first serine residue contained in the limit peptide is indicated in both the c-*erbA* and v-*erbA* sequences. Black box: putative DNA-binding domain in $p46^{c\text{-}erbA}$.

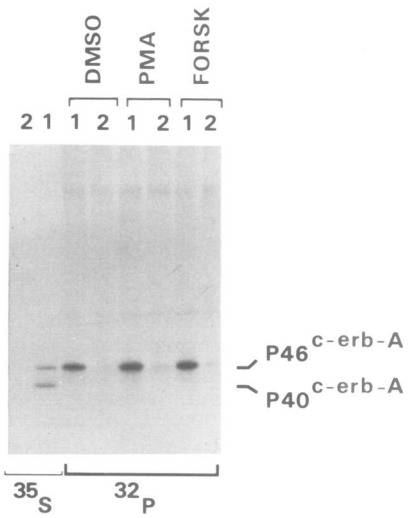

Fig. 7. Phosphorylation of $p46^{c\text{-}erbA}$. 10^6 COS-1 cells (in one 100-mm Petri dish) were transfected with the PKCR3-based c-*erbA* expression vector and 2 days later cells were divided into four dishes. The third day, the cells of one dish were labelled for 60 min with 250 μCi/ml L-[^{35}S]methionine and the three other dishes were labelled for 4 h with 1 mCi/ml [^{32}P]orthophosphate. The [^{32}P]orthophosphate-labelled cells were treated for 30 min with the solvent vehicle DMSO or PMA (40 nM) or forskolin (10 μM). Cells were lysed and immunoprecipitation was carried out on 10^7 acid-precipitable radioactive counts of each lysate. **Lane 1**: immunoprecipitation with serum C. **Lane 2**: immunoprecipitation with serum C blocked by an excess of the bacterial immunogen. Immunoprecipitates were analysed on a 10% polyacrylamide slab gel. The gel was dried, fixed and exposed for 24 h at −70°C with an intensifying screen using Kodak X-AR5 film.

As shown in Figure 4, panel B, purified PKC was found to phosphorylate $P75^{gag-v\text{-}erbA}$ in a Ca^{2+}- and phospholipid-dependent manner, but at a level of only a few percent of that observed with PKA. Part of the low level of phosphorylation by PKC appears to result from a partial inhibition of the enzyme in the presence of *Staphylococcus aureus* immunoadsorbent as shown by the use of chicken 34−36 kd calpactin I/lipocortin II (p34; Figure 4, panel B, compare lanes 5 and 6), a physiological substrate of PKC (Gould *et al.*, 1986). However, comparison of the phosphorylation level of $P75^{gag-v\text{-}erbA}$ as compared to that of p34 or to PKC autophosphorylation (Figure 4, panel B, lane 5), together with the fact that phosphorylation of the 24-residue synthetic peptide by PKC was only observed at peptide concentrations $< 2 \times 10^{-5}$ M, probably reflect the intrinsic inability of Ser-16 and/or Ser-17 to be efficiently phosphorylated by PKC *in vitro*. It remains to be seen whether this *in vitro* situation really reflects the *in vivo* conditions.

