The EMBO Journal vol.15 no.21 pp.5849–5865, 1996

Net (ERP/SAP2), one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix–loop–helix motif

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The three ternary complex factors (TCFs), Net (ERP/SAP-2), ELK-1 and SAP-1, are highly related ets oncogene family members that participate in the response of the cell to Ras and growth signals. Understanding the different roles of these factors will provide insights into how the signals result in coordinate regulation of the cell. We show that Net inhibits transcription under basal conditions, in which SAP-1a is inactive and ELK-1 stimulates. Repression is mediated by the NID, the Net Inhibitory Domain of about 50 amino acids, which autoregulates the Net protein and also inhibits when it is isolated in a heterologous fusion protein. Net is particularly sensitive to Ras activation. Ras activates Net through the C-domain, which is conserved between the three TCFs, and the NID is an efficient inhibitor of Ras activation. The NID, as well as more C-terminal sequences, inhibit DNA binding. Net is more refractory to DNA binding than the other TCFs, possibly due to the presence of multiple inhibitory elements. The NID may adopt a helix–loop–helix (HLH) structure, as evidenced by homology to other HLH motifs, structure predictions, model building and mutagenesis of critical residues. The sequence resemblance with myogenic factors suggested that Net may form complexes with the same partners. Indeed, we found that Net can interact *in vivo* with the basic HLH factor, E47. We propose that Net is regulated at the level of its latent DNA-binding activity by protein interactions and/or phosphorylation. Net may form complexes with HLH proteins as well as SRF on specific promoter sequences. The identification of the novel inhibitory domain provides a new inroad into exploring the different roles of the ternary complex factors in growth control and transformation.

Keywords: bHLH/ELK-1/ets/repressors/SAP-1

Introduction

Mutations of the Ras gene are found frequently in human tumours (Kiaris and Spandidos, 1995). Ras is a component of highly conserved signalling cascades that mediate signal transduction from extracellular signals to intracellular effectors (Cano and Mahadevan, 1995; Seger and Krebs, 1995). Some of these effectors are transcription factors produced by the ets family of oncogenes (B.Wasylyk *et al.*, 1990, 1994). Ets proteins are implicated in many cellular functions, including growth control, cell transformation, development and apoptosis (MacLeod *et al.*, 1992; Seth *et al.*, 1992; Jacknecht and Nordheim, 1993; C.Wasylyk *et al.*, 1994; Bories *et al.*, 1995; Muthusamy *et al.*, 1995). They regulate gene expression by direct or assisted binding of the ets domain to DNA sequences containing the trinucleotide GGA. One subfamily of ets proteins involved in Ras signal transduction is composed of the ternary complex factors (TCFs) ELK-1, SAP-1 and Net [also called ERP (Lopez *et al.*, 1994) or SAP2 (Price *et al.*, 1995)].

The TCFs have three domains, 'A–C' (Figure 1A), with similar sequences and functions. 'A' mediates DNA binding, 'B' interacts with SRF to form ternary complexes with the c-fos serum response element and 'C' activates transcription upon phosphorylation by MAP kinases (Rao *et al.*, 1989; Dalton and Treisman, 1992; Janknecht *et al.*, 1993, 1994, 1995; Marais *et al.*, 1993; Giovane *et al.*, 1994; Hipskind *et al.*, 1994; Kortenjann *et al.*, 1994; Lopez *et al.*, 1994; Hill *et al.*, 1995; Price *et al.*, 1995; Whitmarsh *et al.*, 1995; reviewed by Treisman, 1992, 1994). The D region (Figure 1A) has no known function. Net is the only TCF to have an alanine-rich region that resembles the Krüppel domain (Giovane *et al.*, 1994; Lopez *et al.*, 1994). The TCFs are co-expressed in many cell types and are highly conserved from mouse to man (Giovane *et al.*, 1994, 1995; Price *et al.*, 1995), suggesting that they have distinct yet overlapping roles in the complex signalling cascades that regulate cell division. We previously found that Net inhibits transcription (Giovane *et al.*, 1994), suggesting that repression may be a distinct property of Net.

The mechanisms of transcription repression are relatively less well understood than activation (Johnson, 1995; Hanna-Rose and Hansen, 1996). Repression by DNA-bound proteins can result from a variety of mechanisms. The simplest is competitive DNA binding, in which the repressor excludes an activator from binding to DNA. Many repressors can co-occupy DNA with activators and prevent them from functioning, by masking or quenching interactions with the general transcription machinery. Some appear to bypass activators altogether. They repress basal activity by either direct inhibition of the basal transcription machinery, or recruitment of nucleosomes that inhibit access to the DNA (Cowell, 1994; Kornberg and Lorch, 1995; Hanna-Rose and Hansen, 1996; reviewed by Johnson, 1995).

We show that Net is a DNA-binding repressor that inhibits basal promoter activity *in vivo*. Repression is mediated by a novel inhibitory domain (NID), which appears to contain a helix–loop–helix (HLH) protein–protein interaction motif (reviewed by Murre *et al.*, 1994). The NID sequence most closely resembles the HLH motif

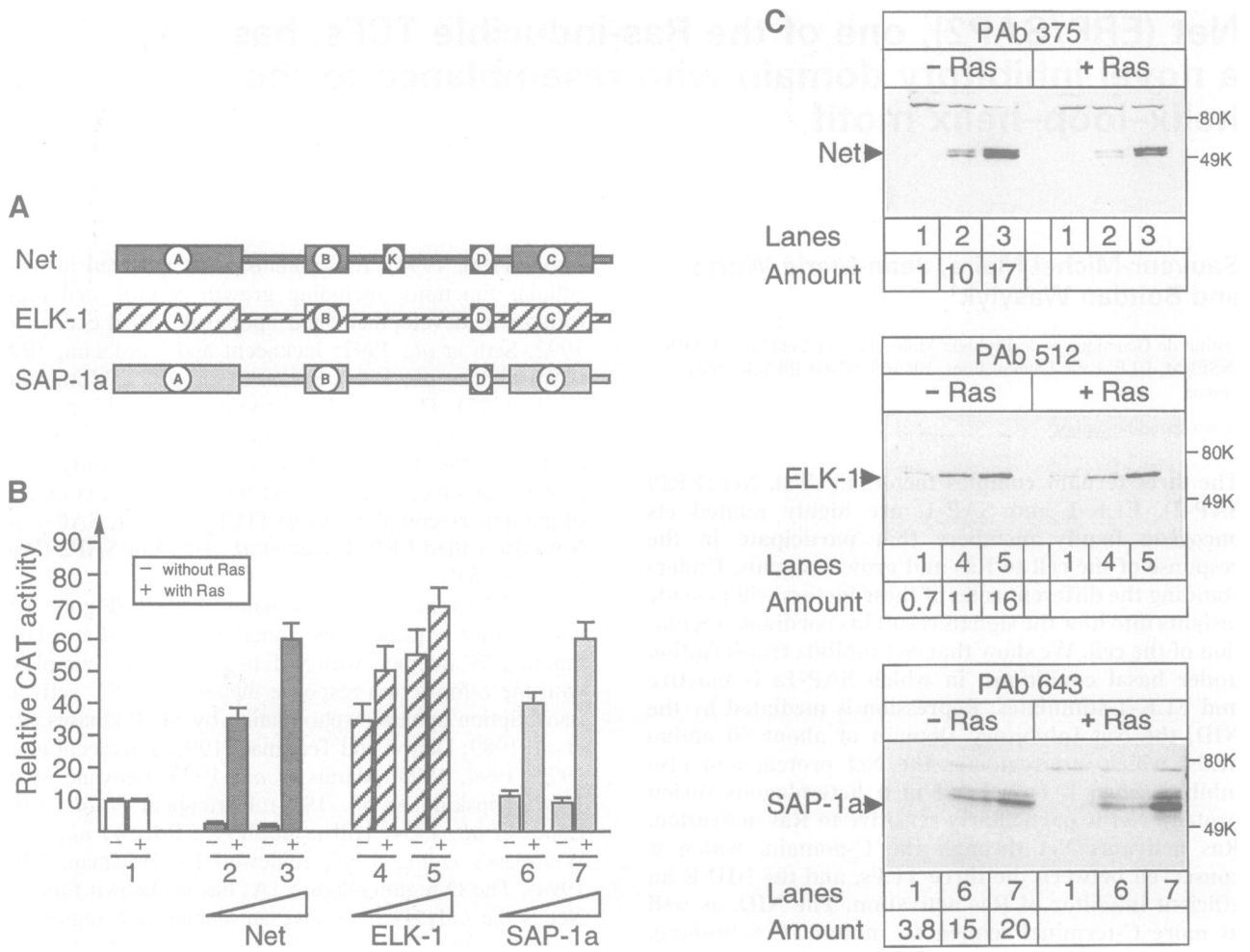

Fig. 1. Comparative transcriptional properties of Net, ELK-1 and SAP-1a. (**A**) Structural homologies between Net, ELK-1 and SAP-1a. The illustration shows the conserved domains A (Ets domain), B (interaction with SRF) and C (activation domain), D (unknown function) and the Net specific region K (Krüppel-like). (**B**) Transcriptional activities of the three homologues and the effects of Ras. NIH-3T3 cells were co-transfected with the ets reporter (5 μg of PALx8–TK–CAT, all samples) and expression vectors for the Ets proteins (murine Net, pTL2–Net, bars 2 and 3; pTL1–Elk1, human ELK-1, bars 4, 5; human SAP-1a, pKOZ1–Sap1a, bars 6 and 7) and Ras (2.5 μg pRCBx2, + bars). The amounts of ets expression vectors were either 0.5 μg (bars 2, 4 and 6) or 2 μg (bars 3, 5 and 7). CAT activities are expressed relative to the reporter alone, arbitrarily set to 10 (bars 1, the basal level). (**C**) Quantitation of protein expression levels. Proteins in 20 μl of whole cell (TGKD) extract of transfected cells (see Materials and methods) were run on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes, which were incubated with specific antibodies for Net (PAb375, upper panel), ELK-1 (PAb512, middle panel) or SAP-1a (PAb643, bottom panel). Equivalent samples have the same numbers in (B) and (C) (Lanes). The numbers (Amount) at the bottom of each panel refer to the amounts of each TCF relative to endogenous Net (considered as 1). The values take into account the relative efficiencies of the three antibodies, determined using known amounts of *in vitro*-translated proteins that were loaded on the same gels used for quantitative Western blotting. Specific bands were scanned with a CS-9000 Shimadzu scanner.

of the myogenic factors. Net interacts *in vivo* with E47, a protein known to heterodimerize with myogenic factors. We propose that interactions of the inhibitory domain with other factors may give a unique role to Net, among the highly related ternary complex factors.

