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Conserved mechanisms of Ras regulation of evolutionary related transcription factors, Ets1 and Pointed P2

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Cell transformation by the Ras oncogene is mediated by members of the ets gene family. To analyse the mechanisms of regulation, we have studied activation of several ets factors by Ras expression. We show that expression of Ha-Ras strongly activates the Ets1 p68 and p54 isoforms and Ets2 in F9 EC cells. We have mapped the Ras responsive elements of Ets1 p68 to two domains, RI + II and RIII. Mutation of threonine 82 to alanine in RI + II abolishes both Ras activation and phosphorylation by MAP kinase. Threonine 82 is part of a sequence that is conserved in Drosophila Pointed P2, an ets protein that has been shown both genetically and biochemically to mediate Ras signalling in Drosophila cells. We extend the comparison of these evolutionary related proteins by showing that Pointed P2 is activated by Ras in mammalian cells and mutation of the homologous threonine abolishes activation. Furthermore, we show that Pointed P2 resembles Ets1, in that it has conserved sequences in a similar position adjacent to the ets DNA binding domain that negatively auto-regulates DNA binding. These results go towards showing that the Drosophila Pointed and vertebrate Ets1 are evolutionary related proteins that have remarkably conserved Ras regulatory mechanisms downstream from MAP kinase.

Keywords: MAP kinase; auto-inhibition; DNA-binding; Drosophila; vertebrates

Introduction

The ras genes were the first activated oncogenes found to be associated with many types of cancers. They have since been shown to be key regulators of mitogenesis and development pathways (Kiaris and Spandidos, 1995). Ras controls several signalling pathways. The best established downstream effector is Raf kinase, which initiates a series of phosphorylation through the MEK and MAPK kinases, leading to the modification of transcription factors (Karin and Hunter, 1995; Cobb and Goldsmith, 1995). Another downstream effector is MEKK1, which is at the beginning of the JNKK/SEK/MKK4 and JNK/SAPK cascade. There is at least one more similar cascade of kinases (Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995). However, there are many other potential downstream targets, including PI(3)K, p120GAP, raIGDS, PKCξ and KSR, although their precise roles still need to be established (Marshall, 1995; Downward, 1995). The Ras/MAP kinase pathway has been studied by biochemical approaches in mammalian cells and genetic studies in Caenorhabditis, Drosophila and yeast. The emerging patterns indicate that certain transcription factor families, such as ets, are evolutionary conserved effectors of the pathways.

The ets gene family is composed of proteins with ets DNA binding domains (MacLeod *et al*., 1992; Seth *et al*., 1992; Janknecht and Nordheim, 1993; Wasylyk *et al*., 1993). The ets domain forms a winged helix–loop–helix (wHTH) structure. It resembles prokaryotic and eucaryotic factors, such as *E. coli* CAP, heat shock factor and HNF-3γ (Donaldson *et al*., 1994; Donaldson *et al*., 1996; Liang *et al*., 1994a), indicating that the roots of the ets gene family go very far back in evolution. However, the ets domain-DNA complex has distinct features, such as the way the HLH element is oriented relative to the major groove and the manner in which a tryptophan intercalates into the minor groove, resulting in a kink and widening of the DNA (Werner *et al*., 1995). The ets family has evolved broadly into subfamilies, that may have distinct physiological roles. Four subfamilies appear to be associated with Ras signaling.

Genetic studies in Caenorhabditis have shown that the ets gene product, Lin-1, acts after the Ras-Raf-MEK-MAPK cascade that mediates development of the vulva. Lin-1 is a negative regulator with multiple potential MAPK phosphorylation sites. The ets domain is most similar to the Elk/SAP1/Net subfamily of mammals, both in sequence and N-terminal position, but it lacks the other similarities outside the ets domain (Beitel *et al*., 1995). In Drosophila, the two ets proteins Pointed P2 and Yan have been shown genetically and biochemically to be targets of the Ras/rolled MAPK pathway (O'Neill *et al*., 1994). Alternative splicing produces two Pointed proteins, P1 and P2, with a common carboxy-terminal ets domain. Both Pointed P2 and Yan have consensus MAPK phosphorylation sites. In transfection assays in Drosphila cells P2 is a weak activator that is stimulated by expression of activated forms of Ras or rolled MAP kinase. Yan is a negative regulator of the constitutively active Pointed P1 isoform, and Ras down regulates Yan (O'Neill *et al*., 1994; Rebay and Rubin, 1995). P2 is phosphorylated *in vitro* by rolled MAP kinase on a single site (Brunner *et al*., 1994).

In mammalian cells the Ras responsive factors include the Elk1/SAP1/Net subfamily, ERF and Ets1/Ets2. The Elk1/SAP1/Net proteins have three related domains. 'A-C', with similar sequences and functions. The amino terminal 'A' or ets domain mediates DNA binding, 'B' interacts with SRF to form ternary

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complexes with the c-*fos* serum response element and 'C' is a Ras inducible activation domain that is phosphorylated by both MAP and SAP kinases (Hill and Treisman, 1995; Treisman, 1994; Janknecht *et al*., 1995; Karin and Hunter, 1995; Price *et al*., 1995; Ross *et al*., 1994; Gille *et al*., 1995; Cavigelli *et al*., 1995). ERF is a distinct factor with an N-terminal ets domain and a C-terminal repressor domain. It inhibits transcription and tumorigenesis, and is regulated by Ras, possibly through phosphorylation of the repressor domain by MAP kinase (Sgouras *et al*., 1995).

The Ets1/Ets2 subfamily proteins are regulated by Ras (Coffer *et al*., 1994; Bradford *et al*., 1995). Ets1 and Ets2 can be subdivided into 6 domains from the N-terminus: RI (or A), RII (or B or Pointed), RIII (or C), D, E and F (Gegonne *et al*., 1992; Schneikert *et al*., 1992; Wasylyk *et al*., 1992; Chumakov *et al*., 1993; Wasylyk and Wasylyk, 1993; Albagli *et al*., 1994; Hahn and Wasylyk, 1994). RI, RII and RIII are involved in transcription activation, D, E and F in DNA binding. In chicken, two products result from alternative splicing of the c-*ets*-1 locus, p68 and p54, that differ only in RI. In mammalian species only the p54 isoform is produced (Leprince *et al*., 1988; Queva *et al*., 1993). The p68 isoform and Ets2 appear to have evolved from a common progenitor, that also gave rise to Pointed P2 (Albagli *et al*., 1994). The RIs of p68 and Ets2 are activation domains with a common function but different sequences, whereas RI of p54 has no known function (Schneikert *et al*., 1992). The RIII regions are activation domains in both Ets1 and Ets2 (Schneikert *et al*., 1992; Gegonne *et al*., 1992; Chumakov *et al*., 1993). The RII regions resemble the 'Pointed' domain of Pointed P2, and are postulated to be both regulatory domains that control the activities of the surrounding activation domains (Schneikert *et al*., 1992) and protein-protein interaction domains (Seth and Papas, 1990). The Ets DNA binding domain or region E is surrounded by sequences that autoregulate DNA binding (Lim *et al*., 1992; Wasylyk *et al*., 1992; Hagman and Grosschedl, 1992; Fisher *et al*., 1994; Hahn and Wasylyk, 1994; Petersen *et al*., 1995).

We have previously shown that the Ras signalling cascade from Ras through Raf and MAP kinase targets Ets1 (Conrad *et al*., 1994; Bradford *et al*., 1995). We report here that the response is mediated by a threonine residue in sequences that are conserved in the Pointed domain of P2. Like its Drosophila homologue, the threonine is essential for Ras activation and is phosphorylated by MAP kinase. Furthermore, P2 has a domain whose sequence is similar to the auto-regulatory inhibitor of Ets1 and which also inhibits DNA binding. The structural and functional similarities between Ets1 and Pointed support the hypothesis that they evolved from a common progenitor, and show a remarkable conservation of regulation by the Ras pathway to the effector in the nucleus.

Results

Specific activation of Ets family members by Ras

The transcription properties of Ets proteins was investigated in transfection assays in F9 embryocarcinoma cells using a reporter containing 8 palindromic ets motifs from the stromelysin promoter upstream from the TK promoter (PALx8). The palindromic element has no effect on the TK promoter in F9 cells in the absence of introduced Ets proteins (results not shown) and is only slightly activated by the expression of different Ets members (Figure 1A, O, white bar). Strikingly, co-expression of activated Ha-Ras with some of the Ets proteins [chicken Ets1 p68 and p54 isoforms, human (Hu) Ets1 p54 and chicken Ets2] strongly activated the PALx8-TK promoter (black bars). Ras had little effect on basal activity due to endogenous factors (see O). Activation of Ets proteins was not an indirect effect due to transcription activation of the expression vector, as judged by semi-quantitative RT-PCR (data not shown). Two other Ets family members, PU.1 and Elf1, were not activated by co-expression of Ras (Figure 1A), even though they are efficiently expressed (data not shown). We examined whether Ets1 p68 alone could be activated by Ras expression in a more natural promoter-setting. The tissue specific rat prolactin promoter is inactive in HeLa cells (Figure 1B, O, white bar). Ets1 p68 expression activated the promoter, and Ras expression further increased activation by Ets1 p68 (Figure 1B). Our results show that Ras expression increases activation of both synthetic and natural promoters by Ets1 p68 in a number of cell types. Ras activation appears to be specific for some members of the Ets family.

Mapping of Ras responsive domains

We chose to map the Ras responsive elements of the p68 isoform of Ets1 because it is structurally and functionally more homologous than p54 to vertebrate Ets2 and Drosophila Pointed, and more directly related to EtsO, the putative precursor of the Ets1/Ets2 subfamily (Lautenberger *et al*., 1992; Laudet *et al*., 1993; Albagli *et al*., 1994). Ets1 p68 is composed of six functional domains (Figure 2) the two activation domains R1 and RIII that surround the 'regulatory' domain RII and the DNA binding domain (ETS) sandwiched between inhibitory domains (Hagman and Grosschedl, 1992; Wasylyk *et al*., 1992; Lim *et al*., 1992; Hahn and Wasylyk, 1994; Fisher *et al*., 1994; Petersen *et al*., 1995). Using progressive deletion mutants (Figure 2) we found that removal of the C-terminal domain that inhibits DNA binding (Hagman and Grosschedl, 1992; Lim *et al*., 1992; Hahn and Wasylyk, 1994) had no effect on fold activation by Ras (ΔB, ΔD, Figure 2), whereas further deletions, that encroach on the DNA binding domain and prevent DNA binding (Δ31, Δ32, (Wasylyk and Wasylyk, 1992; Wasylyk and Wasylyk, 1993)) abolished activation. Starting from the N-terminus, truncation of 56 amino acids did not affect the Ras stimulation (Δ51), further deletion to amino-acid 98 significantly decreased activation (Δ51), further deletion to amino-acid 98 significantly decreased activation (Δ52), three more mutations up to 218 did not decrease activation (Δ53 to Δ55) whereas further deletion reduced activation to low levels (Δ56, N4, Δ57 and N70). These results suggest that there are two Ras inducible domains, one in the RI + II region and a second in RIII.