Phosphorylation of the $p46^{c\text{-}erbA}$ thyroid hormone receptor

The sequence of the phosphorylation site identified in the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$ is highly conserved in the chicken $p46^{c\text{-}erbA}$ protein (Figure 6). We therefore asked whether this site was also phosphorylated in $p46^{c\text{-}erbA}$. As a source of $p46^{c\text{-}erbA}$, we used either chicken embryo fibroblasts infected with a replication-competent retroviral vector containing a chicken c-*erbA* cDNA insert (Sap *et al.*, 1986), or simian COS-1 cells transfected with a recombinant pKCR3 plasmid containing the same cDNA insert. Both of these expression systems yielded identical results. Cells were labelled with either [^{32}P]orthophosphate or [^{35}S]methionine as a control. Immunoprecipitation of the [^{35}S]methionine-labelled cell proteins with the anti-*erbA* serum demonstrated expression of the two previously described c-*erbA* products, $p46^{c\text{-}erbA}$ and $p40^{c\text{-}erbA}$ (Figure 7). Only $p46^{c\text{-}erbA}$ was found to be labelled with [^{32}P]orthophosphate, whereas $p40^{c\text{-}erbA}$ was not. Similar to the results obtained with $P75^{gag-v\text{-}erbA}$, phosphorylation of $p46^{c\text{-}erbA}$ was enhanced 2.5-fold after treatment of the cells with either PMA or forskolin (Figure 7). In both untreated and forskolin-treated cells, phosphorylation of $p46^{c\text{-}erbA}$ exclusively occurs on serine residues (Figure 3, panels F and G). Two-dimensional analysis of the tryptic phosphopeptides of $p46^{c\text{-}erbA}$ isolated from untreated cells is shown in Figure 8 (panel A). In addition to trace amounts of three peptides (peptides 1, 2, 3 in Figure 8, panel F) co-migrating with those found in the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$ (Figure 8, panel D), a major phosphopeptide (peptide 4 in Figure 8, panel F) unique to $p46^{c\text{-}erbA}$ was also detected (Figure 8, compare panels A and D, and mixing experiment in panel E). Treatment of cells with either forskolin or PMA resulted in a similar increase in phosphopeptides 1−3 as had been observed in $P75^{gag-v\text{-}erbA}$ (Figure 8, compare panels A and B,C). We conclude that at least two distinct sites are phosphorylated in the chicken c-*erbA*-encoded thyroid hormone receptor. One site is defined by the limit tryptic peptide ^{28}SSQCLVK; this limit peptide is shared with $P75^{gag-v\text{-}erbA}$. The phosphorylation of this site is strongly enhanced following activation of PKC or PKA. The other site is absent in $P75^{gag-v\text{-}erbA}$; its phosphorylation level is unaffected by a phorbol ester-induced stimulation of PKC activity or by an increase in intracellular cAMP concentration. The location of this second site has not been further investigated here.

Discussion

Three main conclusions can be drawn from the present study. First, at least two phosphorylation sites, defined by two limit phosphopeptides, can be identified in the avian c-*erbA*-encoded thyroid hormone receptor. Second, only the site defined by the limit phosphopeptide SSQCLVK is retained in $P75^{gag-v\text{-}erbA}$, whereas the other site is not. Third, *in vivo*, phosphorylation of this common site is enhanced