Results

Comparative transcriptional analysis of Net, ELK-1 and SAP-1a

Transcription regulation by the three related factors Net, ELK-1 and SAP-1a (Figure 1A) was compared directly in transfection assays in NIH-3T3 cells using a reporter containing ets motifs linked to the thymidine kinase promoter and CAT coding sequences (PALx8–TK–CAT). As shown previously (Giovane *et al.*, 1994), Net expression efficiently inhibited transcription (Figure 1B, compare bars –2 and –3 with –1), whereas ELK-1 activated transcription (bars –4 and –5). In contrast, SAP-1a was found to differ from both, in that it was essentially inactive (bars –6 and –7). The effects were similar at all levels of expression vector tested (0.5 and 2 μg in the results shown in Figure 1B). Protein expression levels were compared by Western blotting using rabbit polyclonal antibodies raised against sequences specific for each protein (Figure 1C). TCF protein levels were estimated by quantitative Western blotting using known amounts of *in vitro*-translated proteins as standards (Figure 1C, numbers at the bottom of each panel). Non-transfected NIH-3T3 cells contained similar amounts of ELK-1 and Net, and about 4-fold more SAP-1a. Transfection resulted in the expression of comparable levels of the TCFs for each amount of expression vector, showing that the differences in transcriptional activity are not due to differences in expres-

sion levels. Net apparently has a unique negative effect on transcription, distinct from ELK-1 and SAP-1a, that cannot be simply explained by levels of expression.

The responses of Net, ELK-1 and SAP-1a to activated Harvey Ras were also compared. Co-expression of Ras activated all three factors (Figure 1B; compare – and +, bars 2–7; note that basal promoter activity does not increase, bars 1). The stimulation was 60-fold for Net, a striking increase because of the low level from which it increases (compare – and +, bars 2 and 3). SAP-1a activity increased to a smaller extent (6-fold, bars 6 and 7), and ELK-1 much less (1.2-fold, bars 4 and 5). Ras did not significantly affect expression levels (Figure 1C, – and +, lanes 1–7), showing that stimulation did not result from changes in the amounts of the factors. Interestingly, Ras has a small but reproducible effect on the mobility of Net on SDS–PAGE. Net migrated as a doublet of ~52 kDa mol. wt under basal conditions. Ras expression increased the relative amount of the slower-migrating band (compare – and +, lanes 2 and 3), and phosphatase decreased the ratio (not shown), as expected for post-translational modification by phosphorylation (see below). In conclusion, Net appears to be particularly sensitive to Ras activation compared with the other TCFs, due to its negative effect on the promoter. However, these differences could be quantitative rather than qualitative in nature.

Localization of an inhibitory domain in Net

An inhibitory domain was 'isolated' from Net using heterologous Gal4–Net fusion proteins (Figure 2A) and a different reporter (pGal–TK–CAT). The Gal4 DNA-binding domain (1–147) was linked to Net sequences that were progressively deleted from either the N-terminus (Gal–N1 to –N6), or the C-terminus (Gal–C7ΔA to Gal–C11ΔA), or both (Gal–NC1 and 2). None of the hybrids contained the ets domain, to avoid potential non-specific effects due to a second DNA-binding domain. Gal4(1–147) alone had no effect on basal reporter activity (Figure 2B, bars –1 and –2), whereas Gal–N1, –N2 and –N3 inhibited (bars –3 to –5) and Gal–N4, –N5 and –N6 were inactive (bars –6 to –8). Gal–C7ΔA, –C8ΔA and –C9ΔA inhibited, in contrast to Gal–C10ΔA and –C11ΔA, which did not (bars –9 to –13). These results show that the Krüppel-like region is not required for inhibition and that there is an inhibitory domain that extends from amino acids 152 to 209. This region alone was sufficient to inhibit (Gal–NC2, bar –15), and the adjacent conserved B-domain did not further affect inhibition (Gal–NC1, bar –14). The proteins were expressed to similar levels, as measured by immunoblotting of transfected cell extracts with anti-Gal4 monoclonal antibodies (Figure 2C), showing that differences in expression levels could not account for the observations. These results identify an inhibitory domain (NID) in Net.

The fusion proteins were used to study the effects of Ras on inhibition. The C region with the flanking C-terminal amino acids was necessary and sufficient for activation by Ras (Figure 2A and B, Gal–N6 and –C7ΔA to –C11ΔA, bars +8 to +13). The Ras response was enhanced 2-fold by the adjoining 219–327 region (Gal–N5 and –N6, bars +7 and 8), suggesting that the C-domain is not sufficient for full induction. The Krüppel-like region did not affect Ras activation (Gal–N4 and –N5; bars +6 and 7). The NID efficiently inhibited Ras-induced activity. It lowered CAT activity by 360 units (Gal–N3 and –N4, bars +5 and +6), whereas the decrease was ~9 units in the absence of Ras (~40-fold less, compare bars –4 and –5). Ras did not affect the expression levels of the Gal fusion proteins (data not shown). These results indicate that Ras does not regulate the inhibitor directly, but rather the ID is a somewhat better inhibitor of Ras-activated Net.

NID inhibits both transactivation and specific DNA binding in the context of the Net protein

ELK-1 does not appear to have an inhibitory function equivalent to Net (see above). ELK-1 sequences were used to create a Net chimera, Net_{elk} (Figure 3A), in which the NID is replaced by a sequence from ELK-1, that comes from an equivalent position just C-terminal to the B-domain, but has no obvious sequence similarity (ELK-1 amino acids 169–228; hatched bar in Figure 3A). In contrast to Net, Net_{elk} did not repress the basal activity of the PALx8–TK–CAT reporter in NIH-3T3 cells (Figure 3B; compare bars –2 to –5), even though it was expressed at equivalent levels (Figure 3C, lanes 1–5). Ras increased Net_{elk} activity to ~4-fold higher levels than Net (Figure 3B, compare bars +2 and +3 with +4 and +5), without affecting overall expression levels (Figure 3C, compare – and +, lanes 2–5). Interestingly, Ras expression had a distinct effect on the mobility of Net_{elk} on SDS–PAGE, resulting in the appearance of several new bands with slower mobility (Figure 3C, lane +5), suggesting that perhaps Net_{elk} is more sensitive than Net to modification, although there are other possibilities (see below and Discussion). We have studied the effects of NID in the context of the ELK-1 protein (data not shown). An ELK_{net} chimera, with NID (amino acids 153–208 of Net) in the place of the equivalently positioned sequence of ELK-1 (168–229) was a less efficient activator than ELK-1 (relative CAT activities: basal = 10; ELK-1 = 35; ELK_{net} = 15) and the m4 mutation in NID (see Figure 7A) restored ELK-1 activity (ELK_{netm4} = 35). Ras expression increased the activity of ELK-1 and ELK_{netm4} to similar extents (both ~50 relative CAT units), but had little effect on both basal activity and ELK_{net} (10 and 15 units, respectively). These results show that the NID represses transcription in the context of the Net protein, as well as in different heterologous fusion proteins.

Net binds relatively inefficiently to various ets motifs compared with the other TCFs (Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995). We have previously shown that Net binds to a consensus ets binding site (PEA3*, Giovane *et al.*, 1994). To examine whether the NID affects specific DNA binding, we used EMSA and a natural Net motif from the IgH enhancer (μE2-π; Lopez *et al.*, 1994). Net did not bind to this probe (Figure 3D, lanes 1 and 2), whereas both Net_{elk} and ELK-1 bound efficiently (lanes 3 and 4). The complexes were specific, since they did not form on a mutated probe (data not shown). The non-specific complexes had different intensities (see upper band in all lanes). These variations were not reproducible and were unrelated to specific complex formation (data not shown). Inhibition of DNA binding by the NID was investigated further with mutants truncated from the C-terminus (Figure 4A). Net and deletion mutants lacking sequences C-terminal to the NID did not bind

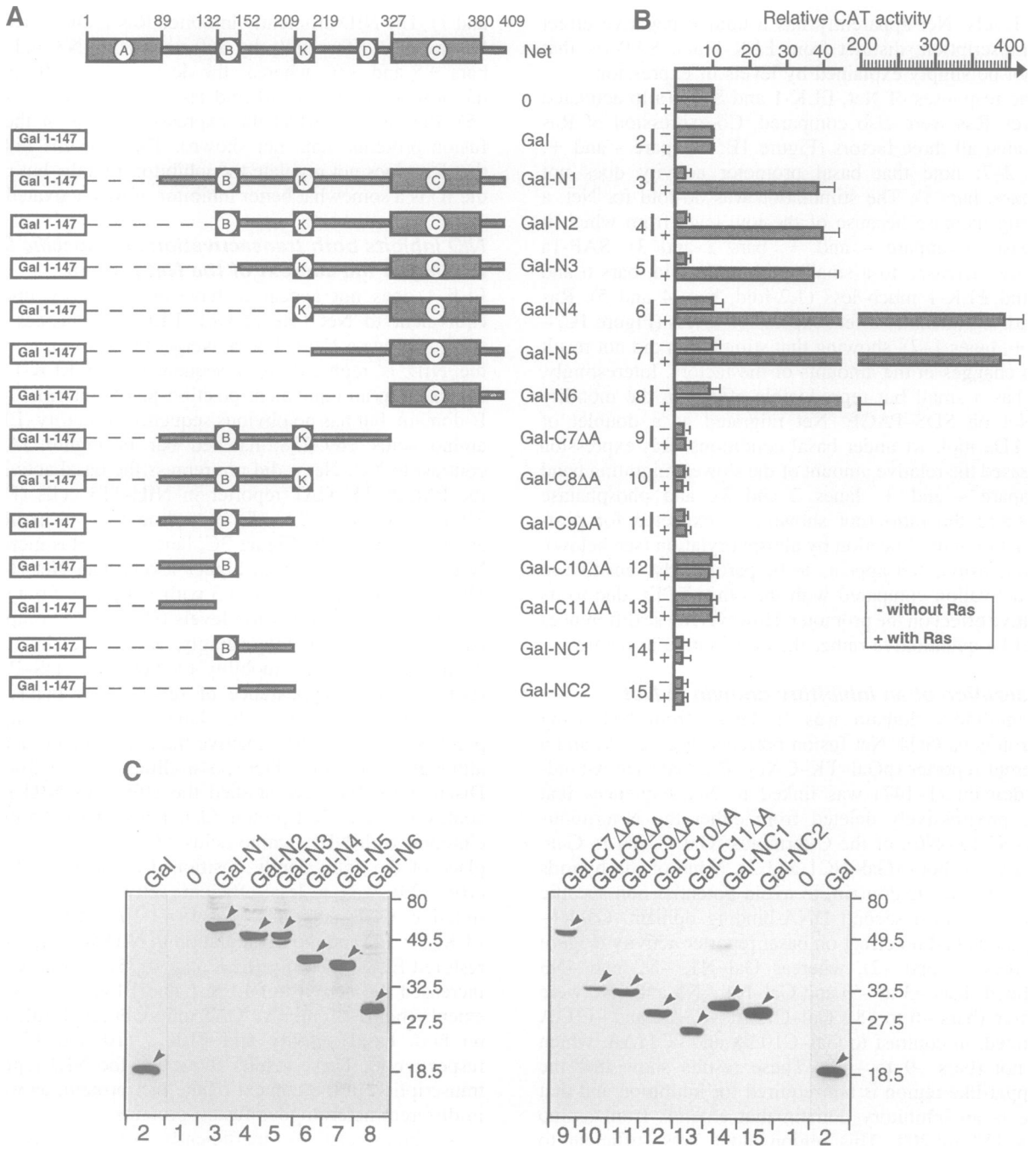

Fig. 2. Localization of an inhibition domain (NID) in Net using Gal4 fusions. (**A**) Protein structure. The fusion proteins are composed of the Gal4 DNA-binding domain (amino acids 1–147) linked to Net deletion mutants lacking the Ets homology (domain A). Numbers refer to amino acids. (**B**) Transcriptional activities of the Gal–Net deletion mutants and the effects of Ras. NIH-3T3 cells were co-transfected with the reporter (5 μg Gal–TK–CAT, all samples) and expression vectors for the illustrated proteins (2 μg; the pSG5 empty vector, pGal, pGal–N1 to –N6, pGal–C7ΔA to –C11ΔA, pGal–NC1 and –NC2; bars 1–15, respectively) and Ras (2.5 μg pRCBx2, + bars). CAT activities are expressed relative to the reporter alone, arbitrarily set to 10 (bars 1, the basal level). (**C**) Quantitation of protein expression levels. Proteins in 20 μl of whole cell (TGKD) extracts of transfected cells [numbered as in (B), without Ras] were loaded on 10% SDS–PAGE gels and transferred to nitrocellulose membranes, which were incubated with Gal4-specific antibodies (mix of MAbs 2GV3 and 3GV2). Arrows indicate bands of the expected size.

detectably (Net and C7 to C9, Figure 4B, panel 1, lanes 1–5), whereas mutants lacking the NID (C10) and in addition sequences up to the ets domain (C11 and C12) all bound efficiently (lanes 6–8).