We investigated Ras activation with fusion proteins between the heterologous DNA binding domain from

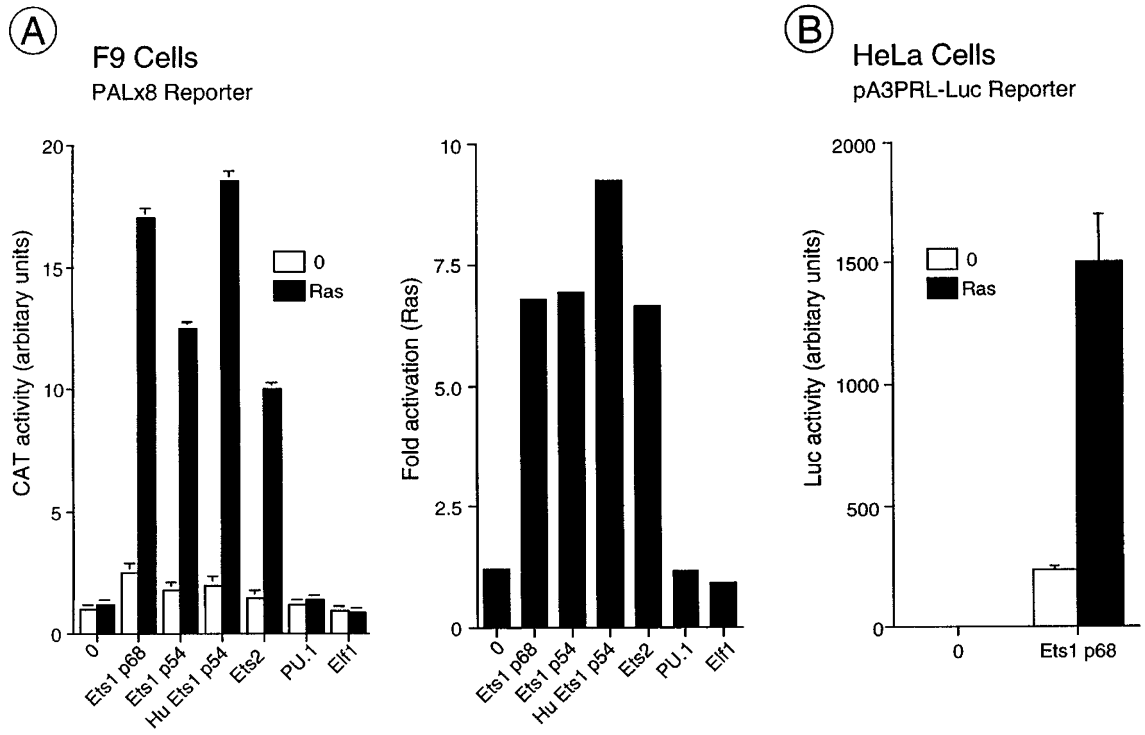


Figure 1 Activation of chicken Ets1 p68 and other Ets-factors by co-expressed Ras. (**A**). Increase in F9 cells of CAT activity from the PALx8 reporter by Ets factors in either the absence (white bars) or presence (black bars) of activated Ha-Ras. PALx8 contains eight tandem repeats of the stromelysin palindromic ets motif upstream from the TK promoter and CAT coding sequences of pBL-CAT4. The graph on the right shows the fold activation by Ras of the different Ets-factors. (**B**). Increase in HeLa cells of luciferase (Luc) activity from the pA3PRL-LUC reporter by Ets1 p68 in either the absence (white bars) or presence (black bars) of activated Ha-Ras. pA3PRL-LUC contains the rat prolactin promoter (−425 to +73) upstream from luciferase coding sequences. The results are the average of five experiments. Error bars indicate the standard deviation. The activity of the reporter in the presence of the empty ets expression vector (0 bars) is set to 1

Ga14 (amino acids 1–147) and various combinations of the RI, RII and RIII regions of Ets1 (p68). Using transfection assays in F9 cells and the UAS-TK-CAT reporter (Webster *et al*., 1988), we found that under basal conditions the complete N-terminal region, RI+II+III, stimulated transcription (G-RI+II+III, Figure 3), whereas the individual activation domains RI and RIII alone had very low activity [less than two-threefold, compare G-RI and G-RIII with GaI4 (1–147) and see (Schneikert *et al*., 1992)]. The regulatory domain RII was inactive on its own (G-RII), whereas combining it with RI or RIII led to a slight increase in the activity of the former (G-RI+II), and a decrease for the latter (G-RII+III). The fusion proteins were expressed at similar levels [data not shown and (Schneikert *et al*., 1992)]. Co-expression of Ras significantly increased the activities of G-RI+II, G-RIII and G-RI+II+III, whereas it had little effect on the other recombinants. A fusion protein containing Ga14 and the HSV VP16 activation domain was not stimulated by Ras expression, showing that the effects were specific (data not shown). These results show that there are two Ras responsive elements in Ets1 (p68), RI+II and RIII.

The role of evolutionary conserved sequences in Ras activation of RI+II

The progressive deletions from the N-terminus suggest that an imporant Ras responsive element is delimited by the mutants Δ51 and Δ52 (Figure 2). Subsequently, we internally deleted amino acids 57–93 of various proteins. This mutation decreased Ras activation of Ets1 by about 50% (compare Δ57–93 and Ets1 p68, Figure 5). The internal deletion decreased the basal activity of both G-RI+II+III (Figure 6A) and G-RI+II (Figure 6B), without effecting the stability of the proteins (Figure 6C, lanes 1, 4, 5 and 7). The deletion decreased Ras induction of G-RI+II+III (Figure 6A), and, strikingly, it abolished activation of G-RI+II (Figure 6B). These results suggest that amino-acids 57–93 are vital for Ras induction of the N-terminal responsive domain, RI+II.

A number of mechanisms could account for Ras activation of Ets1 p68, including direct phosphorylation by the MAP kinases on the Ras pathway. MAP kinase phosphorylation sites are loosely defined as serines and threonines surrounded by prolines (Clark-Lewis *et al*., 1991; Seger and Krebs, 1995; Karin, 1995). There are several candidate serines and threonines, in particular the PLLTPSS sequence in RII, the region that is similar to the Pointed domain of Drosophila Pointed P2. Pointed P2 is regulated by phosphorylation of the threonine in the PPLT*P sequence (O'Neill *et al*., 1994; Brunner *et al*., 1994), that lies in a similar position as PLLT*PSS in Ets1. However, there is another homology that could be a target for phosphorylation, the conserved BEC element (Albagli *et al*., 1994) with a PT sequence (Figure 4). We mutated these sequences in three proteins, Ets1, G-

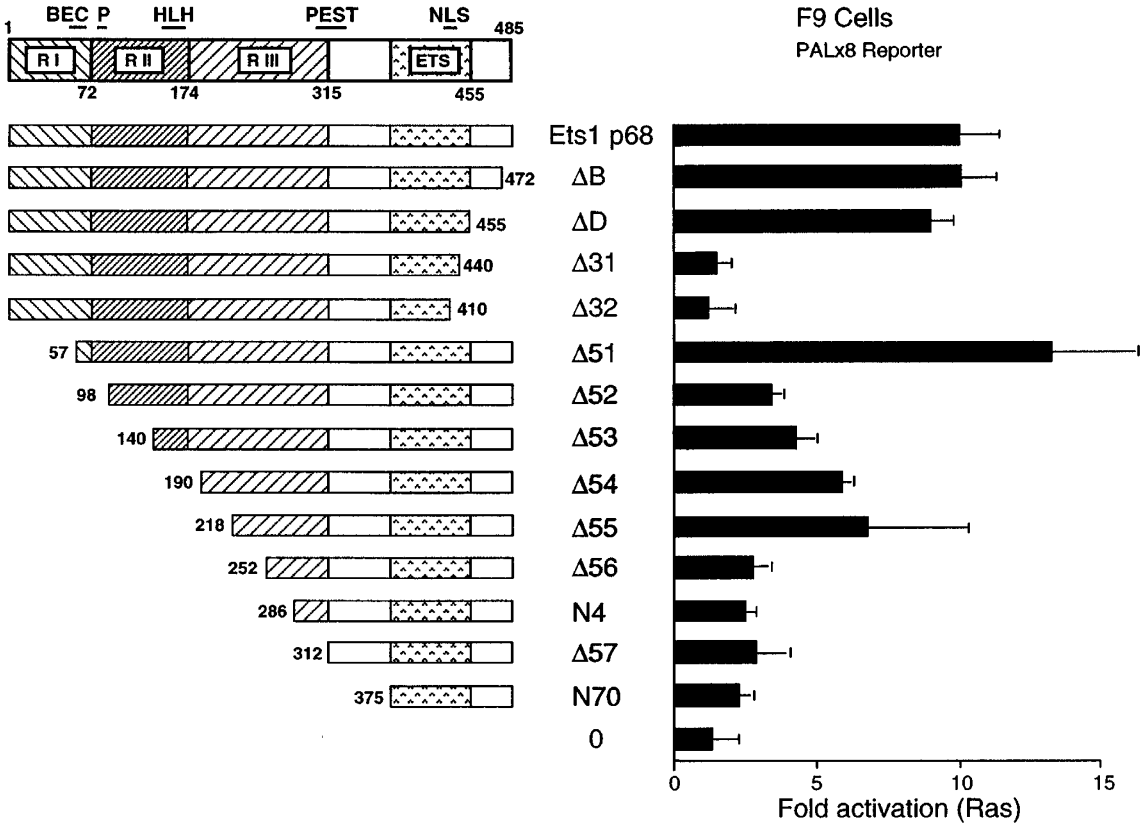


Figure 2 Localisation of the Ras responsive domain of Ets1 p68 using scanning mutants from the carboxy-terminus (3′ deletions) and the amino-terminus (5′ deletions). The graph shows the fold activation in F9 cells of the PALx8 reporter by the combination of activated Ras and Ets1 p68 deletion mutants. The data from three duplicate experiments were averaged and the error bars indicate the standard deviation. The fold activation of the reporter in presence of the empty ets expression vector (0) is set to 1. The features of the Ets1 p68 protein are illustrated, including: the 'RI', 'RII' and 'RIII' regions involved in transcription activation (Schneikert *et al*., 1992); the 'ETS' DNA binding domain (Wasylyk *et al*., 1992); the evolutionary conserved 'BEC' domain identified with the help of the gene structures of ets1, ets2 and pointed in different species (Albagli *et al*., 1992, 1994); the phylogenically conserved 'P' sequence related to the Pointed MAPK phosphorylation site (Brunner *et al*., 1994; O'Neill *et al*., 1994); the 'HLH' helix–loop–helix predicted structure (Seth and Papas, 1990); the 'PEST' region rich in proline, glutamic acid, serine and threonine that might affect protein stability (Seth and Papas, 1990) and the 'NLS' nuclear localisation signal (Boulukos *et al*., 1989)