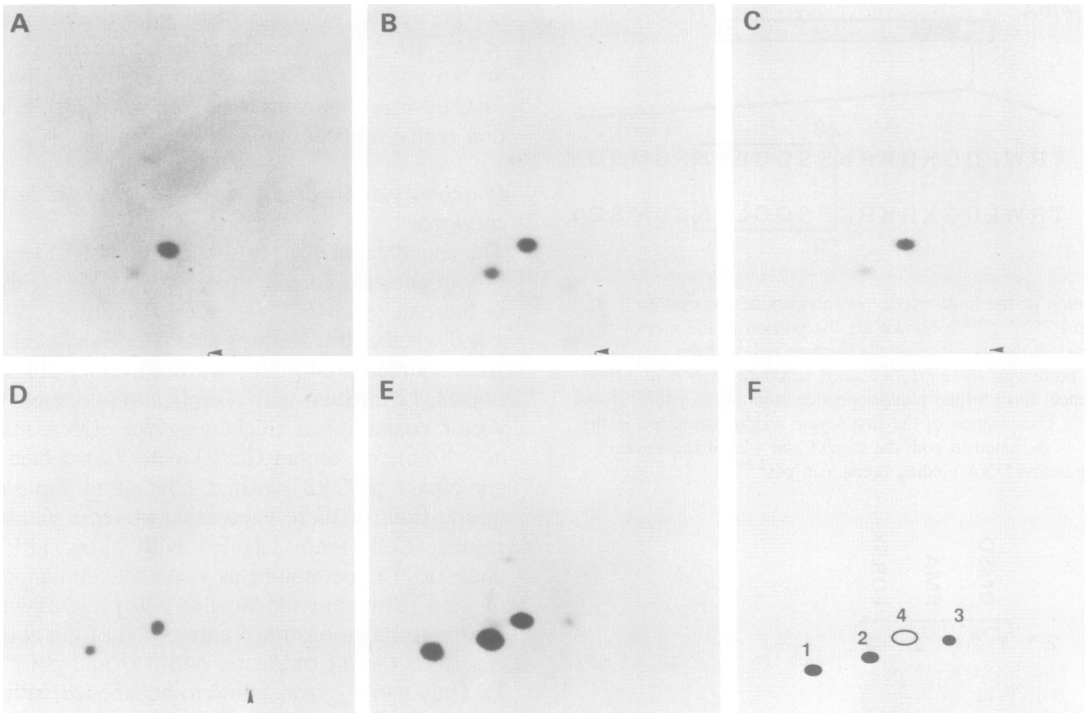

Fig. 8. Two-dimensional tryptic phosphopeptide analysis of $p46^{c\text{-}erbA}$ in unstimulated and stimulated conditions. [^{32}P]Orthophosphate-labelled $p46^{c\text{-}erbA}$ was excised from gels similar to that of Figure 7, eluted, oxidized, digested and analysed as in Figure 3. **Panel A**: $p46^{c\text{-}erbA}$ from untreated cells. **Panel B**: $p46^{c\text{-}erbA}$ from forskolin-treated cells. **Panel C**: $p46^{c\text{-}erbA}$ from PMA-treated cells. **Panel D**: $F45^{v\text{-}erbA}$ from AEV-transformed erythroblasts treated with forskolin. **Panel E**: mixture of the samples used in panels B and D. **Panel F**: schematic drawing of panel E; solid spots (peptides 1, 2, 3) represent peptides common to $p46^{c\text{-}erbA}$ and $F45^{v\text{-}erbA}$ and open spot (peptide 4) represents the peptide unique to $p46^{c\text{-}erbA}$.

10-fold following activation of either PKA or PKC; in contrast, in these conditions, no variation is seen in the phosphorylation of the other, as yet uncharacterized site.

The limit peptide common to $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$, SSQCLVK, contains two contiguous serine residues, corresponding respectively to Ser-28 and Ser-29 in the $p46^{c\text{-}erbA}$ sequence and to Ser-16 and Ser-17 in the sequence of the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$ (Figure 6). Either or both of these two serine residues could be the target of basal, forskolin-stimulated and PMA-stimulated phosphorylation. Discriminating between the two residues will require the development of site-specific *erbA* mutants exhibiting conversion of either serine or an unphosphorylatable residue.

It is intriguing that *erbA* protein phosphorylation is increased following activation of PKC and PKA. These kinases are involved in complex biochemical pathways that transduce growth and differentiation signals received at the membrane to the transcriptional machinery (Rozengurt, 1986). The fact that both PKC and PKA enhance phosphorylation of the same tryptic peptide, in both $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$, suggests that phosphorylation of this region is important in the control of *erbA* protein function. Consistent with this hypothesis, we have obtained evidence that, in the v-*erbA*-encoded domain of $P75^{gag-v\text{-}erbA}$, conversion of both Ser-16 and Ser-17 into alanine by site-directed mutagenesis suppresses phosphorylation of the domain and impairs the differentiation-inhibiting activity of the protein (C.Glineur, H.Beug and J.Ghysdael, preliminary results). It is tempting to speculate that full activity of the c-*erbA*-encoded T_3 receptor may require PKC- or PKA-activating hormonal signals, in addition to thyroid hormone itself. It has recently been proposed that the c-*erbA*-encoded T_3 receptor may play a regulatory role in terminal erythroid differentiation (Hentzen *et al.*, 1987), a process dependent on several soluble factors, including the growth factor erythropoietin (Kowenz *et al.*, 1987). Binding of these factors to their membrane receptors might stimulate, via the PKC or PKA pathways (Nishizuka, 1986; Rozengurt, 1986), the phosphorylation of the c-*erbA*-encoded T_3 receptor, and thereby control its activity.