TCF binding to the c-fos SRE is stimulated by SRF *in vitro*. However, the TCFs may have a role in the absence of SRF. Some studies find that mutating the SRF motif does not abolish activation of the SRE by Ras, whereas TCF-motif mutation does (Gutmann *et al.*, 1991). The 'natural' c-fos SRE probe gave essentially similar results to the IgH ets motif. Net did not bind (Figure 4B, panel 2, lanes 1 and 2), truncating the C-domain and sequences up to the NID led to formation of a weak but detectable complex (C7 to C9, lanes 3–5), while further mutation of NID (C10) and beyond (C11 and C12) generated strong complexes (lanes 6–8). The relative

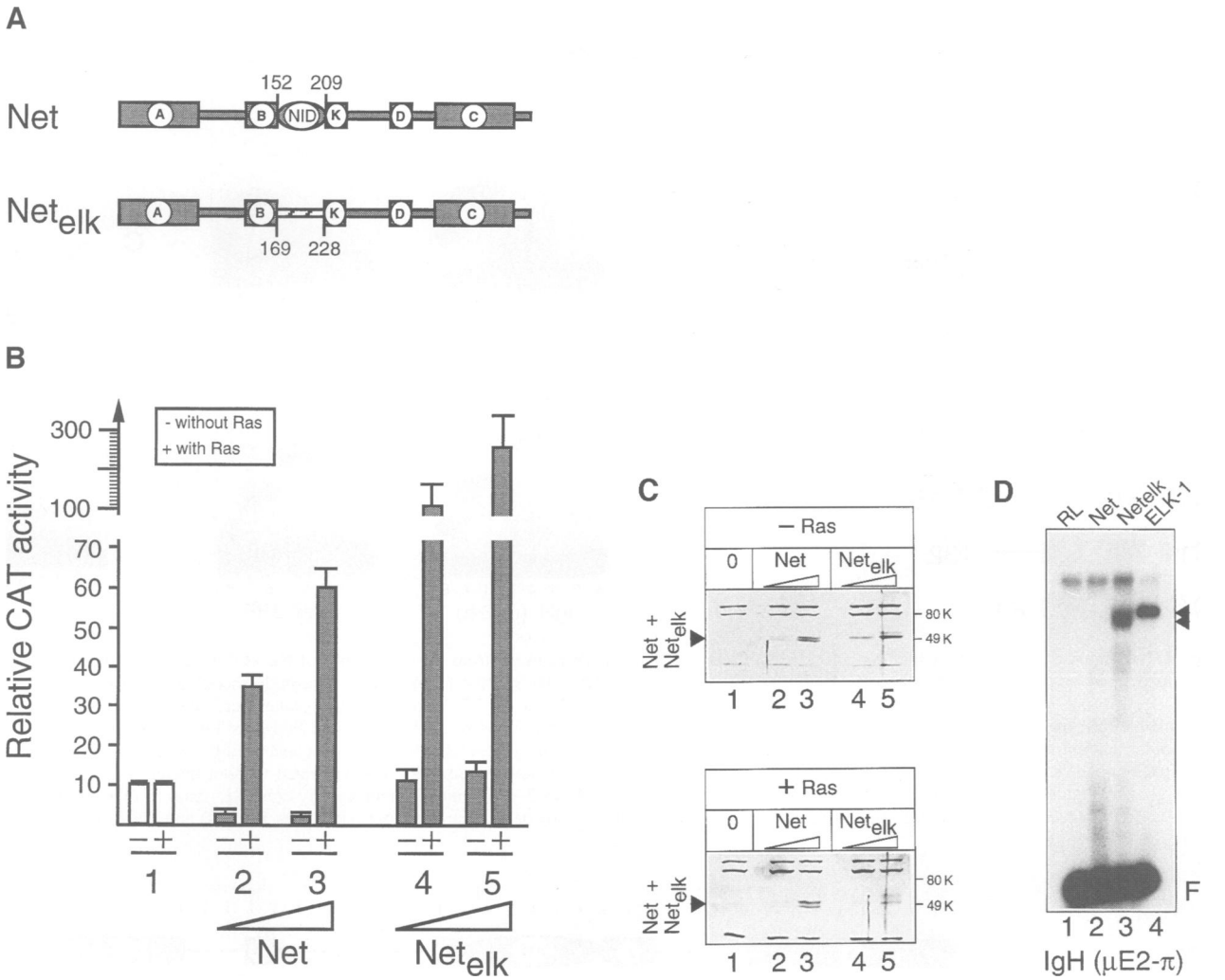

Fig. 3. The NID affects the transcriptional and DNA-binding activities of Net. (**A**) Protein structures. In the Net$_{elk}$ chimera, the Net inhibitory domain (NID, grey oval, amino acids 153–208) is replaced by the corresponding region of ELK-1 (hatched rectangle, amino acids 169–228 of human ELK-1). (**B**) Transcriptional activities of Net and Net$_{elk}$. NIH-3T3 cells were co-transfected with the ets reporter (5 μg of PALx8–TK–CAT, all samples) and expression vectors for Net (pTL2–Net; 0.5 μg, bars 2; 2 μg, bars 3), Net$_{elk}$ (pTL2–Net$_{elk}$; 0.5 μg, bars 4; 2 μg, bars 5) and Ras (2.5 μg pRCBx2, + bars). CAT activities are expressed relative to the reporter in the presence of the empty ets expression vector (pTL2), arbitrarily set to 10 (bars 1, the basal level). (**C**) Quantitation of protein expression levels. Proteins in 20 μl of whole cell (TGKD) extract of transfected cells (see Materials and methods) were loaded on 10% SDS–PAGE gels and transferred to nitrocellulose membranes, which were incubated with PAb375, which recognizes the C-terminal tails of both Net (amino acids 387–409) and Net$_{elk}$. The same numbers in (B) and (C) correspond to equivalent samples. Arrowheads indicate the Net and Net$_{elk}$ sized bands. (**D**) Specific DNA binding. Net, Net$_{elk}$ and ELK-1 were labelled with [^{35}S]methionine during synthesis in rabbit reticulocyte lysates (Promega), quantitated by SDS–PAGE and Phosphor-Imaging (not shown), and equimolar amounts of Net, Net$_{elk}$ and ELK-1 or an equivalent volume of mock reticulocyte lysates (RL) were used in EMSA with the IgH (μE2-π) probe. F, free probe. Arrowheads, specific complexes.

effects of the deletions depended upon the conditions of electrophoresis. When glycerol was included in the gel (Figure 4B, panels 3 and 4), the C-domain truncations (C7 and C8) had a more striking effect on complex formation with both probes, and there was no further effect due to deletion of the NID (C9, C10). Glycerol may stabilize protein–DNA interactions and counteract destabilizing effects of the NID. SRF stimulates DNA binding of full-length Net to form a ternary complex on the SRE (Giovane *et al.*, 1994) showing that NID does not abolish complex formation. In summary, Net apparently has a latent DNA-binding activity, i.e. a 'closed' conformation, due mainly to two domains, NID and C.

Studies with specific DNA-binding proteins such as Ets1 and p53 have shown that antibodies which interact with apparently equivalent inhibitory domains stimulate DNA binding, presumably because they 'open' the structure (Seth *et al.*, 1993; Hupp *et al.*, 1995). We performed similar experiments with antibodies raised against the C-terminus and the NID (PAb375 and PAb376, respectively). The antibodies against both regions induced DNA binding by Net (Figure 4C, lanes 2, 3, 5 and 6), whereas non-specific antibodies had no effect (lanes 1 and 4). These results suggest that Net has two domains that inhibit DNA binding, reminiscent of Ets1 (Hagman and Grosschedl, 1992; Lim *et al.*, 1992; Wasylyk *et al.*, 1992; Hahn and Wasylyk, 1994). Overall, our results, both *in vivo* and *in vitro*, show that the NID is a critical domain of Net. NID inhibits basal promoter activity and Ras-induced transcription activation *in vivo*. It lowers specific DNA binding *in vitro*.