RI+II+III and G-RI+II, by either deletion of the BEC sequences (Figure 4, amino acids 55 to 67, ΔBEC) or the Pointed homology (amino acids 77–85, ΔPointed), or by point mutation of threonine 82 to an alanine (T82A). In full length Ets1, the deletions and mutations reproducibly decreased Ras activation by between 25 and 50% (Figure 5), in agreement with the effect of the larger deletion [The proteins were expressed at similar levels in F9 cells in the presence of Ras (data not shown)]. In G-RI+II+III, the T82A and the ΔBEC mutations decreased both basal and Ras induced activity (Figure 6A). In contrast, with the isolated RI+II responsive element (G-1+II), the T82A mutation prevented Ras induction, whereas the ΔBEC mutation did not, even though both mutations decreased basal activity (Figure 6B). The proteins were synthesised at similar levels, as shown by Western blotting of expressed proteins (Figure 6C, lanes 1–3, 5, 6, 8). These results show that T82 in the regulatory domain is required for Ras induction in three different settings. The effect of the T82A mutation on basal activity presumably results from partial activation of RI+II by signals from the endogenous Ras pathway, which may be partially active even under non-induced conditions. Our results suggest that there is a remarkable conversation between Drosophila and vertebrates of Ras inducible elements, in the similar Pointed/RII domains.

The BEC sequence appears to have different roles, depending on the context. These differences were reproduced in different cell types and with different reporters. In GH4 pituitary cells and with the natural prolactin cell-specific promoter, Ras superactivates exogenous Ets1 p68, as we have reported previously (Bradford *et al*., 1995). Ras induction was diminished to background levels by the ΔBEC and T82A mutations, as well as the Δ57–93 deletion encompassing both domains (Figure 5A). In HeLa, compared to F9 cells, G-RI+II had a higher constitutive activity and the fold activation by Ras was lower (Figure 6B2). However, as in F9 cells, basal activity was decreased by mutating either BEC, T82 or deleting amino acids 57–93, and Ras activation was lost in Δ57–93 and T82A, but not with ΔBEC (Figure 6B2). These results show that the BEC region is important for Ras activation in the context of the whole molecule, but not in the context of the RI+II region. The results resemble previous data for Ets1 that show that functions such as

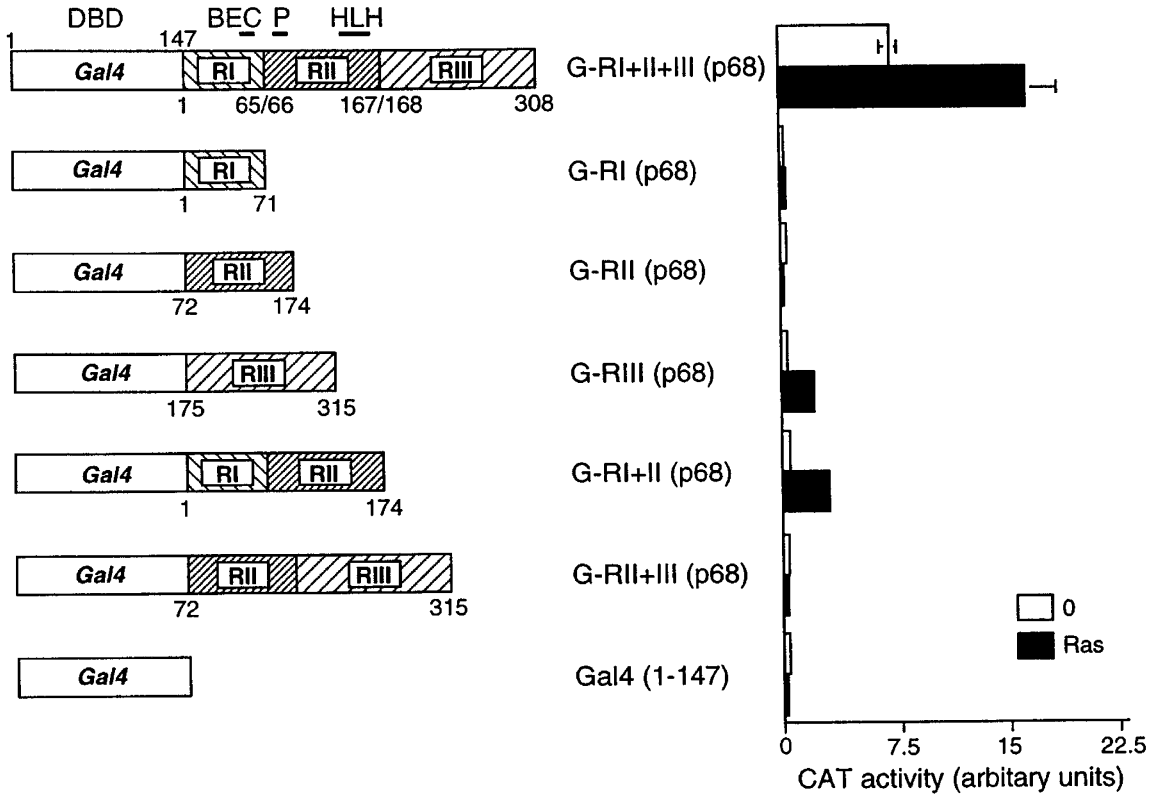


Figure 3 Localisation of the Ras responsive domain using fusion proteins between different regions of Ets1 p68 (RI, RII and RIII) and the GA14 DNA binding domain (DBD). The graph shows the CAT activity generated in F9 cells from the UAS-TK-CAT reporter by the heterologous fusion proteins in either the absence (0, white bars) or presence (Ras, black bars) of activated Ha-Ras. The data from three duplicate experiments were averaged and the error bars indicate the standard deviation. The features and structures of the fusion proteins are illustrated. The heterologous yeast Gal4 1–147 region, containing the DNA binding domain, is fused to various regions of Ets1 p68 [R1, amino-acids 1–71; RII, amino-acids 72–174; RIII, amino acids 175–315, (Schneikert *et al*., 1992)] or left unfused [Gal 4 (1–147)] 'BEC', 'P' and 'HLH' are defined in Figure 2

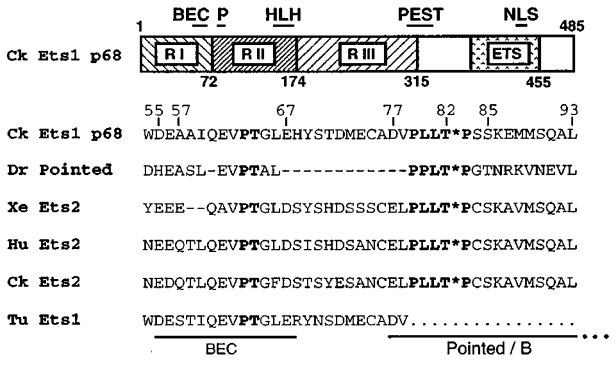


Figure 4 N-terminal sequences in chicken Ets1 p68 conserved during evolution from Drosophila to man. Illustration of the chicken (Ck) Ets1 p68 protein with the conserved BEC domain and the threonine [T*(82)] at position 82 in the MAP kinase consensus sequence that is homologous to the Pointed MAPK kinase phosphorylation site (Brunner *et al*., 1994; O'Neill *et al*., 1994). Comparisons between Ets1, Ets2 and Pointed proteins from man (Hu), Xenopus (Xe), chicken (Ck), turtle (Tu) and Drosophila (Pointed) identifies the BEC sequence and a conserved MAPK consensus (bold letters) in the Pointed (RII, B). Numbering on the Ck Ets1 sequence localise mutations in ΔBEC (55–67), ΔPointed (77–85), Ets1 p68 T82A (82) and Δ57–93 (57–93)

DNA binding and trans-activation are different in the context of individual function elements compared to larger fragments or full length protein and that there are 'cis' interactions between domains in the protein.

The results of the N-terminal deletion mutants (Figure 2) and the GaI4-RIII construct (Figure 3) suggests that there is a second Ras inducible element in RIII, that requires the region delimited by Δ55 and Δ56. This region could either be an inducible element, or a constitutive element that is enhanced through induction. To study its functions, we constructed F1–5 (Figure 5B), in which amino acids 217 to 258 were deleted (see Materials and methods). The basal activity of F1–5 was lower than both full length Ets1 and Δ57–93 (Figure 5C), but the fold activation by Ras was similar to Ets1 p68 and higher than Δ57–93. These results suggest that the F1–5 region is important for basal activity rather than Ras activation. To confirm this conclusion we used scanning mutagenesis across the region, mutating 8 amino acids at a time (F1 to F5, Figure 5B). All the mutants were inducible by Ras to the same extent as the wild type protein, suggesting the larger deletion in F1–5 did not somehow mask the effects of a shorter inducible element. These results suggest that the Ras inducible element is not in the F1–5 region. To exclude that the presence of the inducible element in the 57–93 region masked the effect of the F1–5 mutation, we deleted both regions. The double mutant, Δ57–93F1–5 (Figure 5B), was activated by Ras to a similar extent as Δ57–93 (Figure 5C). These results show that amino acids 218 to 225 are important for the constitutive activity of Ets1 68, and support the conclusion that an element other than the 57–93 region in RI+II is

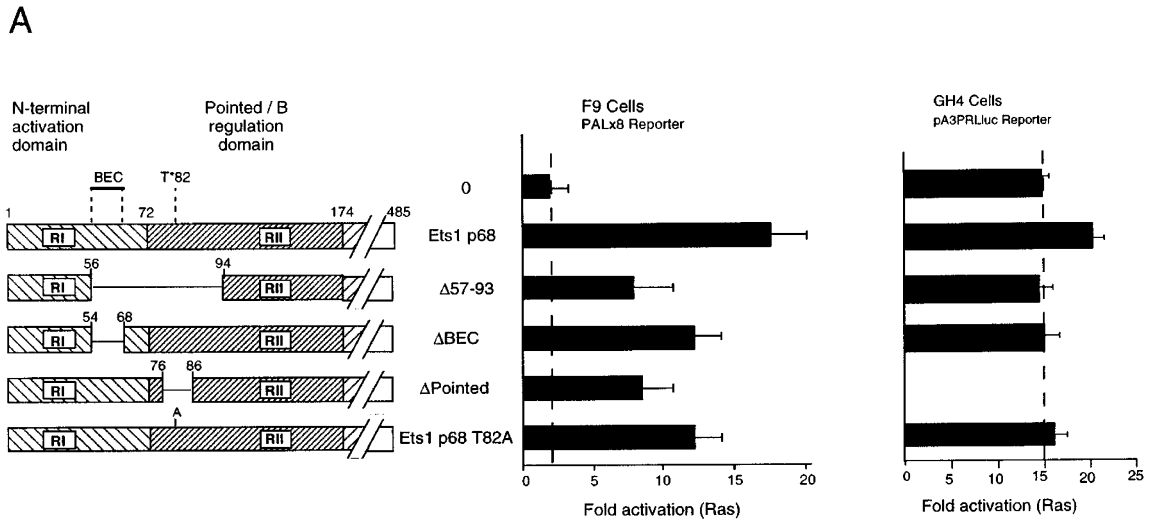
required for full Ras induction of cEts1 p68. A second Ras inducible element has not been detected in Pointed P2. We studied Pointed P2 in mammalian cells, to show that the mechanisms of induction are conserved and to establish whether the difference was due to the choice of experimental systems.