Data obained in other systems suggests that phosphorylation of *trans*-acting nuclear factors may play an important role in controlling their activity, notably in response to membrane signals. Transcriptional activation of certain genes following stimulation of PKC, PKA and other kinases has been shown to depend on specific nuclear proteins binding to *cis*-acting DNA elements (Sen and Baltimore, 1986; Angel *et al.*, 1987; Montminy and Bilezikjian, 1987). It has been postulated that, in several cases, phosphorylation is the key event regulating the activity of these nuclear proteins (Sen and Baltimore, 1986). The necessity of PKA catalytic activity for cAMP-dependent transcriptional activation was recently demonstrated in the case of the cAMP-responsive human enkephalin gene promoter (Grove *et al.*, 1987) and cAMP-dependent phosphorylation was directly observed in the nuclear factor that binds to the somatostatin gene cAMP-responsive element (Montminy and Bilezikjian, 1987). Interestingly, a number of genes are transcriptionally responsive to both PKA and PKC, and in at least one of these genes transcriptional activation was recently shown to be mediated by a single nuclear protein, the AP-2 nuclear factor (Imagawa *et al.*, 1987). Our data raise the possibility

that the c-*erbA*-encoded T_3 receptor may mediate some of the epigenetic effects resulting from stimulation of PKA and PKC.

Steroid hormone receptors, whose structural and functional properties are closely related to those of thyroid hormone receptors (Weinberger *et al.*, 1985; Green *et al.*, 1986), also appear to be phosphorylated. The role of these phosphorylation events has not been fully clarified. Phosphorylation was reported to correlate with hormone-binding capacity of the oestrogen receptor (Miggliaccio *et al.*, 1984) and with hormone-induced transformation of the progesterone receptor to a high nuclear affinity state (Logeat *et al.*, 1984). In the avian c-*erbA*-encoded T_3 receptor, forskolin- or PMA-induced phosphorylation affect neither hormone-binding capacity nor hormone affinity, as shown by binding studies using ^{125}I-labelled T_3, and by the observation that the unphosphorylatable $p40^{c\text{-}erbA}$ can be covalently labelled with hormone *in vivo* (unpublished data). Conversely, hormone binding causes no change in $p46^{c\text{-}erbA}$ phosphorylation, since culturing cells for 48 h in T_3-depleted serum (Samuels *et al.*, 1979), or for 24 h in serum-free medium, affected neither basal phosphorylation nor forskolin- or PMA-stimulated phosphorylation (unpublished data).

The phosphorylation site common to $p46^{c\text{-}erbA}$ and $P75^{gag-v\text{-}erbA}$ is located in the amino-terminal domain of the molecules, 21 amino acid residues upstream of the DNA-binding domain (Figure 6). Phosphorylation of this site may conceivably influence affinity of the molecules for specific DNA sequence elements. Proper testing of this hypothesis will require the identification of specific target genes, the transcription of which is regulated by $p46^{c\text{-}erbA}$ and/or $P75^{gag-v\text{-}erbA}$. Phosphorylation does not seem to affect general DNA affinity, since forskolin-stimulated phosphorylation caused no gross change in the DNA-binding ability of $p46^{c\text{-}erbA}$, as probed by the retention of immunoprecipitable $p46^{c\text{-}erbA}$ on a DNA–cellulose column (our unpublished data). There is no apparent relationship either between phosphorylation and nuclear localization, since in AEV-transformed erythroblasts, the lower amount of $P75^{gag-v\text{-}erbA}$ found in the cytoplasm exhibits the same v-*erbA* versus *gag* phosphorylation ratio as does the nuclear $P75^{gag-v\text{-}erbA}$, whether in unstimulated or in stimulated conditions (unpublished results).