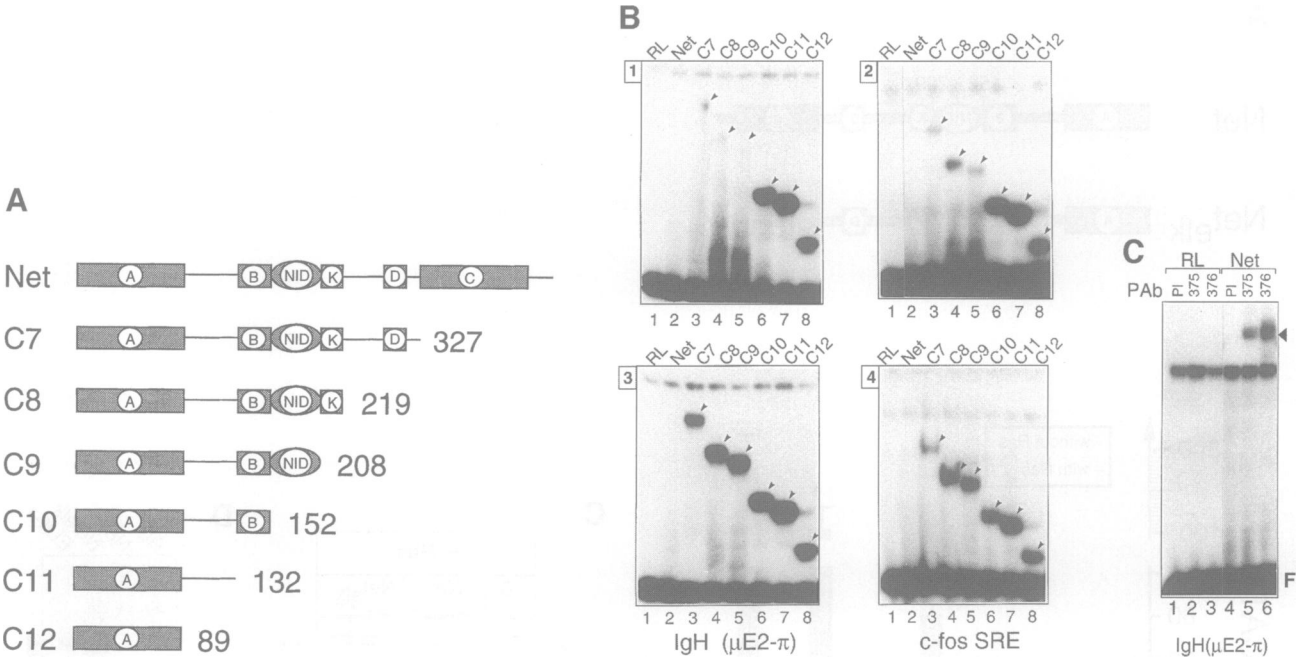

Fig. 4. NID inhibits specific DNA-binding activity. (**A**) Protein structures. Truncations from the C-terminus of Net (C7–C12) progressively delete regions defined by homology or function (C, K, ID, B and A; see Figure 1A). (**B**) Specific DNA binding. Plasmids encoding Net (pTL2–Net) and C7–C12 (pKOZ–C7 to –C12, respectively) were cut with *Xba*I and transcribed with T7 RNA polymerase. Proteins were labelled with [^{35}S]methionine during synthesis in rabbit reticulocyte lysates (Promega) and quantitated by SDS–PAGE and Phosphor-Imaging (not shown). Equimolar amount of Net (lanes 2) and C7–C12 (lanes 3–8), or mock reticulocyte lysates (RL, lanes 1), were analysed by EMSA using either the IgH (μE2-π; panels 1 and 3) or the c-fos SRE (panel 2 and 4) probes in 4% polyacrylamide gels co-polymerized without (panels 1 and 2) or with 2.5% glycerol (panels 3 and 4). (**C**) Supershifts. Equimolar amount of Net (lanes 3–6) or mock reticulocyte lysates (RL, lanes 1–3) were analysed by EMSA. Binding reactions contained PAb375 (lanes 2 and 4), PAb376 (lanes 3–6) or preimmune serum (PI, lanes 1 and 4) and the IgH (μE2-π) probe.

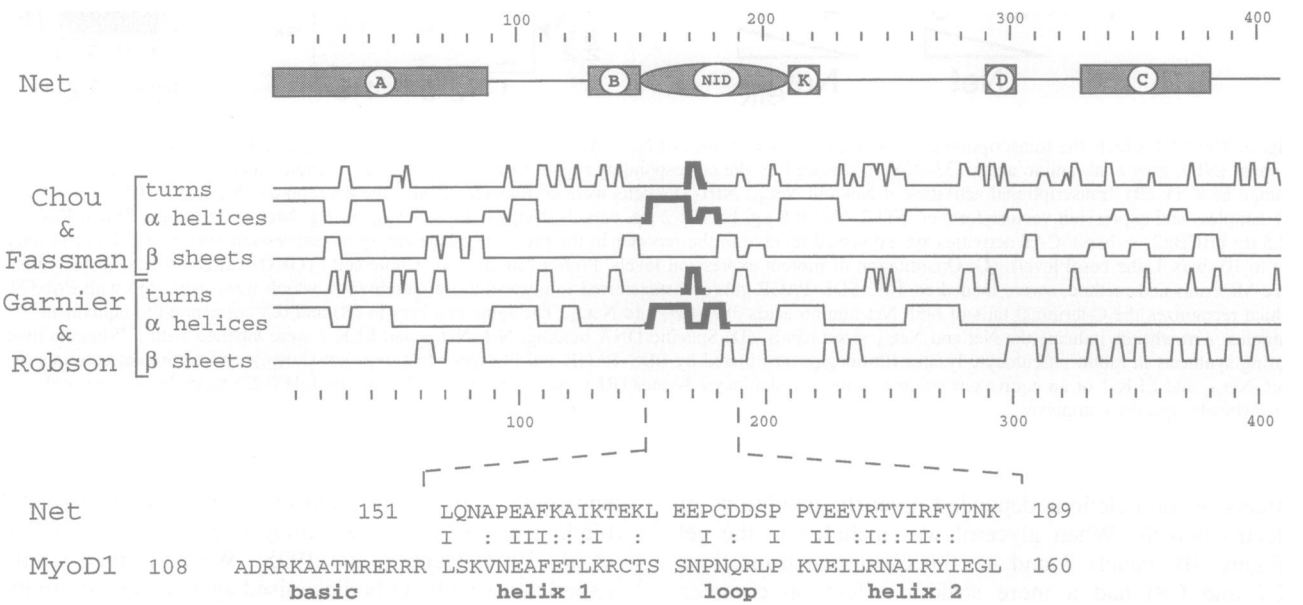

Fig. 5. Structure predictions and homology to MyoD1. The NID contains a putative helix–loop–helix (HLH) structure with homology to MyoD1. The top panel illustrates the functional domains of Net. The middle panel shows the secondary structures of Net (turns, α helices and β sheets) predicted by both the Chou and Fassman and the Garnier and Robson algorithms, using the PEPTIDESTRUCTURE program and displayed with PLOTSTRUCTURE program (UWGCG Package). A putative HLH domain in the NID is indicated in bold. The bottom panel displays the homology alignment between Net and MyoD1 generated by searching through the GenEMBL databank with the FASTA program (UWGCG) and the NID.

NID contains a putative HLH motif and resembles myogenic factors

The secondary structure of the NID was analysed with two different algorithms, Chou and Fassman and Garnier and Robson (see Figure 5). Significantly, both predicted a HLH structure for the NID (Figure 5, bold lines). Homology searches in the GenEMBL data bank with NID sequences (FASTA, UWGCG Package) revealed resemblances with the HLH-motifs of MyoD1 (Figure 5) and other myogenic factors (myf3, myogenin, myf5; see

Figure 6A, but also data not shown). There is a large family of proteins with HLH motifs that mediates protein–protein interactions (Murre *et al.*, 1994). Some have an N-terminal basic domain (the bHLH factors) that mediates DNA binding. The putative HLH and adjoining N-terminal sequence of the NID were compared with HLH proteins in the data bank and used to generate a dendogram (DISTANCES and GROWTREE, UWGCG Package). The Net sequence was found to be most closely related to the myogenic factors (mNET, mMYF5, mMYOD, mMYF6, mMYOG; data not shown), and in turn this group resembles the HLH proteins that lack a basic domain (demc, mID1–4; Murre *et al.*, 1994). The close sequence resemblance to the myogenic proteins led us to use the known structure and function relationships in this sub-family of proteins to study the properties of NID.

The three-dimensional structure of MyoD1 has been determined by X-ray crystallography (Ma *et al.*, 1994). The critical residues that stabilize the MyoD1 HLH motif are extremely well conserved among members of the HLH family (Figure 6A, white columns; Phillips, 1994). Related amino acids are present in Net, except for E178 (red box) that replaces I149 in MyoD1. The three-dimensional structure of MyoD1 was used to model the HLH of Net NID as a monomer. The sequence alignment shows that the HLH motifs of Net and MyoD1 have the same length. Consequently, an initial model was generated using the Cα-trace of the MyoD1 HLH as a template on which the side-chains were positioned (Holm and Sander, 1992). This model was refined by minimization (Van Gusteren and Berendsen, 1987) to correct the geometry. A molecular dynamics simulation was then carried out, in which the Cα atoms of helices 1 and 2 were maintained fixed and their side-chains and the loop segment were allowed to move, in order to relax their geometry (Van Gusteren and Berendsen, 1987). The last conformer of the simulation was then minimized, first with the helices' Cα-atoms constrained and then without constraint, giving the final structure shown in Figure 6B. The ID–HLH homodimer model (data not shown) was generated by superposing the Cα atoms of helices 1 and 2 with those of the MyoD1 homodimer (Ma *et al.*, 1994).

In the monomer (Figure 6B), residues 154–187 form two helices (in green and blue) connected by an eight-residue loop (in pink). The monomer is stabilized by numerous hydrophobic contacts in the interior (I161, T181, V182, F185, coloured black in Figure 6B). These hydrophobic residues are conserved in other HLH proteins (Figure 6A, white bars). Interestingly, the highly conserved F158 in helix 1 makes a hydrophobic contact with P175 at the beginning of helix 2. F185 at the end of helix 2 stacks against the hydrophobic side-chain of E164 at the end of helix 1 and caps its C-terminus. Net is unusual in that it has charged residues, K165 and E178 (boxed in red in Figure 6A), in the place of hydrophobic amino acids in other HLHs. In the model, the long aliphatic chains of these residues contribute to the hydrophobic core of the monomer and ionic interactions between them potentially stabilize the HLH structure and constrain the ends of the loop (Figure 6B). The loop forms an extended structure different from MyoD1. It is mainly composed of acidic side chains that are accessible to the solvent. The loop backbone packs around the salt bridge formed by K165 and E178, and the side chains of C170 and S173 are orientated towards the core. In human Net there is a Pro in the place of Ala154 of mouse Net. This amino acid is at the beginning of the first helix and does not disrupt the structure. The NID–HLH dimer (data not shown) is a compact four-helix bundle that is stabilized by numerous hydrophobic contacts (A157, F158, A160, I161, V176, V179, V182, F185 and V186) between amino acids that are highly conserved. Interestingly, as in MyoD1 and E47, F158 in helix 1 of each monomer forms van der Waals interactions across the dimer interface. In the same way, at the C-terminus of helix 2, F185 and V186 form hydrophobic dimer contacts.

The model of the Net HLH is of good quality. In a Ramachandran plot, 89.3% (25) of non-proline residues of the NID HLH are in most favoured regions, and 10.7% (3) in allowed regions, which compares with 90% and 10% for MyoD1. Good-quality models are expected to have ~90% or more in the favoured regions, as deduced from the analysis of 118 structures of at least 2.0 Å resolution and R-factors not greater than 20%. These results strongly suggest that the NID adopts a HLH structure.