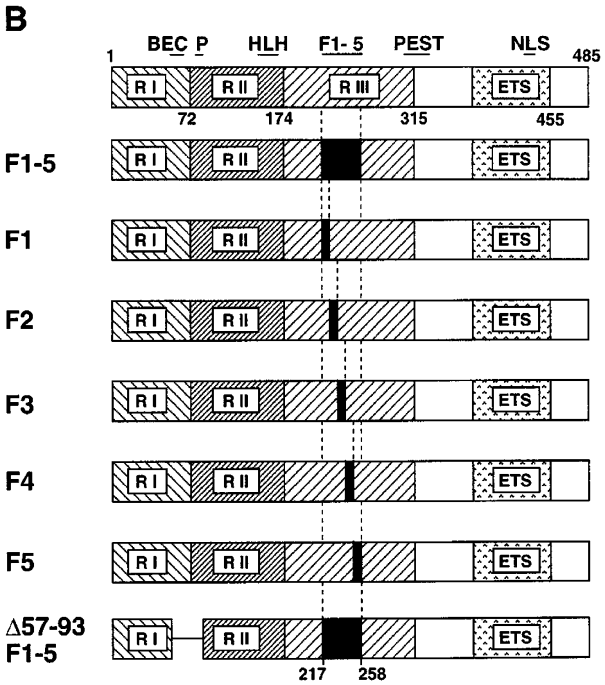
Ras induction of Drosophila proteins in mammalian cells

In Drosophila cells, the P1 isoform of Pointed is constitutively active, whereas P2 is inactive and Ras inducible. Mutation of T151 to A prevents Ras activation, whereas mutation to E produces a constitutively active factor (O'Neill *et al*., 1994; Brunner *et al*., 1994). In F9 cells, using the PALx8 reporter and mammalian expression vectors (see Materials and methods), we found that under basal conditions P1 activated transcription whereas P2 and the T151A mutant were essentially inactive (Figure 7; compare P1 WT, P2 WT T^{15} and P2 $MutA^{151}$). The T151E mutation did not activate P2 (Figure 7, P2 $MutE^{151}$), suggesting that there are differences between, organisms perhaps at the level of the basal transcription machinery. Ras expression stimulated P2, whereas it had no significant effect on either P1 or the two mutant P2 proteins (Figure 7, compare with 0 due to endogenous proteins). These results show that there is a remarkable conservation of the mechanisms of Ras activation of Drosophila and vertebrate Ets proteins, that implicate related domains of both proteins. However, there are also differences, since Ets1 has a second Ras inducible domain, in a region of the protein that has a different sequence.

MAP kinase phosphorylation of similar sequences in related vertebrate and Drosophila Ets proteins

Activated rolled MAPK stimulates Pointed P2 in transfection assays and phosphorylates it *in vitro* on a unique site, T151 (O'Neill *et al*., 1994; Brunner *et al*.,





1994). We found in transfection assays that MAP kinase p44 expression stimulated G-RI+II (results not shown), suggesting that T82 is a target for MAP kinase phosphorylation. To test this directly, RI+II wild type and T82A were produced as GST-fusion proteins in *E. coli* and purified. Equal quantities of GST-RI+II (p68), G-RI+II T82A and GST alone (Figure 8a, lanes 1–3) were phosphorylated with MAP kinase p44 and γ[^{32}P]ATP *in vitro* and analysed by both SDS–PAGE and phosphopeptide mapping (see Materials and methods). GST-RI+II was phosphorylated by MAP kinase (Figure 8A, lane 4), and gave one major labelled tryptic phosphopeptide on the map (Figure 8B, left panel), whereas neither the mutant nor GST alone were phosphorylated (Figure 8a and b, right panel). These results show that T82 is a MAP kinase phosphorylation site and it is the only site in the RI+II Ras inducible domain.

Conserved sequences in Pointed and Ets1 p68 inhibit specific DNA binding

The conservation of phosphorylation targets for MAP kinase suggested that there may be other similarities between the two proteins. Sequence alignment with the Bestfit program (UWGCG Package) showed that around 100 amino-acids adjacent to the ets domain are about 70% similar or identical between Pointed and Ets1 (Figure 9a). Secondary structure prediction with the PHDsec program (Internet, Rost@EMBL-Heidelberg. DE; (Rost and Sander, 1993; Rost *et al*., 1994; Rost and Sander, 1994)) indicated that both regions have a similar extended loop structure with a α helix lying just next to the ets domain (data not shown). This homology was particularly striking because it corresponds almost precisely to the domain we and others showed previously to inhibit DNA binding by Ets1 (Lim *et al*., 1992; Wasylyk *et al*., 1992; Hagman and Grosschedl, 1992; Fisher *et al*., 1994; Hahn and Wasylyk, 1994; Leprince *et al*., 1992, 1993; Petersen *et al*., 1995). To investigate whether the Pointed sequences have a similar function, we generated deletion mutants which are equivalent to those we used previously, according to the sequence alignment (P-N4 and P-N70, Figure 9b; (Wasylyk *et al*., 1992)). The proteins were synthesised in reticulocyte lysates in the presence of [^{35}S]methionine, quantitated by SDS–PAGE and a Phospho-Imager and equal molar quantities were used for EMSA with a probe containing a consensus ets binding site (PEA3*), as described previously (Wasylyk *et al*., 1992). In agreement with our previous findings, Ets1 p68 and N4 bound poorly relative to N70 (Figure 9c, lanes 5–8, arrows). Note that inhibition is sufficiently marked that

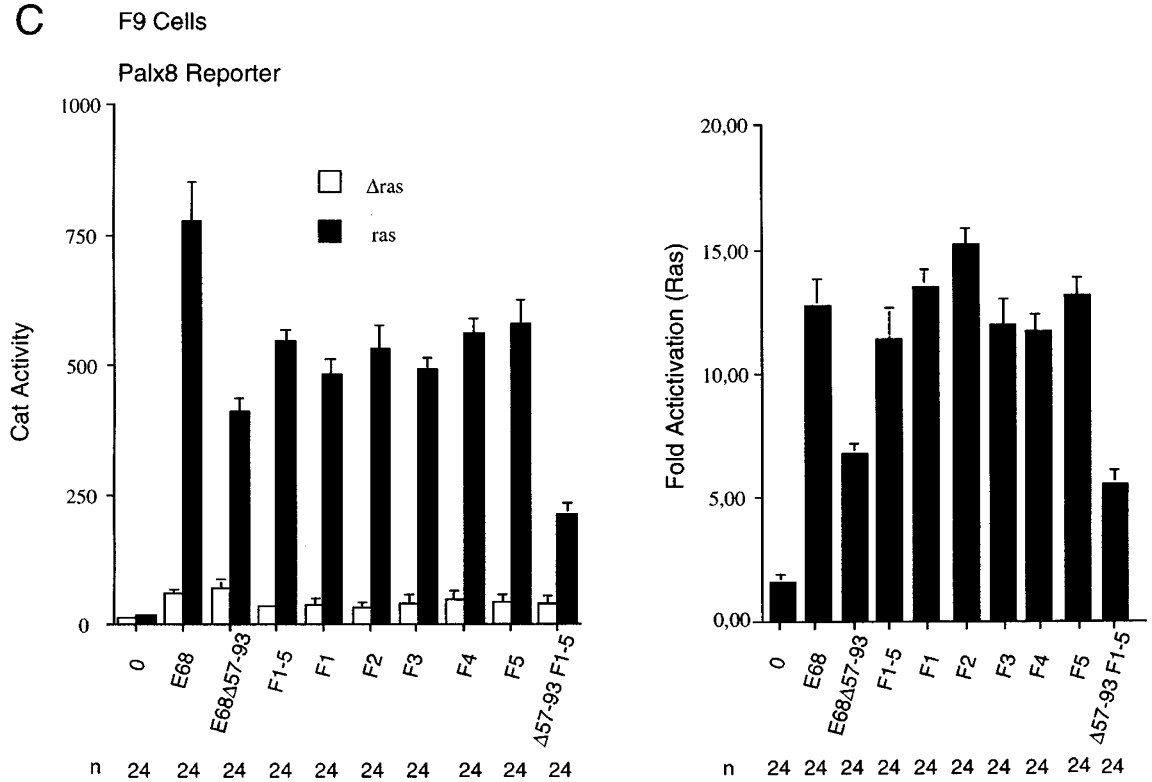
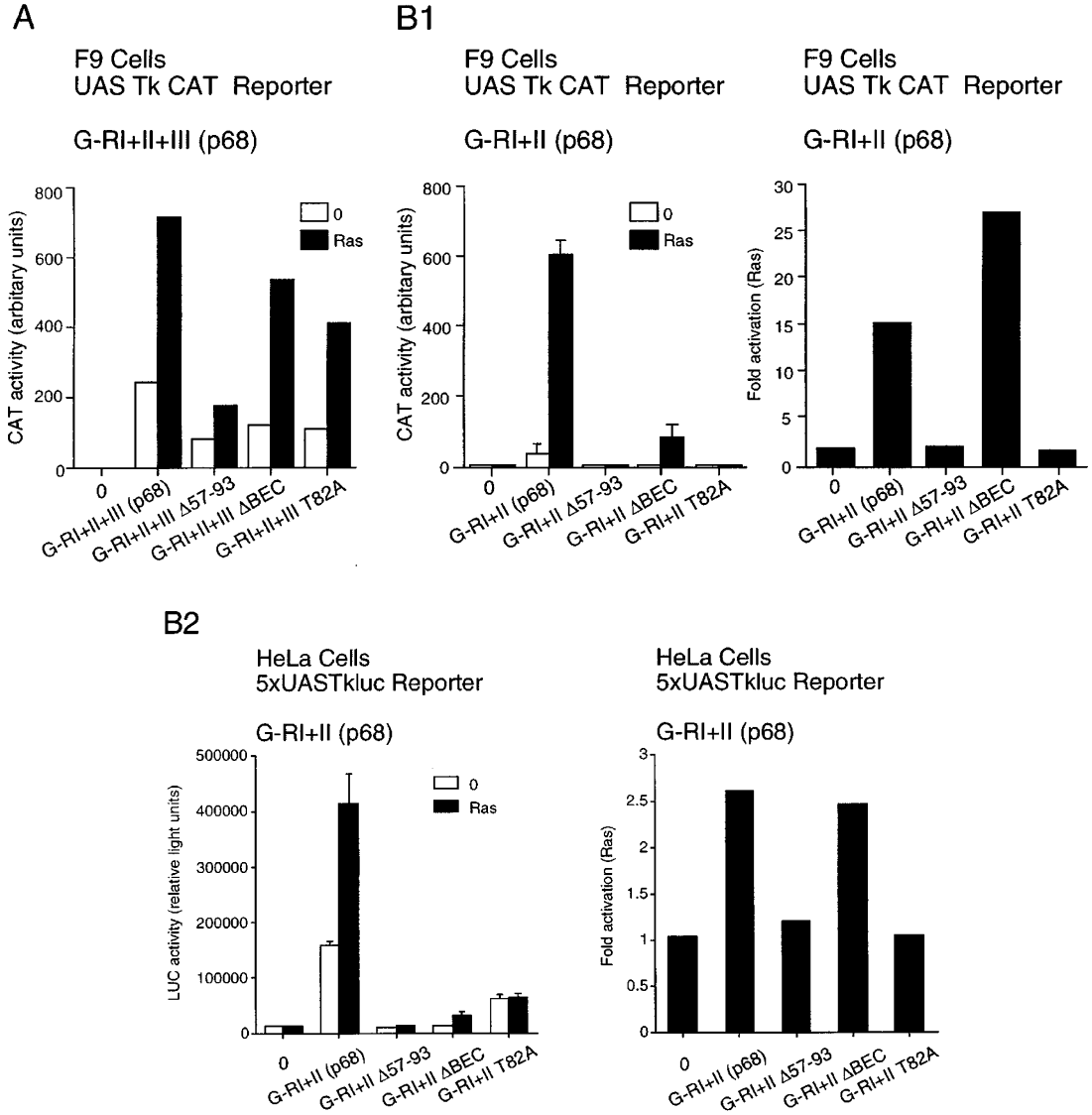