The variability of the amino-terminal domain is a noticeable feature of the T_3 receptor family (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Thompson *et al.*, 1987; Benbrook and Pfahl, 1987). No site homologous to Ser-28 and/or Ser-29 of $p46^{c\text{-}erbA}$ is found in the other members of the family sequenced to date (Weinberger *et al.*, 1986; Thompson *et al.*, 1987; Benbrook and Pfahl, 1987). In a human protein related to the hTRα of Benbrook and Pfhal (1987), phosphorylation does exist but is not modified by PKA or PKC activation (unpublished observations). The diversity of the amino-terminal domains of different T_3 receptors may contribute to target gene specificity; while recognition of receptor-specific target DNA sequences is probably mediated by the DNA-binding domain (Green and Chambon, 1987), the amino-terminal domain may be involved in transcriptional activation (Giguère *et al.*, 1986; Gronemeyer *et al.*, 1987), possibly through protein–protein interactions with nuclear factors which may be different for different receptor species. Phosphorylation of the amino-terminal domain may regulate this kind of specific protein–protein interaction. Phosphorylation-defective *erbA* protein mutants should help discern better the possible role of phosphorylation in *erbA* protein function.

Finally, it is at present unknown what protein kinases are directly responsible for phosphorylation of $p46^{c\text{-}erbA}$ and the v-*erbA* domain of $P75^{gag-v\text{-}erbA}$. Purified PKA was found to be able to efficiently phosphorylate the SSQCLVK region of $P75^{gag-v\text{-}erbA}$ and $p46^{c\text{-}erbA}$ *in vitro*. Since activation of PKA *in vivo* is accompanied by translocation of the catalytic subunit into the nucleus (Nigg *et al.*, 1985), it is conceivable that PKA could directly phosphorylate $P75^{gag-v\text{-}erbA}$ and $p46^{c\text{-}erbA}$ *in vivo*. In our experimental conditions, we found the SSQCLVK region of *erbA* molecules to be inefficiently phosphorylated by purified PKC *in vitro*. Additional experiments using *erbA* molecules as substrate in a soluble form and the use of specific inhibitors of endogenous PKC are required to definitively assess whether these molecules are direct substrates of PKC in cells. Alternatively, the increased phosphorylation of *erbA* molecules after PKC stimulation may result from a PKC-initiated cascade of phosphorylation events.

Materials and methods

Cells and viruses

The AEV (ES4)-transformed erythroblast line 6C2 (a gift from Dr T.Graf, EMBL, Heidelberg) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Gibco). AEV (ES4)-transformed chicken embryo fibroblasts and normal chicken embryo fibroblasts infected with a c-*erbA*-expressing retroviral vector (Sap *et al.*, 1986) were grown in the same medium. Simian COS-1 cells (Gluzman, 1981) were grown in DMEM supplemented with 5% FCS.

Construction of bacterial erbA expression vectors

Two fragments of the v-*erbA* gene and one fragment of the c-*erbA*-related human gene were inserted into the pPLc24 expression vector (Remaut *et al.*, 1981). Cloning steps were performed according to standard procedures (Maniatis *et al.*, 1982), using the lysogenic strain *Escherichia coli* L392 (λ).

Construction of pPLc24 veA1. Plasmid pPLc24ex1 (Ferré *et al.*, 1987) is a derivative of pPLc24. pPLc24ex1 was cleaved with *Cla*I (near the 3′ end of the ex1 insert) and the extremities were filled using the Klenow fragment of DNA polymerase I. The plasmid was then cleaved with *Xho*I. Plasmid pAE11 (Vennström *et al.*, 1980), a pBR313 derivative which contains a complete, biologically active AEV (ES4) genome, was digested with *Sal*I, the extremities were filled using the Klenow enzyme, and the plasmid was next cleaved with *Xho*I. The 1.3 kb *Xho*I–*Sal*I fragment, which includes the v-*erbA* sequence 5′ to *Sal*I, was purified and inserted between the *Xho*I site and the filled-in *Cla*I site of pPLc24ex1. The resulting plasmid was cleaved with *Xho*I and *Bcl*I, leaving in the plasmid the *Bcl*I–*Sal*I segment of v-*erbA*; the extremities were filled using the Klenow enzyme, and the plasmid was religated, yielding pPLc24veA1.