Mutations expected to disrupt the NID HLH structure affect inhibition

The HLH motifs of MyoD1 and E47 have been studied by point mutation (Davis *et al.*, 1990; Voronova and Baltimore, 1990). Equivalent mutations, that are expected to affect the structure, were introduced in Gal–NC2 (Figure 7A; m1, m3 and m4, cf. MyoD1, Davis *et al.*, 1990; m2, cf. E47, Voronova and Baltimore, 1990). All of the mutations decreased inhibition of the pGal–TK–CAT reporter in NIH-3T3 cells (Figure 7B). Gal–NC2 m1 and m2 lost ~50% of the inhibition (compare bars –3 and –4, respectively, with –2 and –1), whereas m3 and m4 lost all of it (bars –5 and –6). The proteins were expressed at similar levels (Figure 7C, lanes 1–6). In the context of the whole Net protein, the m4 mutation abolished inhibition of transcription from the PALx8–TK–CAT reporter (Net m4, Figure 7A and B, bars –7 to –11), and raised Ras-stimulated activity to ~4-fold higher levels than Net (compare + and –, bars 7 to 11), without affecting overall expression levels (Figure 7C, – and + Ras, lanes 7 to 11). Interestingly, the mutant migrates differently upon SDS–PAGE, somewhat reminiscent of Net_{elk}. The post-translationally modified form of Net, which migrates more slowly in the doublet, increased in relative amount upon Ras expression (Figure 7C, – Ras, lane 8 and + Ras, data not shown), and decreased with phosphatase treatment (data not shown). The ratio of the slower- to faster-migrating forms was higher for the mutant compared with the wild-type protein in the absence of Ras (–Ras, lanes 10 and 11), and the ratio for the wild-type protein only became similar after Ras expression (+Ras, lanes 8 and 9), suggesting that Net m4 is more sensitive to post-translational modification under basal conditions.

We also studied the effect of the mutation on specific DNA binding. Net m4 forms a specific complex with the IgH (μE2-π) probe similar to ELK-1, and in contrast to Net (Figure 7D, lanes 1–4); there is an endogenous binding activity that migrates above the Net complex that is also present with mock RL. The properties of Net m4 are

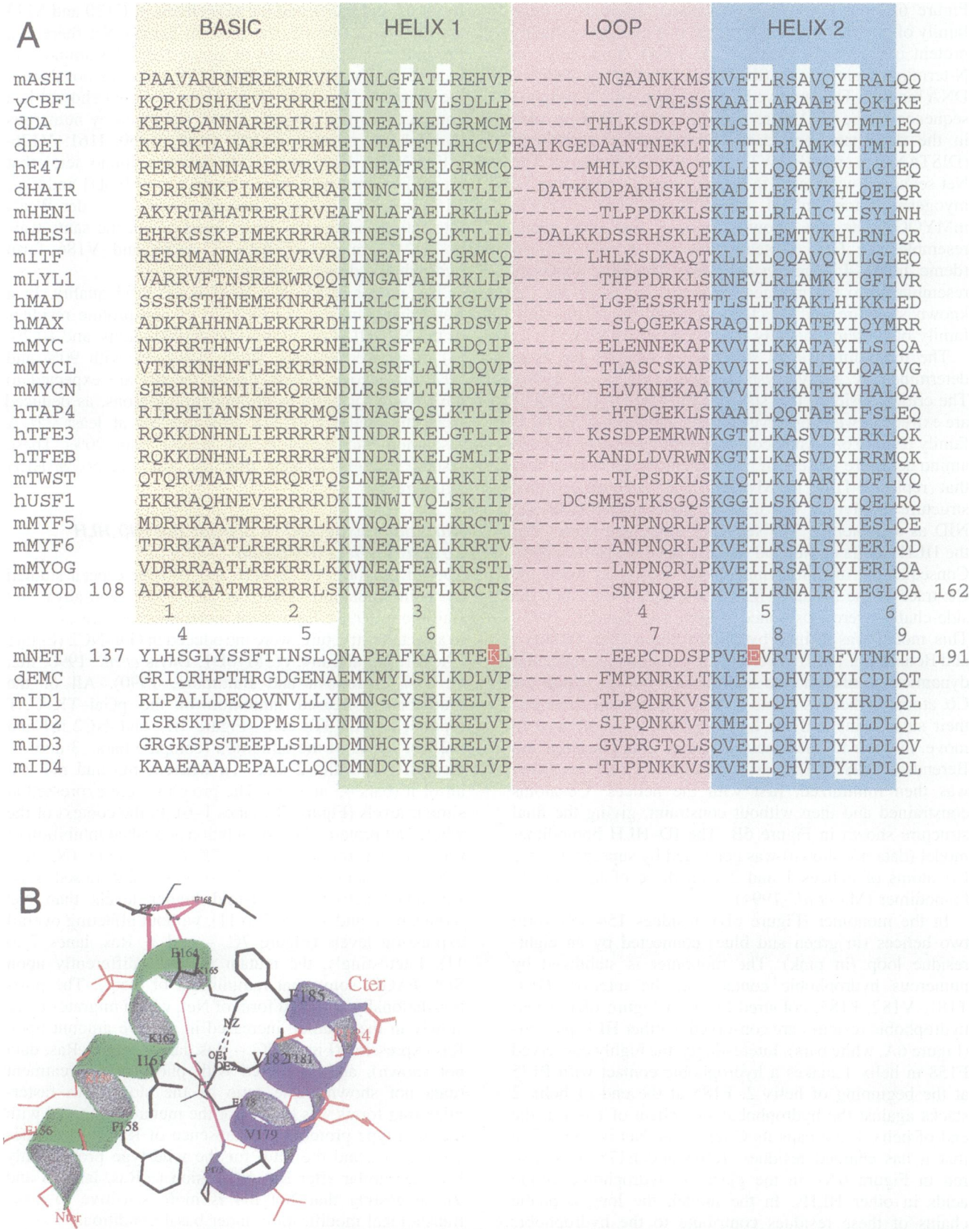

Fig. 6. Multiple alignment of HLH proteins (A) and three-dimensional models of the Net–HLH monomer. (**A**) The CLUSTALW program (Thompson *et al.*, 1994) was used to align selected HLH proteins from the GenEMBL database. For highly homologous proteins from different species, the closest to mouse was chosen. The basic regions of the bHLH proteins are indicated in yellow, whereas non-basic sequences in an equivalent position are left uncoloured. Helix 1 is coloured in green, the loop in pink and helix 2 in blue. Amino acids in the helices that stabilize the structure of the MyoD1 HLH and are conserved are left uncoloured. K165 and E178 of Net that are predicted to form a salt bridge are boxed in red. (**B**). Ribbon backbone representation of the Net–HLH monomer. The colours are as in (A), except that buried side chains that potentially stabilize the structure are in black. Dashed lines indicate the K165–E178 salt-bridge. The picture was generated with Molscript (Kraulis, 1991).

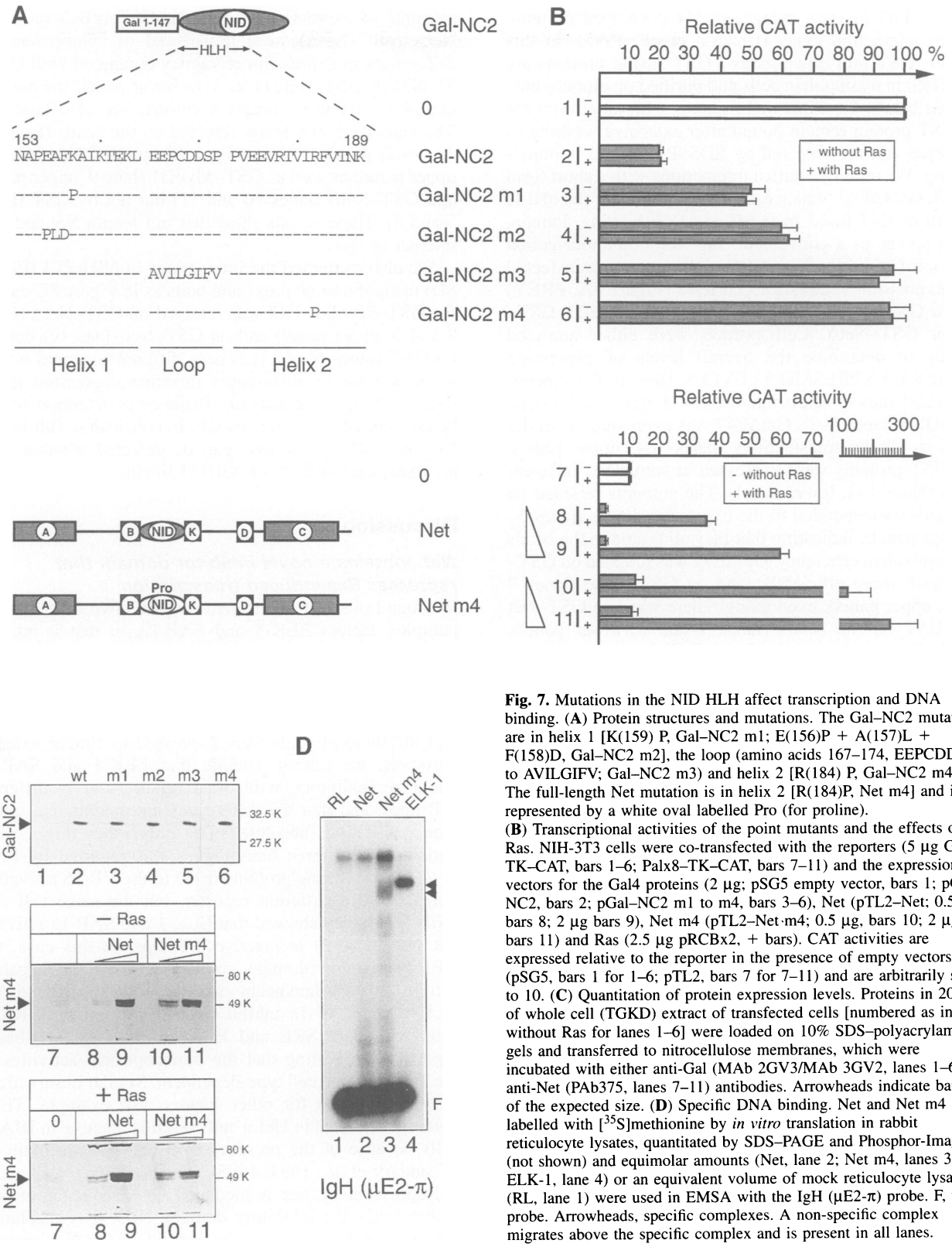

Fig. 7. Mutations in the NID HLH affect transcription and DNA binding. (**A**) Protein structures and mutations. The Gal–NC2 mutations are in helix 1 [K(159) P, Gal–NC2 m1; E(156)P + A(157)L + F(158)D, Gal–NC2 m2], the loop (amino acids 167–174, EEPCDDSP to AVILGIFV; Gal–NC2 m3) and helix 2 [R(184) P, Gal–NC2 m4]. The full-length Net mutation is in helix 2 [R(184)P, Net m4] and is represented by a white oval labelled Pro (for proline).
(**B**) Transcriptional activities of the point mutants and the effects of Ras. NIH-3T3 cells were co-transfected with the reporters (5 μg Gal–TK–CAT, bars 1–6; Palx8–TK–CAT, bars 7–11) and the expression vectors for the Gal4 proteins (2 μg; pSG5 empty vector, bars 1; pGal–NC2, bars 2; pGal–NC2 m1 to m4, bars 3–6), Net (pTL2–Net; 0.5 μg, bars 8; 2 μg bars 9), Net m4 (pTL2–Net·m4; 0.5 μg, bars 10; 2 μg, bars 11) and Ras (2.5 μg pRCBx2, + bars). CAT activities are expressed relative to the reporter in the presence of empty vectors (pSG5, bars 1 for 1–6; pTL2, bars 7 for 7–11) and are arbitrarily set to 10. (**C**) Quantitation of protein expression levels. Proteins in 20 μl of whole cell (TGKD) extract of transfected cells [numbered as in (B), without Ras for lanes 1–6] were loaded on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes, which were incubated with either anti-Gal (MAb 2GV3/MAb 3GV2, lanes 1–6) or anti-Net (PAb375, lanes 7–11) antibodies. Arrowheads indicate bands of the expected size. (**D**) Specific DNA binding. Net and Net m4 were labelled with [^{35}S]methionine by *in vitro* translation in rabbit reticulocyte lysates, quantitated by SDS–PAGE and Phosphor-Imaging (not shown) and equimolar amounts (Net, lane 2; Net m4, lanes 3; ELK-1, lane 4) or an equivalent volume of mock reticulocyte lysate (RL, lane 1) were used in EMSA with the IgH (μE2-π) probe. F, free probe. Arrowheads, specific complexes. A non-specific complex migrates above the specific complex and is present in all lanes.

strikingly similar to Net_{elk}, in which the whole of the NID was replaced by sequences from ELK-1 (see above). These results suggest that the important structural feature of the NID is an HLH motif, which may mediate interactions with other proteins such as repressors and bHLH transcription factors.