Figure 5 Effects of BEC, Pointed or threonine 82 to alanine mutation of cETs1 p68 on Ras activation. (**A**). The diagrams show the features and structures of the recombinants and the graphs the fold activation of Ets1 proteins by Ras. BEC is described in Figure 2 and T*82 in Figure 4. ΔBEC lacks amino acids 55–67, ΔPointed lacks amino acids 77–85, Ets1 p68 T82A has threonine 82 mutated to an alanine and Δ57–93 lacks amino acids 57–93. The results in the centre are for F9 cells and the PALx8 reporter and are the mean standard deviation of 4 transfections. The results on the right are for the rPRL promoter and GH4 cells and are the mean standard deviation of 6 transfections. The enhancement of the Ras response by Ets-1p68 is statistically significant, $P<0.05$. The numerical fold activation in each case is for 0: 14.8; Ets1 p68: 20.2; Δ57–93: 14.4; ΔBEC: 15, Ets1 p68 T82A: 16.5 (**B**) Structure of the scanning mutants of Ets-1 p68. The sequences between 217 and 258 indicated in black in F1–5 and F1 to F5 were replaced with the flag epitope. (**C**) Activity and fold Ras activation in F9 cells with the PALx8 reporter of the mutants illustrated in B. The error bars indicate the mean standard deviation of 24 transfections (n)

a minor product, resulting from initiation at a second ATG, was the major binding form in the N4 synthesis (lane 7, band labelled with a star and see (Wasylyk *et al*., 1992)). Pointed P2 and P-N4 bound very poorly compared to P-N70 (lanes 1–4, the broken arrrows indicate specific bands visible on longer exposure).



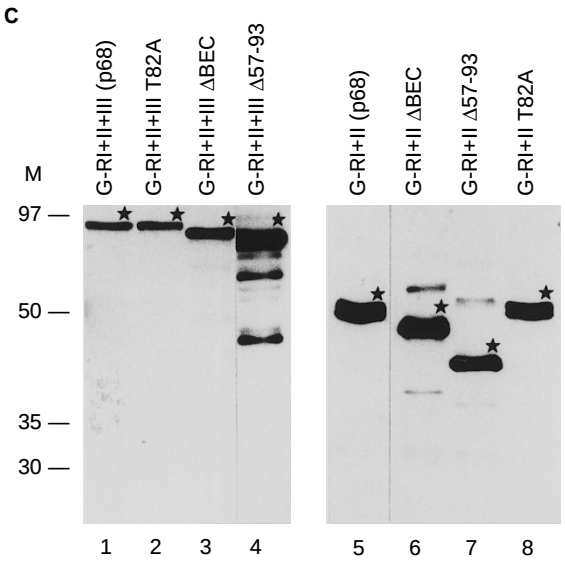


Figure 6 Effects of BEC, Pointed or threonine 82 to alanine mutation of cEts1 p68 on Ras activation in the context of Ga14 fused to either RI + RII + III (G-RI + II + III, A) or RI + II (G-RI + II, B **A** and **B**). The mutations generated in Ets1 p68 were transferred into G-RI + II + III (A) or G-RI + II (B1 + 2) and analysed for their ability to activate in the absence or presence of Ras the UAS-TK-CAT reporter in F9 cells (B1) or the 5XUATkluc reporter (5μg) in HeLa cells (B2, 2μg Ras). The error bars indicate the mean standard deviation of 4 transfections. (**C**). The mutations do not significantly affect protein stability. Proteins were extracted from transfected F9 cells, quantified by the Bradford reaction and separated on a SDS–PAGE gel. Western blots of total cell extracts (100 μg per lane) were probed with specific antibodies against Ga14 and detected with the ECL kit (Amersham). Stars indicate proteins of the expected size

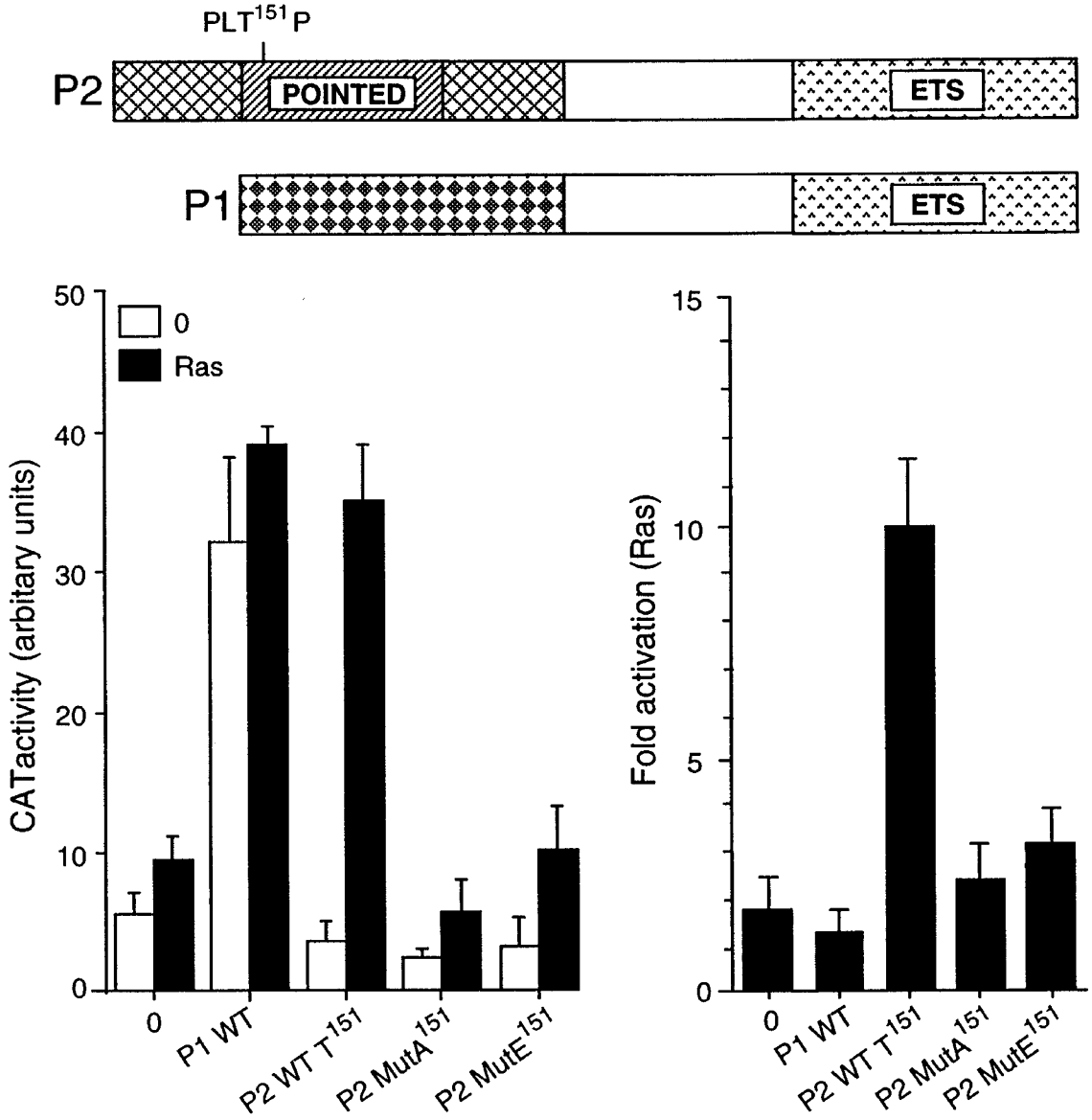


Figure 7 Activity of the Drosophila Ets proteins Pointed P1 and P2 in mammalian cells and effect of mutation of the MAP kinase phosphorylation site in P2 on Ras activation. P1 or P2 Pointed proteins were expressed in F9 cells from the same vector as Ets1 p68 and their activity was measured in the absence or presence of Ras using the PALx8 reporter. The data is compiled from three separate experiments and the error bars indicate the standard deviation

These results show that the Pointed P2 protein has a latent DNA binding activity due to sequences that have been conserved between the Drosophila and vertebrate proteins.