Construction of pPLc24veA2. Plasmid pPLc24ex1 was cleaved with *Xho*I and *Hpa*I. Plasmid pAE11 was digested with *Sal*I and *Stu*I. The 0.8 kb *Sal*I–*Stu*I fragment, which includes all of the v-*erbA* sequence 3′ to *Sal*I, was purified and inserted between the *Xho*I and *Hpa*I sites of pPLc24ex1, yielding pPLc24veA2.

Construction of pPLc24heA1. Plasmid pPLc24 was cleaved with *Bam*HI, the extremities were filled using Klenow enzyme, and the plasmid was next digested with *Hin*dIII. Plasmid pKH47heA (a gift from Dr S.Saule) contains a human genomic insert which encodes a sequence highly similar to that comprised between Asp-229 and Ser-325 of $p46^{c\text{-}erbA}$. This insert was excised with *Dpn*I and *Hin*dIII, and inserted between the filled-in *Bam*HI site and the *Hin*dIII site of pPLc24, yielding pPLc24heA1.

Mass production of erbA polypeptides and immunization of rabbits

Plasmids pPLc24veA1, pPLc24veA2 and pPLc24heA1 were transfected into *Escherichia coli* K12ΔH1ΔTrp cells (Bernard *et al.*, 1979). Expres-

sion and purification of the three corresponding proteins (called respectively bp22$^{MS2\text{-}erbA}$, bp31$^{MS2\text{-}erbA}$ and bp18$^{MS2\text{-}erbA}$) were carried out as previously described (Ghysdael *et al.*, 1986a). Rabbits were immunized against the purified proteins, as described (Ghysdael *et al.*, 1986a). Sera A, B and C are raised against bp22$^{MS2\text{-}erbA}$, bp31$^{MS2\text{-}erbA}$ and bp18$^{MS2\text{-}erbA}$ respectively.

Construction of a c-erbA-expressing pKCR3 vector

The c-*erbA* cDNA insert was excised from the pF1Δ vector (Sap *et al.*, 1986) by *Eco*RI digestion, and inserted at the *Eco*RI site of the SV40-derived pKCR3 vector (Breathnach and Harris, 1983).

Transfection of COS-1 cells

Monkey COS-1 cells were plated at 10^6 cells per 100-mm Petri dish, 24 h before transfections. Cells were transfected using the DEAE dextran method with 10 μg plasmid DNA as described by Gorman (1985). Following DEAE−dextran/DNA treatment, chloroquine was added for 2−3 h to increase efficiency of transfection (Luthman and Magnusson, 1983). If needed, cells were divided 2 days after transfection. Metabolic labelling was performed 3 days after transfection.

Cell labelling and immunoprecipitation procedures

For L-[^{35}S]methionine labelling, cells were rinsed with methionine-free DMEM (Gibco) supplemented with 5% dialysed FCS, then incubated in the same medium with L-[^{35}S]methionine, as indicated in the figure legends.

For [^{32}P]orthophosphate labelling, cells were incubated for 2−4 h in phosphate-free DMEM (Gibco) supplemented with 5% dialysed FCS, then incubated for 4−20 h in the same medium with [^{32}P]orthophosphate, as indicated in the figure legends. When [^{32}P]orthophosphate-labelled proteins were prepared for tryptic peptide mapping analysis, the radioactive concentration was raised to 2 mCi/ml. At the end of the labelling period, cells were rinsed in ice-cold phosphate-buffered saline, and lysed in 50 mM Tris−HCl (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 10 mM sodium fluoride, 40 mM *p*-nitrophenylphosphate, 1% aprotinin (Sigma), 0.1 mg/ml PMSF and 1 μg/ml leupeptin (Sigma). The lysates were centrifuged at 100 000 *g* for 60 min and immunoprecipitations were carried out as described previously (Ghysdael *et al.*, 1979), except that 10 mM sodium fluoride was included in all buffers. Immunoprecipitates were analysed on 10% polyacrylamide slab gels (acrylamide:bisacrylamide 30:0.4) in the presence of 0.1% SDS. Gels were fixed in methanol:water:acetic acid (5:5:1), dried and autoradiographed.