Net interacts with E47 in vivo

The sequence resemblance between the NID and the HLH of MyoD1 suggests that they may interact with the same proteins. MyoD1 forms heterodimers with the E2A gene products E12 and E47 more efficiently than homodimers with itself (Murre *et al.*, 1994). We tested whether Net also

binds to E47 *in vivo*, using a newly developed protein–protein interaction assay (Chatton *et al.*, 1995). In this *in vivo* 'GST pull down assay', GST fusion proteins are expressed in mammalian cells and purified on glutathione–agarose beads. Co-expressed proteins with an affinity for the GST protein remain bound after extensive washing of the beads and are detected by SDS–PAGE and immunoblotting. We initially studied interactions with a short form of E47, Gal4ΔE47, which essentially consists of the bHLH domain of E47 fused to the Gal4 DNA-binding domain, which serves as a convenient 'tag' for immunodetection with anti-Gal antibodies. COS cells were co-transfected with expression vectors for GalΔE47 (Figure 8A, PREY) and the GST proteins (BAITS): GST, GST–MyoD1, GST–Net or GST–NetΔ. Cell extracts were either analysed directly to determine the overall levels of expression (Figure 8A, EXPRESSION LEVELS, lanes 1–4), or incubated with beads to detect interactions (Figure 8A, INTERACTIONS, lanes 5–8). GalΔE47 was expressed at similar levels in all the transfections (lanes 1–4, upper panel). The GST proteins were expressed at somewhat different levels (lanes 1–4, lower panel). The amounts retained on the beads corresponded to the expression levels (lanes 5–8, lower panel), indicating that they all bound to the beads with equivalent efficiency. GalΔE47 was retained on GST–Net much more efficiently than on GST alone (lanes 7 and 5, upper panel), even though there was less GST–Net than GST on the beads (lanes 7 and 5, lower panel). GalΔE47 was also retained on GST–NetΔ (a deletion mutant of Net) much more efficiently than on GST (lanes 8 and 5, upper panel), the two proteins were expressed at similar levels (lanes 8 and 5, lower panel). The greater amount of GalΔE47 retained on GST–NetΔ compared with GST–Net could be a consequence of the amounts of the GST–proteins on the beads (lanes 7 and 8, lower panel). GalΔE47 was retained on GST–MyoD1 more efficiently than on GST–Net (lanes 6 and 7, upper panel), even though the GST proteins were expressed at similar levels (lanes 6 and 7; lower panel). We conclude that the bHLH domain of E47 can interact with Net *in vivo*, albeit with a lower affinity than MyoD1.

To test whether this interaction still occurs when ΔE47 is the bait rather than Net or NetΔ, and that it requires the NID, we performed similar experiments using GST or GSTΔE47 (Figure 8B, BAITS) and Net, Net_{elk} or Net m4 (Figure 8B, PREYS). These preys and baits were expressed at similar levels (EXPRESSION LEVELS, lanes 1–6, upper and lower panels) and the preys were retained on the beads in similar amounts (INTERACTIONS, lanes 7–12, lower panel). GSTΔE47 efficiently retained Net (INTERACTIONS, lane 10, upper panel) but not Net_{elk} nor Net m4 (lanes 11 and 12, upper panel). This interaction is specific, since no binding was seen with GST alone (lane 7, upper panel). These results show that the ΔE47–Net interaction can be detected when either protein is directly bound to the beads and the interaction requires an intact NID and the bHLH region of E47.

We also tested whether the full-length proteins interact. COS cells were co-transfected with expression vectors for E47 (Figure 8C, PREY) and GST–Net or the control baits GST, GST–MyoD1 or GST–NetΔ (Figure 8C, BAITS). E47 was equally expressed in all transfections (EXPRESSION LEVELS, lanes 1–6, upper panel). Two different amounts of expression vectors for GST (pBC) or GST–NetΔ (pBC–NetΔ) were transfected to compensate for differences in expression efficiency compared with GST–MyoD1 or GST–Net (lanes 1–6, lower panel: the band in lane 4 is visible on longer exposure; see also lane 10). The baits were efficiently retained on the beads (lanes 7–12, lower panel). E47 was retained by GST–Net (lane 12, upper panel) as well as GST–MyoD1 (lane 9, upper panel) and GST–NetΔ (lanes 10 and 11) but not by GST (lanes 7 and 8). These results show that full-length Net and E47 interact *in vivo*.

We also examined the interactions in NIH-3T3 (Figure 8D) using the same preys and baits as in Figure 8C except for GST–MyoD1. E47 was retained on GST–NetΔ (lanes 8 and 9, upper panel) and on GST–Net (lane 10) but not on GST (lanes 6 and 7). These differences could not be accounted for by differences in either expression levels (lanes 1–5, lower panel) or efficiency of retention on the beads (lanes 6–10, lower panel). In conclusion, full-length Net and E47 interactions can be detected whether they are expressed in COS or NIH-3T3 cells.

Discussion

Net contains a novel inhibitor domain that represses Ras-induced transcription

We found that Net is different from the two other ternary complex factors ELK-1 and SAP-1a, in that it inhibits promoter activity, whereas SAP-1a has no effect and ELK-1 activates it. This difference is not an indirect consequence of expression levels, since it was observed over a wide range of concentrations and despite the fact that the three proteins were expressed to similar extents. However, we cannot exclude that ELK-1 and SAP-1a could be inhibitors, with other reporters or in different cell lines, and that the differences are quantitative rather than qualitative (absolute). The only other direct comparison of the three homologues was reported by Price *et al.* (1995). Using proteins fused to the VP-16 activation domain and a different reporter, but the same cell line (NIH-3T3), they showed that ELK-1 and SAP-1a activate, but Net (SAP-2) is inactive. Perhaps, in this case, the VP16 activation domain compensated for the negative activity of Net. Janknecht *et al.* (1993, 1995) found that ELK-1 and SAP-1a inhibit basal transcription in RK13 cells with both SRE and EBS (ets binding site)-based reporters, suggesting that the transcriptional activities of the TCFs may be cell type-dependent. Similar observations have been made for other factors. For example, TEF1 potently activates in HeLa cells but it is inactive in BJA-B cells because of the presence of a cell-specific inhibitor (Chaudary *et al.*, 1994, 1995).

Repression by Net is mediated by a sequence of ~50 amino acids, the inhibitory domain, NID. It is absolutely required for inhibition in the context of the Net protein, and it can be isolated and confer repression when fused to a heterologous DNA-binding domain. Repression apparently requires binding to a specific DNA sequence, since control reporters lacking the corresponding specific binding motifs were not inhibited (Giovane *et al.*, 1994; see Results, also data not shown). Furthermore, expression of the NID alone without a DNA-binding domain did not inhibit PALx8–TK–CAT, even though NID was localized