Discussion

Ets1 p68 has two Ras regulated activation domains

Ets1 and Ets2 have a relatively high constitutive activity when expressed in a number of cell-lines in culture, such as HeLa and NIH3T3 cells, and are relatively insensitive to Ras activation ((Wasylyk *et al*., 1990; Giovane *et al*., 1994); data not shown). In contrast, we found that the Ets proteins have a low basal activity and are sensitive to Ras activation in several EC cell-lines (F9, PC19). Similar observations have been made with other Ras responsive factors (e.g. Jun), suggesting that the Ras signalling pathway itself has a low basal activity in EC cells. However, Ras activation is observed in HeLa cells with the prolactin promoter (Figure 1; (Bradford *et al*., 1995)). In NIH3T3 cells, the repressor ets factor Net has low activity and is activated by Ras expession, in contrast to Ets1 p68 (Giovane *et al*., 1994). With 'pure' reporters, containing only ets binding sites, we found that Ets2 and the two Ets1 isoforms (p54 and p68) are responsive to the Ras signal, whereas two other ets factors, PU.1 and Elf1, are not. However, we cannot exclude that they could be induced in other cell types. We previously showed that Ets1 p68 can cooperate with Pit-1 for Ras activation of the rat prolactin promoter in a pituitary cell specific manner, whereas Ets2 cannot (Bradford *et al*., 1995). This may result from the inability of Ets2 to interact with GHF-1/Pit-1 and hence to be recruited to the prolactin promoter (A Bradford, in preparation). Comparing the effects of

Ras expression in various cell-types may reveal different facets of the complex Ras signalling pathways (Marshall, 1996; Downward, 1995).

We identified two Ras responsive domains in Ets1 p68, the combination of regions RI + II and RIII. The regions RI, RII and RIII have been defined by sequence comparisons and functional dissection of related factors (Ets1, Ets2, Pointed, Yan-Pok, Tel, GABP, Fli1, Erg1; (Schneikert *et al*., 1992; Gegonne *et al*., 1992; Chumakov *et al*., 1993; Albagli *et al*., 1994; Hahn and Wasylyk, 1994; MacLeod *et al*., 1992; Seth *et al*., 1992; Janknecht and Nordheim, 1993; Wasylyk *et al*., 1993)). Interestingly, deletion analysis and heterologous fusions show that RI is inactive in F9 cells, whereas it is active in a number of other cell-types (Schneikert *et al*., 1992; Albagli *et al*., 1994). Neither RI nor RII alone can be activated by Ras, but fusion confers Ras responsiveness. RIII alone is inducible, but this activity also appears to depend upon other factors, since equivalent mutants were not inducible in pituitary GH4 cells (Bradford *et al*., 1995). The p54 isoform of Ets1 is inducible by Ras, even though it has a different sequence in the place of RI. This is presumably due to the RIII domain, but we cannot exclude that the alternative RI is involved in induction.

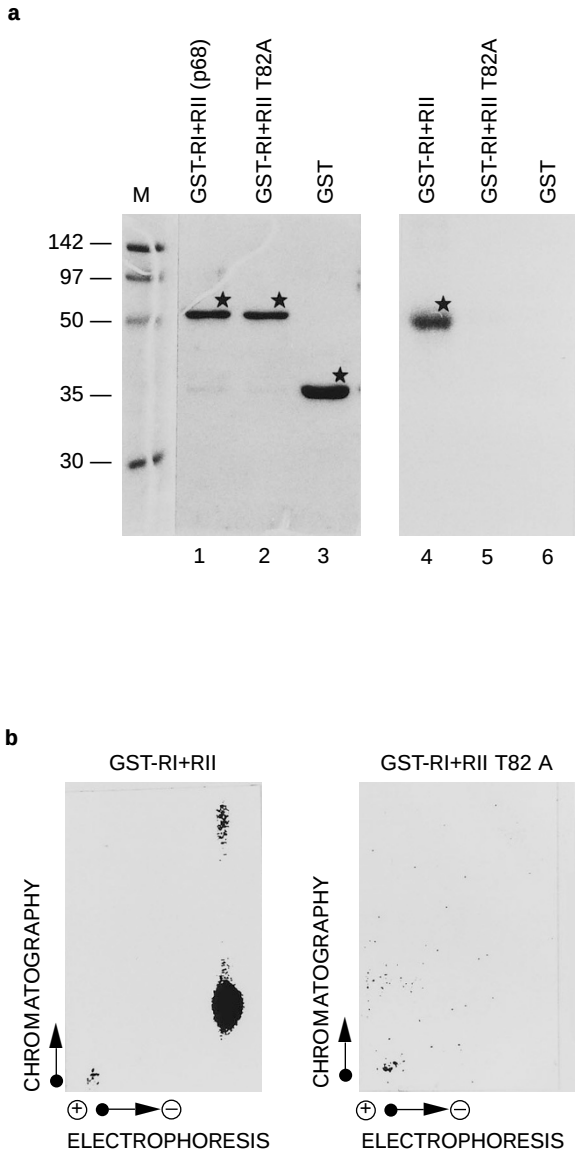


Figure 8 Threonine T82 of Ets1 p68 is phosphorylated by activated p44 MAP Kinase. SDS–PAGE analysis. GST-RI + II, GST-RI + II T82A and GST alone were expressed in *E. coli*, purified on Glutathione Sepharose beads. (**A**) SDS–PAGE. The bacterial proteins were either analysed by SDS–PAGE followed by Coommassie Blue staining (lanes 1–3) or phosphorylated with MAP Kinase-mpk.p44 (Upstate) and γ-[^{32}P]ATP, and analysed by SDS PAGE followed by autoradiography (lanes 4–6). The bands of the expected size are indicated by a star (**B**). Phosphopeptide mapping. After SDS–PAGE the radioactive bands corresponding to GST-RII + II and GST-RI + II T82A were cut out and the proteins were eluted, proteolytically digested with TPCK-treated trypsin and oxidised. The peptides were then separated by electrophoresis in the first dimension at pH 1.9 followed by chromatography in the second dimension. The phosphorylated peptides were visualised using a Phospho-Imager. The origins are indicated by Xs. The sequence of the expected tryptic peptide is SCCPPWDEAAIQEVPTGLEHYSTDMECADVPLLT*PSSK

An evolutionary conserved MAP kinase phosphorylation site is required for Ras activation of RI + II

Studies in different systems (see Results (Bradford *et al*., 1995)) suggested that a common mechanism of Ras activation is mediated by a short sequence in RI + II. Intriguingly, this sequence turned out to be conserved in the Drosophila Pointed P2 protein and in vertebrate Ets2. In Drosophila, development of the R7 photoreceptor is regulated by the Ras/MAPK pathway. Two ets factors are targets of this Ras controlled developmental pathway, Pointed P2 and Yan-Pok. Pointed P2 transcription activity is stimulated by the Ras pathway, and mutation of a MAP kinase phosphorylation site abrogates the response (O'Neill *et al*., 1994; Brunner *et al*., 1994). Yan is a negative factor, and Ras signalling overcomes its inhibitory activity (Rebay and Rubin, 1995). In vertebrates, structural and functional homologies in the Ets1/Ets2 subfamily have led to a model for the evolution of Ets factors (Lautenberger *et al*., 1992; Laudet *et al*., 1993; Albagli *et al*., 1994) that proposes that an ancestral Ets0 duplicated in early vertebrates, leading to the Ets1 and Ets2 proteins. The ets1 gene acquired the additional I^{54} exon, resulting in the production of two isoforms, p54 and p68 in birds. The exons coding for the p68 isoform were then lost along the mammalian branch, presumably due to redundancy with Ets2. The Pointed P2 protein is proposed to have originated from the same ancestral gene, as witnessed by the conservation of the BEC sequence. Intriguingly, the sequence next to BEC is the MAP kinase target sequence. The Ets1 57–94 sequence we identified as required for Ras activation (Bradford *et al*., 1995) contains both the BEC sequence and a homology to the MAPK phosphorylation site. Remarkably, mutation of the MAPK homology abolished activation of the RI + II responsive unit, whereas a BEC mutation was still inducible even though the basal activity was reduced. We have shown that MAP kinase uniquely phosphorylates T82 in RI + II, indicating that it is a primary target for the Ras signaling cascade. BEC contributes to the basal activity of the RI + II unit but is not required for Ras induction. However, it somehow affects Ras activation of Ets1. In the complete protein, mutation of either element lowers activation, whereas mutating both has a larger effect. This points to complex interactions between the two