Digestion with p15^{gag}

Preparation of crude virionic p15^{gag} and digestion of P75$^{gag-v\text{-}erbA}$ immunoprecipitates were carried out as described by Beug *et al.* (1980).

Peptide synthesis

The peptide TRWLDGKHKRKSSQCLVKSSMSGY was synthesized according to Merrifield *et al.* (1982), using Boc tyrosine (*O*-dichlorobenzyl) bound to a 1% cross-linked styrene-divinyl benzene resin. *N*-α-Boc protection was used and trifunctional amino acids were protected as follows: Asp (*O*-cyclohexyl), Ser (Bzl), Thr (Bzl), Lys (Cl-Z), Arg (Tos), His (Tos), Cys (acetamidomethyl), Trp (unprotected). After methionine incorporation a 2% solution of 1,2-dimercaptoethane was added during the Boc deprotection steps. Coupling of amino acids was performed as their hydroxybenzotriazole esters, using a 2.5-fold excess. After incorporation of *N*-α-Boc His (Tos), coupling was performed by pre-formed symmetrical anhydride, using amino acids and dicyclohexylcarbodiimide in a 2:1 ratio. After cleavage and deprotection by anhydrous hydrogen fluoride, the crude peptide was extracted in 5% acetic acid and purified by gel filtration on TSK W40 superfine (Merck). Purity of the peptide was checked by TLC, gradient reversed phase HPLC and amino acid analysis.

Peptide mapping

[^{32}P]Orthophosphate-labelled proteins were eluted from gel slices, precipitated, oxidized, and digested to completion with L-1 tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. The resulting tryptic peptides were analysed as described previously (Ghysdael *et al.*, 1986b) except that separation of the phosphopeptides in the first dimension was run for 30 min at 1000 V at pH 2.2 in water:formic acid:acetic acid (90:2:8). Plates were exposed to Kodak X-AR5 film at −70°C with an intensifying screen.

Phosphorylation of P75$^{gag-v\text{-}erbA}$ and synthetic peptide

AEV-transformed erythroblasts were lysed as described before, and aliquots of lysate equivalent to 10^6 cells were immunoprecipitated with serum B. The protein A−Sepharose or *S.aureus* cell pellets containing immunoprecipitated P75$^{gag-v\text{-}erbA}$ were washed as for standard immunoprecipitation assay and used as a substrate for either the catalytic subunit of cAMP-dependent protein kinase (Sigma) in 10 mM Tris−HCl (pH 7.2), 6.25 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP (sp. act. 1.5 Ci/mmol), and 2 μg/ml catalytic subunit; or protein kinase C (PKC, kindly provided by Dr E. Chambaz) in 10 mM Tris−HCl (pH 7.2), 6.25 mM $MgCl_2$, 0.625 mM $CaCl_2$, 25 μM [γ-^{32}P]ATP (sp. act. 1.5 Ci/mmol), 0.5 mg/ml phosphatidylserine and 400 ng/ml purified PKC. Reactions were carried out for 10−30 min at 30°C. Synthetic peptide phosphorylation by PKA was carried out in similar conditions except for the use of 250 μM [γ-^{32}P]ATP and 10 μg/ml PKA. The reactions were stopped by boiling in gel loading buffer, and analysed on a 10% polyacrylamide slab gel. In control reactions that did not involve P75$^{gag-v\text{-}erbA}$ immunoprecipitates, unreacted protein A−Sepharose was included to minimize difference in incubation conditions.

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