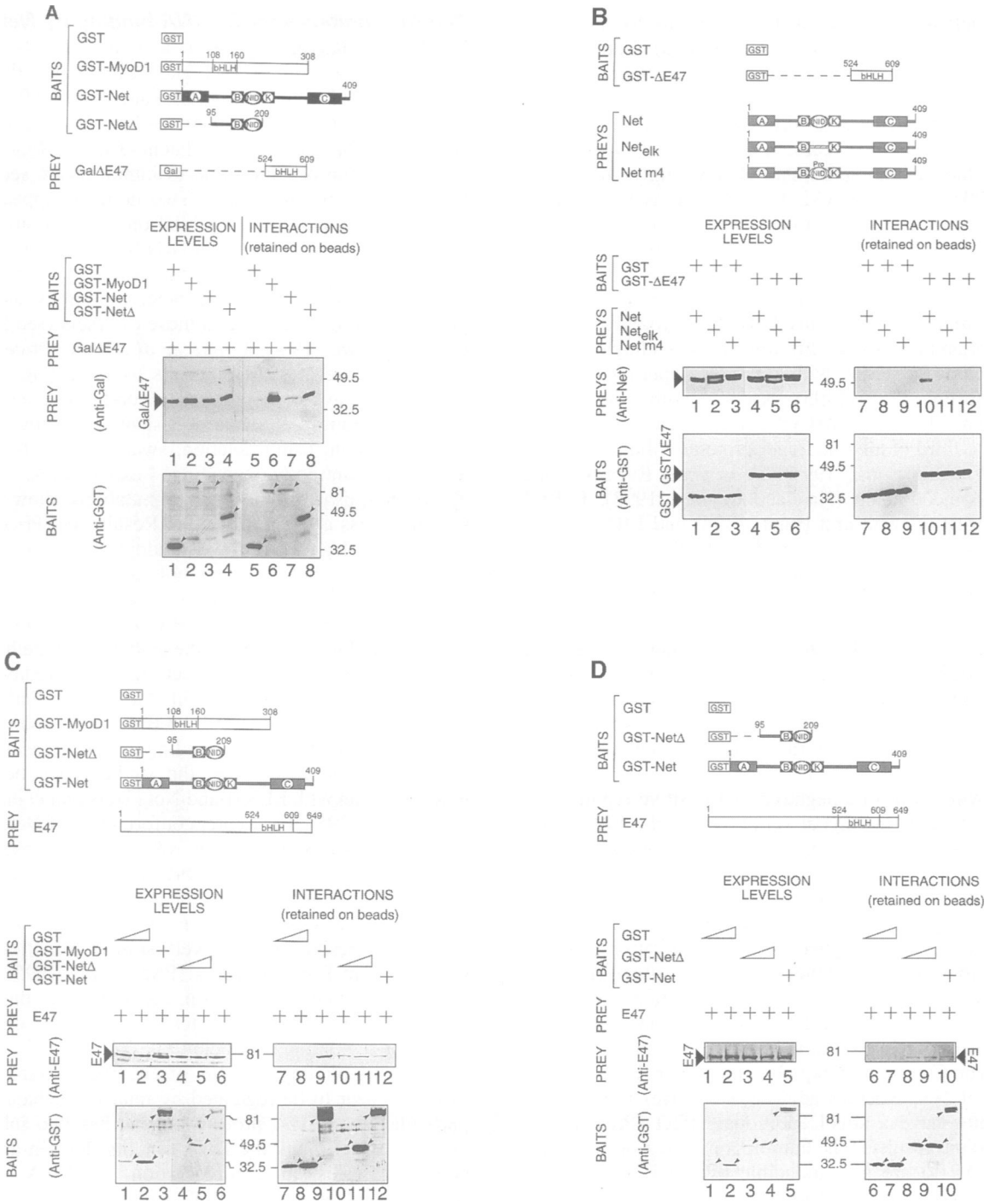

Fig. 8. *In vivo* interactions between Net and E47. The upper panels represent the structures of the proteins used in the interaction assay shown in the bottom parts. The BAITS consist of GST alone or GST fused to either full-length MyoD1 (1–308), full-length Net (1–409), NetΔ (95–209) or ΔE47 (524–609). The PREYS were: GalΔE47 (524–609 of E47 fused to the Gal4 DNA-binding domain), full-length E47 (1–649) and full-length Net, Net_{elk} or Net_{m4}. Expression vectors encoding the baits and the preys were co-transfected either in COS (A, B and C) or NIH-3T3 (D) cells and extracts were analysed either directly (EXPRESSION LEVELS) or incubated with glutathione–Sepharose beads (INTERACTIONS) before analysis to determine the amounts bound either directly to the beads (lower blots) or indirectly through protein–protein interactions (upper blots). Proteins were electrophoresed on 10% SDS–PAGE and transferred to nitrocellulose membranes. The preys were first revealed with specific antibodies (see below) and then the GST fusion proteins were detected with anti-GST (MAb 1D10) on stripped membranes. (**A**) Net and NetΔ interact with ΔE47. COS cells were co-transfected with 5 μg of pGalΔE47 and 5 μg of either pBC (lanes 1 and 5), pBC–MyoD1 (lanes 2 and 6), pBC–Net (lanes 3 and 7) or pBC–NetΔ (lanes 4 and 8). Gal–ΔE47 was revealed with MAbs 2GV3/3GV2 (anti-Gal). (**B**) The interaction between Net and ΔE47 requires an intact NID. COS cells were co-transfected with 5 μg of either pBC (lanes 1–3 and 7–9) or pBCΔE47 (lane 4–6 and 10–12) and 5 μg of either pTL2–Net (lanes 1, 4, 7 and 10), pTL2–Net_{elk} (lanes 2, 5, 8 and 11) or pTL2–Net_{m4} (lanes 3, 6, 9 and 12). All the preys were detected with PAb375 (anti-Net). (**C**) Full-length Net and E47 interact. COS cells were co-transfected with 5 μg of pRcCMVE47 (all lanes) and either pBC (1 μg, lanes 1 and 7; 5 μg, lanes 2 and 8) or 10 μg of pBC–MyoD1 (lanes 3 and 9), pBC–NetΔ (1 μg, lanes 4 and 10; 5 μg, lanes 5 and 11) or 10 μg of pBC–Net (lanes 6 and 12). E47 was revealed with MAb Yae (anti-E47). (**D**) Net and E47 interact when expressed in NIH-3T3 cells. NIH-3T3 (C11 clone) cells were co-transfected with 5 μg of pRcCMVE47 (all lanes) and either pBC (1 μg, lanes 1 and 6; 5 μg, lanes 2 and 7), pBC–NetΔ (1 μg, lanes 3 and 8; 5 μg, lanes 4 and 9) or 10 μg of pBC–Net (lanes 5 and 10). E47 was revealed with the MAb Yae (anti-E47).

in the nucleus as well as in the cytoplasm, as shown by immunocytochemistry (data not presented). Cahill *et al.* (1994) have proposed that an important mechanism of control could be regulatory squelching, i.e. regulated inhibition without DNA binding. Net does not repress by regulatory squelching because it necessitates DNA binding but not the activation domain. Interestingly, Janknecht *et al.* (1995) found that SAP-1a inhibits transcription, and, even though they dissected the protein in similar ways as Net, they did not identify a distinct inhibition domain. Perhaps SAP-1a inhibits by more complex mechanisms that may require more than one region of the protein. Distinct repression domains have been found in many transcription factors with different DNA-binding domains. They include Mad/Max with a bHLH–zipper (Ayer *et al.*, 1995; Hurlin *et al.*, 1995), Krüppel with zinc fingers (Licht *et al.*, 1994), the KRAB-domain factors comprising about one-third of all zinc finger proteins (Margolin *et al.*, 1994; Witzgall *et al.*, 1994), *Drosophila* Even-Skipped with a homeodomain (Han and Manley, 1993), E4BP4 with a bZIP (Cowell and Hurst, 1994) and ERF with an Ets domain (Sgouras *et al.*, 1995). Like the NID, some of these domains have been shown to repress when fused to a heterologous DNA-binding domain (Krüppel, Licht *et al.*, 1994; KRAB proteins, Witzgall *et al.*, 1994; Margolin *et al.*, 1994; Even Skipped, Han and Manley, 1993; E4BP4, Cowell and Hurst, 1994; ERF, Sgouras *et al.*, 1995). Interestingly, there is no obvious homology between the NID and previously described active repressor domains, suggesting that the NID is a new inhibition domain.

Net switches from a negative to a positive regulator in response to the Ras signal (Giovane *et al.*, 1994). Our results show that this is not a consequence of Ras directly regulating repression by the NID. Ras does not affect repression of basal activity by the NID fused to the Gal4 DNA-binding domain. In fact, deleting or mutating the NID leads to much higher levels of activation, both in heterologous fusion proteins and in Net, suggesting that the NID is an inhibitor of Ras activation. However, inhibition is not specific for Ras activation since basal activity was also inhibited, although apparently to a lesser extent. The C homology region is the major Ras-inducible domain of Net, whereas adjacent sequences towards the N-terminus have a small additional effect (Price *et al.*, 1995 and our results). The homologous C regions of ELK-1 and SAP-1a are also inducible by the Ras signalling pathway (Gille *et al.*, 1992, 1995; Hill *et al.*, 1993; Janknecht *et al.*, 1993, 1994, 1995; Marais *et al.*, 1993; Kortenjann *et al.*, 1994; Price *et al.*, 1995). There is an interesting resemblance between the regulatory mechanism of Net with those of c-Fos and c-Jun. c-Fos contains an inhibitor domain, ID1, whose mutation enhances the ability of c-Fos to activate transcription (Brown *et al.*, 1995). ID1 inhibits the HOB1-containing activation domains from c-Fos or c-Jun, that are inducible by the Ras/MAPK signalling cascade (Baichwal *et al.*, 1991; Bannister *et al.*, 1994). c-Jun contains a bipartite inhibitor domain (delta plus epsilon), that modulates the activation domain (Baichwal *et al.*, 1992). Indirect evidence suggests that these inhibitor domains interact with repressor proteins, whose identities remain to be established (Baichwal *et al.*, 1992; Brown *et al.*, 1995).

The NID inhibits specific DNA binding by Net

Net binds relatively poorly to a number of ets-binding sites compared with ELK-1 and SAP-1a, whether it is expressed *in vitro* or *in vivo* (Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995; see Results; also, data not shown). Net appears to have a latent DNA-binding activity or 'closed' conformation due to inhibition by sequences C-terminal to the B-domain. Two domains appear to be particularly important for inhibition, the C-domain and the NID. We have shown that NID has an effect on DNA binding under particular conditions. The effects of the C-terminus are highlighted in other conditions, as shown previously in our studies and those of others (see Results; Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995). We interpret these results as indicating that the band-shift assays under the various conditions are sensitive to different ranges of affinity constants. We show that the ID is important using deletion, swap and point-mutants, and with antibody 'opening' experiments. Similar approaches, using antibodies and mutations, show that the C-domain has a similar role (see Results and Price *et al.*, 1995). ELK-1 and SAP-1a also adopt analogous 'closed' conformations, but they are not as refractory to DNA binding as Net (Treisman *et al.*, 1992; Janknecht *et al.*, 1994; Price *et al.*, 1995). Interestingly, a different region inhibits binding of ELK-1, the B-domain together with sequences up to the Ets domain (Rao and Reddy, 1992; Treisman *et al.*, 1992; Janknecht *et al.*, 1994). Perhaps the interaction between SRF and the B-domain is particularly important for ELK-1. DNA binding by SAP-1a is inhibited by the C-domain, although the studies have been less extensive than with ELK-1 and Net (Treisman *et al.*, 1992; Price *et al.*, 1995). The tighter conformation of Net relative to ELK-1 and SAP-1a may reflect the presence of a more extensive bipartite inhibitory domain. The tighter conformation could account for the lower transcriptional activity in transfection assays of Net under activating conditions, despite the relatively similar activities of the C-domains in fusion proteins (Price *et al.*, 1995). These data indicate that changes from the 'closed' to the 'open' DNA-binding competent states could be an important target for regulation (see below).

The DNA-binding activities of a number of transcription factors appear to be regulated by related mechanisms. In particular, the cEts-1 proto-oncogene has two inhibitory domains which flank the DNA-binding domain and have concerted roles in autoregulation of DNA binding (Hagman and Grosschedl, 1992; Lim *et al.*, 1992; Wasylyk *et al.*, 1992; Fischer *et al.*, 1994; Hahn and Wasylyk, 1994; Peterson *et al.*, 1995). Significantly, the C-terminal inhibitor is modified in the viral oncogenic form (Hagman and Grosschedl, 1992; Leprince *et al.*, 1992; Hahn and Wasylyk, 1994). The solution structure of part of the ets protein shows that the C-terminal sequence packs against the ets domain and is positioned to interact with the N-terminal inhibitory sequence, suggesting that they may interact and thereby have a concerted effect (Peterson *et al.*, 1995; Donaldson *et al.*, 1996). The N-terminal domain is subject to regulation by phosphorylation (Rabault and Ghysdael, 1994) and alternative splicing (Wasylyk *et al.*, 1992). Antibodies against this region stimulate the latent DNA-binding activity (Seth *et al.*, 1993). However, sequences beyond the essential flanking

domains appear to contribute to the overall closed conformation (Lim *et al.*, 1992). DNA binding by the p53 tumour suppressor is inhibited by C-terminal sequences. Again, interactions with other factors, which can be mimicked by antibodies against the inhibitory region, as well as phosphorylation, affect the affinity for DNA (Hupp *et al.*, 1995).

The HLH motif of the NID

The NID most probably has a HLH conformation, as shown by sequence comparisons, secondary structure prediction, three-dimensional model building and mutagenesis of critical residues. Furthermore, as predicted from the homology, Net can interact with E47. We are currently screening on a more random basis for the physiological partners of Net. Further confirmation of the structure will require X-ray crystallography or NMR studies.