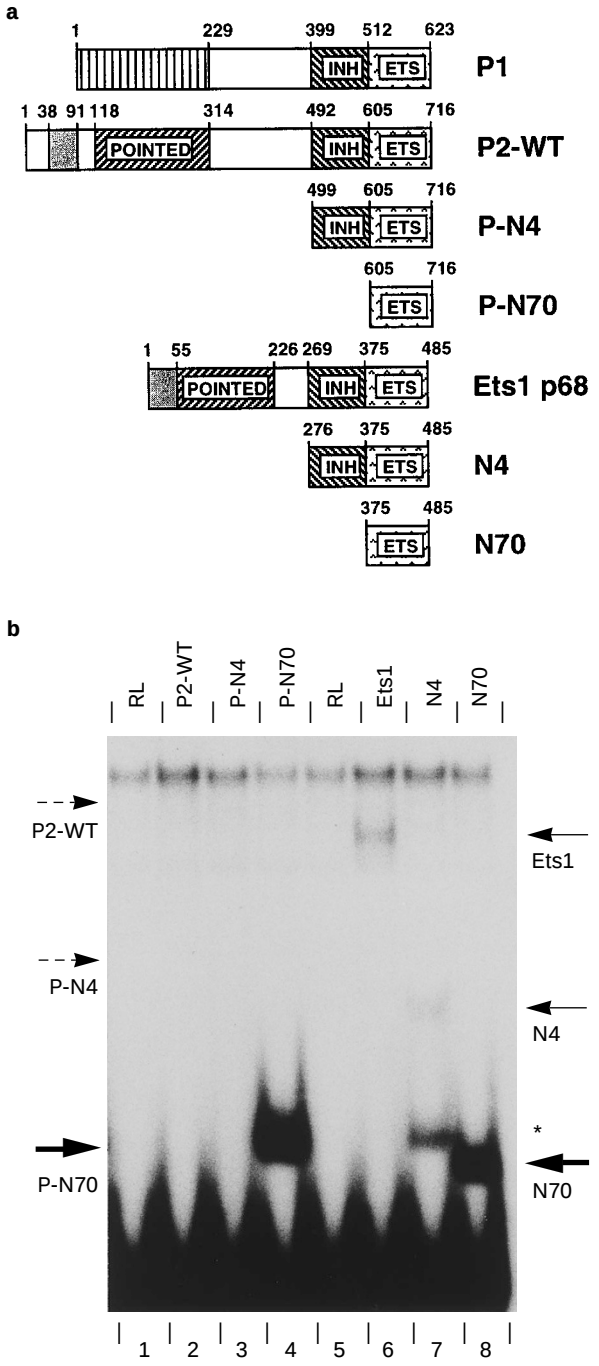


Figure 9 Specific DNA binding is auto-regulated by inhibitory sequences that are conserved between Ets1 p68 and Pointed P2. (**A**) Sequence homology and structures of proteins used for EMSA. The sequenced were compared by the Bestfit program (GCG package, UWGCG Package). Related sequences are indicated by the use of the same shading, and unshaded regions are unrelated between the three proteins. Identity between Pointed P1 and P2 begins just after the 'POINTED' sequence homology between Ets1 and P2. The 'INH' sequence homology between Ets1 p68 and Pointed corresponds almost precisely the DNA-binding inhibitory domain we defined previously (Wasylyk *et al*., 1992). 'ETS' indicates the Ets DNA binding domain. Ets1 and P2 are also similar in the 'POINTED' domain. (**B**) EMSA of Pointed P2 and Ets1 p68. The proteins were synthesised in reticulocyte lysates in the presence of [^{35}S]methionine, analysed by SDS–PAGE, specific protiens were quantitated with a Phospho-Imager, quantities were adjusted with mock incubated reticulocyte lysates, taking into account the number of methionines, and equal quantities used for EMSA with the PEA* probe containing a consensus ets binding site as described previously (Wasylyk *et al*., 1992). Arrows point to specific complexes and broken arrows to complexes detected with more protein on longer exposition of the autoradiograms. *Indicates a minor N-terminally shortened protein produced during *in vitro* synthesis of N4. It binds very efficiently because it lacks the inhibitor of DNA binding (Wasylyk *et al*., 1992)

inducible units. Our results indicate that there is a remarkable conservation of part of the Ras signaling cascade downstream from MAPK, in the RI+II sequence.

The second Ras responsive element in Ets1

The RIII region appears to contain an independent Ras responsive element. Deletion of sequences from the N-terminus between 218 to 252 in Ets1 decreases stimulation to a very low level, suggesting that this sequence could contain an inducible element, possibly a phosphorylation site for MAP kinase. There are 9 serine/threonines in this region, several of which are adjacent to prolines (two PS and one PXSS), but there are no consensus MAP kinase sites (PXT/SP; (Clark-Lewis *et al*., 1991)). Extensive mutagenesis showed that this region is not directly required for induction, but rather for the basal activity of the protein, in agreement with previous studies with Ets1 p54 (Suzuki *et al*., 1995). Several studies have pinpointed phophorylation sites in RIII. Valentine *et al*. (1995) showed that Ser-156 in the p54 isoform (equivalent to serine-200 in p68), as well as Thr-73, are phosphorylated in response to the increased intracellular calcium following ligation of IgM on B lymphocytes. Rabault and Ghysdael (1994) showed using T cells that calcium and anti-CD3 induce phosphorylation of four sites, two of which are in RIII (Ser-251 and Ser-257, equivalent to Ser-295 and Ser-301). Ets1 has also been reported to be hyperphosphorylated during mitosis in T cells (Fleischman *et al*., 1993) and in response to neuro-transmitters in astrocytes (Fleischman *et al*., 1995), although the phosphorylation sites were not mapped. It remains to be seen how these phosphorylations affect the activity of RIII, or if dephosphorylation is involved, and if they are involved in any way in Ras signaling.

Auto-regulation of binding of Pointed to DNA by conserved sequences

A comparison of the sequences of Pointed with Ets1 showed that it has a second homologous region, adjacent to the ets domain, which corresponds closely in size and position to the auto-inhibitor of DNA binding of Ets1 (Lim *et al*., 1992; Wasylyk *et al*., 1992; Hagman and Grosschedl, 1992; Fisher *et al*., 1994; Hahn and Wasylyk, 1994; Petersen *et al*., 1995). A Pointed mutant retaining just this domain and the Ets domain was refractory to DNA binding, whereas the Ets domain alone bound efficiently. Petersen *et al*. (1995) have shown using physical studies that there is an α helix in the auto-inhibitor that unpacks from the ets domain upon DNA binding. Structure predictions indicate that Pointed P2 and Ets1 p68 have similarly positioned α helices (amino acids 579-583 of P2 spaced by 26 amino acids from helix 1 of the ets domain, and 349-354 of Ets1 p68 spaced by 24; PHDsec program through Internet, Rost@EMBL-Heidelberg. DE; (Rost and Sander, 1993; Rost *et al*., 1994; Rost and Sander,

1994)). Structure determination will be required to establish if the auto-inhibitor of Pointed folds in a similar manner. The Ets1 auto-inhibitor is regulated by phosphorylation, notably by calcium signaling (Rabault and Ghysdael, 1994; Fleischman *et al*., 1993, 1995). However, the Drosophila protein lacks the consensus $^{D}/_{E}S^{F}/_{Y}^{D}/_{E}$ of the four principle phosphorylation sites of Ets1 (Rabault and Ghysdael, 1994); suggesting that it is not subject to the same regulation.

Structural and functional conservation in the ets family

Structural studies have shown that the ets domain adopts a winged–helix–loop–helix DNA binding motif and belongs to a large structural family that includes for example *E. coli* CAP, heat shock factor and HNF-3γ (Donaldson *et al*., 1994, 1996; Liang *et al*., 1994a, 1994b).

Subfamilies have arisen during evolution that contain additional conserved elements. The subfamily specific elements that are found in Ets1 include the Pointed domain, the auto-inhibitor and BEC. The Pointed (RII, B) domain is found in nine proteins: vertebrate Ets1, Ets2, Fli1, Erg, GABPα, Tel and Drosophila Pointed, Yan-Pok and Elg. The auto-inhibitor domain is present in four proteins: Ets1 p68, Ets1 p54, Ets2 and Pointed. The BEC domain is part of three: Ets1 p68, Ets2 and Pointed (MacLeod *et al*., 1992; Seth *et al*., 1992; Janknecht and Nordheim, 1993; Wasylyk *et al*., 1993; Watanabe *et al*., 1993; Golub *et al*., 1994). In contrast, the elk1 subfamily (Elk1, Net and SAP1) has two subfamily specific sequences that mediate specific interactions with the accessory factor SRF and Ras inducible transactivation (Treisman, 1994; Janknecht *et al*., 1995).

The Pointed domain is particularly interesting because it is conserved in evolution, and is critical for the oncogenic activation of Tel in human tumours. The Pointed domain of Tel is retained in fusion proteins produced by chromosomal translocations to various loci (Golub *et al*., 1994, 1995; Buijs *et al*., 1995; Papadopoulos *et al*., 1995). The Tel-PDGFβ receptor and Tel-cAbl fusion proteins are constitutively active kinases, possibly due to dimerization mediated by the Pointed region (Golub *et al*., 1994; Papadopoulos *et al*., 1995). In the Tel-AML1 fusion protein, the Pointed region of Tel is linked to the DNA binding and activation domains of AML1 (Golub *et al*., 1995). Interestingly, our work suggests that the Pointed domain may in fact comprise two sub-domains. The MAP kinase phosphorylation site and flanking sequences (approximately 75–100 of p68) are only found in Ets1, Ets2 and Pointed P2 (Figure 4), whereas the second part of the domain (approximately amino acids 100–175) is conserved in all the proteins with a Pointed domain. The second part contains the helix–loop–helix that Seth and Papas (1990) have proposed may mediate interactions with other proteins.

The Ets1 p68, Ets2 and Pointed subfamily have two specific elements, the BEC domain and the auto-repressor that lie in similar positions in the proteins. Going from the N-terminus of these proteins, there is a divergent sequence, followed by the BEC, the MAP kinase homology, the rest of the Pointed domain, a second divergent domain, the auto–inhibitor and finally the ets domain. We previously showed that the divergent domains RI and RIII in Ets1 and Ets2 are transcription activation domains (Schneikert *et al*., 1992). The Ets1 RIII is inducible by Ras. We note that the RIII of Ets2 has a consensus MAPK site, suggesting that it may also be Ras inducible. The equivalent region of Pointed P2 is apparently not inducible, since mutation of the unique MAP kinase site abolishes activation in both Drosophila (O'Neill *et al*., 1994; Brunner *et al*., 1994) and in mammalian cells (this work). It remains to be seen if the RI and RIII equivalents of Pointed P2 are independent activation domains.

Conservation of Ras/MAP kinase targets in evolution

Previous work in one of our laboratories identified the functional components of the Ras signaling pathway as consisting of Ras-Raf-MAP kinase and Ets (Conrad *et al*., 1994). Subsequent work (Bradford *et al*., 1995) localised an essential region in Ets1 p68 that mediates the response, and we have now narrowed this down to an essential threonine residue. The remarkable aspect is the convergence with genetic studies in Drosophila. We show that the Ras signaling pathway converges on an evolutionary conserved site in a related factor, Pointed P2 (Dickson, 1995). There are other similarities in regulation by Ras. In mammalian cells Ets1 co-operates with Jun in transcription activation through Ras responsive elements (Bortner *et al*., 1993; Chambers and Tuck, 1993), and they physically interact (Bassuk and Leiden, 1995). In Drosophila Jun cooperates with Pointed to induce photoreceptor R7 fate (Treier *et al*., 1995). These results go further towards extending the classical Ras/MAP kinase cascade to evolutionary related nuclear targets, such as Ets and Jun, that may in turn orchestrate conserved and co-ordinate responses of gene expression in recognisable patterns.

Materials and methods

Cell Culture, transfections and reporter assays

Cell culture F9, HeLa and GH_4T2 rat pituitary tumor cells were maintained in Dulbecco's modified Eagle medium (DMEM,Gibco) supplemented with 10% fetal calf serum (Hyclone) and penicillin-streptomycin at 37°C and either 7% (F9) or 5% (HeLa) CO_2.

Transfections in F9 cells F9 cells were transfected with calcium phosphate precipitates containing 20 μg total plasmid DNA per 10 cm dish, composed of 4 μg of the internal control β-galactosidase expression vector (pSG5-LacZ), 2.5 μg reporters, 1 μg of expression vectors for the Ets proteins or 100 ng for the Gal4-fusions, 2 μg of expression vectors for activated Ha-Ras vector or its control (ΔRas), and the appropriate amount of empty vector (pSG5). The cells were split 2 h before transfection, plated at 30% confluence, incubated with the pecipitated DNA for 16 h, washed extensively with DMEM, incubated in complete medium for 24 h, scraped, freeze-thawed three times in solution A (15 mM Tris-HCl pH 7.9, 60 mM KCl, 2 mM EDTA, 0.15 mM spermine, 1 mM DTT and 0.4 mM PMSF) and centrifuged. Before CAT assays the extracts were heated for 10 min at 65°C. Appropriate amounts of extracts and incubation times were used to give measurable, non-saturated levels of [^{14}C]acetylated chloramphenicol

col. After thin layer chromatography with 95% chloroform and 5% methanol, radioactivity was quantitated with a Fuji Phospho-Imager.

Transfections of HeLa and GH_4 T2 cells by electroporation Aliquots containing $2-4\times10^6$ cells were added to plasmid DNA and transfected by electroporation at 220 V and 500 mmF using a BioRad Gene Pulser. All transfections included 0.5 μg pCMVβ as an internal control for transfection efficiency. Electroporations were performed in triplicate for each condition within a single experiment and were repeated several times using different plasmid preparations for each construct. The cells were harvested in PBS containing 3 mM EDTA, freeze-thawed three times in 10 mM potassium phosphate, 1 mM DTT with vortexing betwen each cycle and centrifuged at 10 000 *g* for 10 min at 4°C. Luciferase was assayed as previously described (Conrad and Gutierrez-Hartmann, 1992) in 100 mM potassium phosphate pH 7.8 1 mM DTT, 15 mM $MgSO_4$, 5 mM ATP and 0.2 mM luciferin. Samples were measured in duplicate using a Monolight Luminometer (Analytical Luminescence Laboratories, San Diego, CA, USA).

Reporters and expression vectors

Full details of the materials and methods used to construct the recombinants are available on request.

- *PALx8* contains eight copies of the stromelysin palindromic ets motif upstream from the TK promoter and CAT coding sequences of pBL-CAT4.
- *UAS-TK-CAT* contains a 360 bp fragment from the Gal1-Gal10 promoter inserted upstream from the TK promoter of pBLCAT8+ (Webster *et al*., 1988).
- *pA3 rPRL-LUC* (Conrad and Gutierrez-Hartmann, 1992) contains a 498 bp fragment from positions −425 to +73 of the rPRL promoter ligated upstream of the firefly luciferase gene.
- *pSG5-cEts1 p68* (chicken Ets1 p68 isoform), *pSG5-cEts1 p54* (chicken Ets 1 p54 isoform), *pSG5-cEts2* (chicken cEts2), *pSG5-hu-Ets1* (human), *pSG5-PU1, pSG5-Elf1* (mouse) contain the indicated cDNAs under control of the SV40 early promoter of pSG5 (Wasylyk *et al*., 1990, 1992).
- *Δ51 to Δ57, Δ31, Δ32, ΔB, ΔD, N4 and N70* are pSG5 based vectors that express progressive deletion mutants from the N- and C-termini of Ets1 p68 (Schneikert *et al*., 1992; Wasylyk *et al*., 1992; Wasylyk and Wasylyk, 1993).
- *pSG5-ΔBEC, pSG5-ΔPointed, pSG5-Δ57–93 and pSG5-Ets1 p68 T82A* (Figure 5) express cEts1 p68 proteins which lack internal sequences (ΔBEC, 55–67; ΔPointed, 77–85; Δ57–93, 57–93) or have a point mutation (Ets1 p68 T82A with alanine at position 82 instead of threonine, see Figure 4). The mutations were created by PCR in DNA fragments containing unique restriction sites [Bbs1(1080)-PmlI(1480) in chicken cEts1 p68], which were used to replace the equivalent fragment of pSG5-cEts p68. The fragments created by PCR were accurately sequenced.
- *F1–5, F1, F2, F3, F4, F5 and Δ57–93 F1–5* (Figure 5B) are pSG5 p68 Ets1 based vectors which express Ets1 p68 with scanning mutations in RIII that replace natural sequences with the FLAG TAG epitope (DYKDDDDK). The deleted sequences are: F1 (Δ218–225=EFSEPSFI); F2 (Δ226–233=TESYQTLH); F3 (Δ234–241=PISSEELL); F4 (Δ242–249=SLKYENDY); F5 (Δ250–257=PSVILRDP); F1–5 (Δ218–257); and Δ57–93 F1–5. Mutated fragments were made by PCR, accurately sequenced and EcoNI (1570) - AflIII (1953) unique fragments were swapped into chicken cEts1 coding sequences.
- *pSG5-G-RI+II WT, pSG5-G-RI+II ΔBEC pSG5-G-RI+II T82A, pSG5-G-RI+II Δ57–93, pSG5-G-RI+II+III WT, pSG5-G-RI+II+III ΔBEC, pSG5-G-RI+II+III T82A, pSG5-G-RI+II+III Δ57–93*, express fusion proteins between Gal4 (1–147) and Ets1 p68 regions RI+II or RI+II+III with the corresponding mutations (see above and Figures 3–5). The vectors were constructed by replacing restriction fragments of pSG5-G-RI+II and pSG5-G-RI+II+III (Schneikert *et al*., 1992) with the corresponding fragments from pSG5–ΔBEC, pSG5-ΔPointed, pSG5-Δ57–93 and pSG5-Ets1 p68 T82A. The transferred fragments were re-sequenced.
- *pSG5-P1 WT, pSG5-P2 WT T151, pSG5-P2 MutA151 and pSG5-P2 MutE151* express Drosophila Pointed P1 and P2, P2 $MutA^{151}$ and P2 $MutE^{151}$ (Brunner *et al*., 1994) from pSG5 based vectors. They were constructed by transferring BamHI and Asp718 fragments from vectors described by Brunner *et al*. (1994) into pTL1 or pTL2 derivatives of pSG5.
- *pGEX4T3-RI+II and pGEX4T3+RI+II T82A* are vectors that express *E. coli* fusion proteins between glutathione-S-transferase (GST) and either wild type or mutant (T82A) Ets1 p68 regions RI+II+III. Restriction fragment with the appropriate sequences were produced by PCR and transferred into the EcoRI site of pGEX4T3 (Pharmacia). All the clones were fully sequenced.
- *pSG5-K1-P2-WT, pSG5-K1-P2-N4, pSG5-P2-N70* express Pointed P2 wild type and N-terminal deletion that lack amino-acids 1–488 and 1–605. They were constructed by transferring PCR fragments into the KpnI and BamHI sites of KOZ1 (Wasylyk *et al*., 1992).

Protein blots

Total cell extracts were prepared by freeze-thawing the cells twice in TGK buffer (10 mM Tris HCl pH 7.8; 10% glycerol; 0.6M KCl; 5 mM DTT; 0.4 mM PMSF and a protease inhibitor cocktail). Cells debris were pelleted by centrifugation at 10 000 *g* for 20 min at 4°C. Proteins were quantitated by the Bradford assay. 100 μg proteins were electrophoresed on 10% SDS–PAGE gel, blotted to nitrocellulose membrane and incubated with Gal4 specific mono-clonal antibodies and with the ECL detection kit (Amersham).

GST protein preparation

GST-RI+II (p68), GST-RI+II T82 and GST-RI+II T82A fusion proteins were prepared as described by Pharmacia. DH5α cells transformed with pGEX-fusion recombinants were grown in 2 x YTG at 30°C with vigorous agitation to 0.8 OD at 600 nm, induced by adding 2 mM IPTG and incubated for 3 h at 25°C. The bacterial pellets were washed with ice-cold PBS, resuspended in lysis buffer (PBS, 1% Triton X100, 1% NP40, 10 mM DTT, 0.4 mM PMSF and protease inhibitor cocktail), agitated gently for 30 min at 4°C, sonicated four times for 15 s in ice, centrifuged and the supernatant mixed with glutathione Sepharose 4B equilibrated with PBS for 2 h at 4°C with gentle mixing. The beads were washed 4 times with ice-cold lysis buffer. The fusion proteins were quantitated by the Bradford assay and verified by SDS–PAGE.

Phosphorylation assay

10 μg of GST fusion proteins pre-bound to glutathione-Sepharose beads were washed twice with buffer A (20 mM HEPES pH 7.6, 50 mM NaCl,2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X100, 0.4 mM PMSF) and twice with kinase buffer (20 mM HEPES pH 7.6, 20 mM $MgCI_2$, 20 mM glycerophosphate pH7,0.5 mMNaF,0.1 mM Na_3VO_4, 2 mM DTT, 10 mM okadaic acid). Kinase assays were performed in 50 μl kinase buffer with 20 μM cold ATP and 5 μCi/ml [γ-^{32}P]ATP and 1 μl purified active MAP kinase p44 (Upstate Biotechnology Incorporated) at 30°C for

20 min. The reaction was stopped by washing the beads four times with 1 ml cold buffer A and the labeled proteins were analysed by SDS–PAGE.

Two-dimensional phosphopeptide mapping

Proteins were resolved by SDS–PAGE and transferred to nitro–cellulose membranes. The piece of membrane containing the labeled band was soaked in 0.5% PVP 250 in 100 mM acetic acid for 30 min at 37°C, washed five times with 1 ml H_2O and twice with 0.05 M NH_4HCO_3. The immobilised proteins were eluted by digestion with 10 mg TPCK-treated trypsin for 4 h in 200 μl freshly made 0.05 M NH_4HCO_3 at 37°C and then overnight with an additional 10 μg of fresh enzyme. 600μl H_2O was added, the sample was centrifuged, the liquid transferred to a new tube and dried in a Speed-Vac. The dried tryptic peptides were oxidised in 50 μl performic acid for 60 min at 37°C, diluted with 1 ml of H_2O and dried in a Speed-Vac. Salt was removed by repeated lyophilization. The digests were applied to thin-layer cellulose plates for two dimension peptide mapping by electrophoresis at pH 1.9 for 35 min at 1250 V in the first dimension, and chromatography in phosphochromo buffer (Boyle *et al*., 1991). The maps were analysed with a Phospho-Imager.

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