Transcription factors with bHLH domains have been linked to diverse biological processes such as neurogenesis (for a review see Campuzano and Modolell, 1992), myogenesis (reviewed by Olson and Klein, 1994), segmentation (Rushlow *et al.*, 1989), transcriptional regulation (Schlissel *et al.*, 1991), oncogenesis (Mellentin *et al.*, 1989; Chen *et al.*, 1990) and mesoderm formation (Thisse *et al.*, 1988). The HLH motif mediates protein–protein contacts, which mainly lead to homo- (Murre *et al.*, 1989) and hetero- (Davis *et al.*, 1990) dimerization, but also mediates interactions with non-HLH proteins. For example, the HLH of MyoD1 interacts with the basic domain of Jun (Bengal *et al.*, 1992), and the HLH of Id2 with the pocket of Rb (Iavarone *et al.*, 1994). There are indications that Ets and HLH proteins synergize. The ets factor PU.1/Spi1 (Moreau-Gachelin, 1994) forms a ternary complex with an unknown HLH factor on the FcγRIIIA promoter (Feinman *et al.*, 1994). Erg3 or Fli-1 synergize with E12 or E47 in activation of a reporter containing both the μE2 E-box and the π (μA) ets site of the IgH enhancer in HeLa cells (Rivera *et al.*, 1993). The π sequence appears to be a natural binding site for Net (Libermann and Baltimore, 1993; Lopez *et al.*, 1994). Net is expressed in pre-B cells, but not at later stages of B-cell development (Lopez *et al.*, 1994). Interestingly, the E2A gene products (E47 and E12) are required for B-cell development and immunoglobulin gene rearrangement (Bain *et al.*, 1993; Zhuang *et al.*, 1994), suggesting links with Net. It will be interesting to study the role of Net during B-cell development, and its interactions with the many different factors that regulate the IgH enhancer (Ernst and Smale, 1995).

A model for the regulation of Net

We propose that Net exists in a 'closed' conformation and that the transition to an 'open' DNA-accessible structure is an important point of regulation by protein–protein interactions or phosphorylation (Figure 9). Opening is driven by protein–protein interactions between the NID and members of the HLH family, possibly between the B-domain and SRF, and also the C-domain with other proteins (which are mimicked by antibodies that stimulate DNA binding). The NID lies next to the B-domain, suggesting that it could also affect specificity for members of the SRF family (Shore and Sharrocks, 1995), a number of which are expressed in both muscle and non-muscle

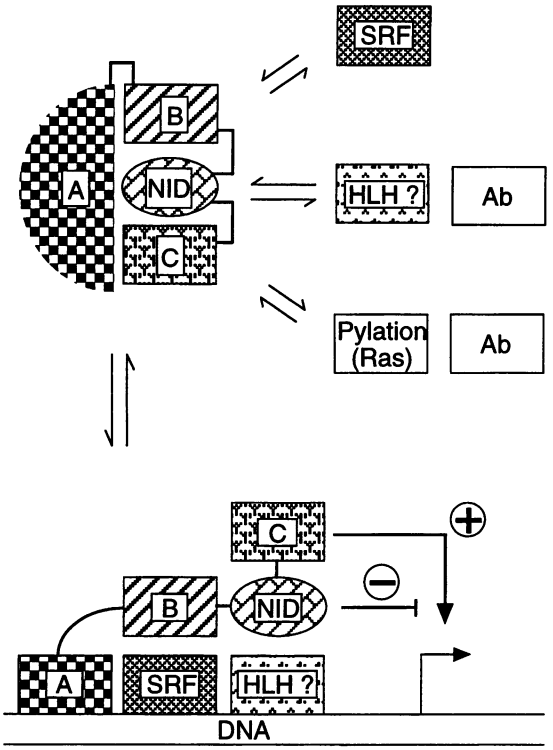

Fig. 9. A model for Net regulation. Net has a latent DNA-binding activity due to its 'closed' compact conformation (upper illustration) that can be 'opened' by various stimuli directed to different parts of the protein, including: interaction of SRF with the B-domain, interaction of HLH or other proteins (?) with the NID, and phosphorylation (Pylation) of the C-domain by components of the Ras signalling pathway. Protein–protein interactions are mimicked by antibodies (Ab). The DNA-bound complex involves interactions between the B-domain and SRF and the NID and a HLH or other protein. The NID inhibits basal activity (–), even in the presence of the Ras signal, whereas the C-domain stimulates (+). NID activity could be affected by protein interactions or modification (not illustrated).

cells (Dodou *et al.*, 1995). Phosphorylation through the Ras signalling pathway appears to be the major regulatory mechanism for the C-domain (Price *et al.*, 1995). Net, which has been forced open by mutation in the NID, appears to be more sensitive to modification (see Results), suggesting that 'opening' would make it more responsive to fine regulation by mechanisms such as phosphorylation. In the model, Net 'opening' leads to the formation of a complex on DNA.

The DNA-bound protein complex is proposed to be stabilized by the interactions of Net with SRF and/or bHLH proteins and to be subject to regulation. There are interesting parallels. On muscle cell differentiation, MyoD1 represses the *c-fos* promoter through an E-box binding site in the SRE (Trouche *et al.*, 1993). A possibility is that Net forms ternary complexes with related bHLH factors, which are present in the cells where it is abundant (e.g. NIH-3T3 cells; Giovane *et al.*, 1994). Myogenic bHLH factors have been shown to interact physically with SRF (Groisman *et al.*, 1996). The NID could participate in these interactions, either with the myogenic factors, with SRF, or with both. DNA-bound Net inhibits transcription by unknown mechanisms that could involve recruiting negative coactivators and nucleosomes, analogous to α2

and MCM1 (Cooper *et al.*, 1994), the SRF homologue of yeast (Shore and Sharrocks, 1995). However, many mechanisms of repression have been described (Cowell, 1994; Kornberg and Lorch, 1995; reviewed by Johnson, 1995). Repression by DNA-bound Net could be subject to regulation (see, for example, Perlmann and Vennström, 1995). The NID is located in sequences that differ between the ternary complex factors. An interesting possibility is that the divergent sequences of ELK-1 and SAP-1a could also mediate specific functions of these otherwise highly related proteins.

Materials and methods

Plasmids

All plasmids were constructed by standard methods. Full details are available on request.

Expression vectors

Gal4–Net fusions. (pGal–N1 to –N6; pGal–C7ΔA to –C11ΔA; pGal–NC1 and –NC2; pGal–NC2 m1 to m4). *Kpn*I flanked PCR fragments were cloned in the *Kpn*I site of pG4MpolyII (Webster *et al.*, 1988), which encodes the Gal4 DNA-binding domain (amino acids 1–147). Clones were screened for the correct orientation and sequenced. Point mutations were introduced by the two-round PCR mutagenesis technique.

Net, ELK-1 and SAP-1a. pTL2–Net and pTL1–Elk1 encoding complete murine Net and human ELK-1, respectively, are described elsewhere (Giovane *et al.*, 1994). pKOZ1–Sap1a, encoding complete human SAP1a, was obtained by PCR subcloning from pT7–SAP-1a (Dalton and Treisman, 1992) into pKOZ1 (Wasylyk *et al.*, 1992).

C-terminal Net deletion mutants and Net_{elk}. (pKOZ–C7 to C11 and pTL2–Net_{elk}). PCR products were cloned in pKOZ1 or pTL2.

Eukaryotic GST fusion proteins. pBC–MyoD contains the 1.8 kbp *Nde*I–*Bam*HI fragment (full-length MyoD1) from pVP–MyoD (Finkel *et al.*, 1993) in the corresponding sites of pBC (Chatton *et al.*, 1995). pGal–ΔE47 encoding the 85 C-terminal amino acids of E47, including the bHLH domain was described elsewhere (Hsu *et al.*, 1994). pBC–ΔE47 was generated by subcloning the 0.4 kbp *Nde*I–*Sac*I from pGal–ΔE47 into corresponding sites of pBC. pBC–Net and pBC–Net (95–209) were generated by PCR cloning. pRcCMV–E47 was a generous gift from Dr X.H.Sun and encodes full-length E47 protein (1–649).

Reporters

Palx8–TK–CAT4. Eight copies of the palindromic ets site from the stromelysin promoter are inserted upstream from the thymidine kinase (TK) promoter of pBL–CAT4.

Gal–TK–CAT. A synthetic Gal4 binding site is located upstream from the TK promoter of pBL–CAT8+ (Webster *et al.*, 1988).

Cell culture and transfections

NIH-3T3 (C11) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical, St Louis, MO, USA) containing 10% fetal calf serum (FCS) and transfected by the BBS calcium phosphate method (Chen and Okayama, 1987) in 100 mm dishes with 20 μg of DNA. 20 h later the cells were washed twice with DMEM, incubated in DMEM containing 0.05% FCS for 24 h, scraped in 1 ml of PBS and 700 μl was used for CAT assays and 300 μl for Western blots. CAT assays are described in Giovane *et al.* (1994).

Western blots and antibodies

Whole-cell extracts. Transfected cells (300 μl) were centrifuged at 1500 r.p.m. for 5 min at 4°C, resuspended in 100 μl of TGKD buffer (10 mM Tris–HCl, pH 7.8, 10% glycerol, 0.5 M KCl, 1 mM DTT and a protease inhibitor cocktail), lysed by three cycles of freeze–thawing and spun at 10 000 r.p.m. for 10 min at 4°C.

Western blots. Proteins (40–50 μg) in 20 μl of the whole cell (TGKD) extract were fractionated by SDS–PAGE on 10 or 12% polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked in PBSTM (PBS with 0.1% Tween 20 and 5% milk) either overnight at 4°C or 30 min at room temperature, incubated in PBSTM with specific primary antibodies (diluted 1/2000) for 2 h at room temperature, washed four times in 100 ml of PBST, incubated with secondary antibodies coupled to peroxidase (diluted 1/5000) in PBSTM for 1 h at room temperature, washed four times and revealed with the ECL detection kit (Amersham, Ref. RPN 2106).

Primary antibodies. Anti-Net: rabbit polyclonal PAb375 and 376 (Giovane *et al.*, 1994), respectively, raised against amino acids 385–409 and 151–176 of mouse Net. Anti-ELK-1: rabbit polyclonal PAb512 (Giovane *et al.*, 1994) raised against amino acids 411–427 of human ELK-1. Anti-SAP-1a: rabbit polyclonal PAb643 raised against the ovalbumin-coupled peptide (C)NTSNKKLFKSIKIENPAEKLAEK corresponding to amino acids 131–153 of murine SAP-1a [200 μg/injection, cysteine (C) was added for coupling]. Anti-Gal: mix of two monoclonal antibodies, MAb 2GV3 and 3GV2 (White *et al.*, 1992). Anti-GST: monoclonal 1D10 antibody (Y.Lutz, unpublished results). Anti-E47: either polyclonals A1(C) /A1(N) kindly provided by Dr M.Walker, or the monoclonal Yae antibody (Santacruz Biotechnology, Inc., Ref. SC 416)

Secondary antibodies. GAR.PO: goat-anti-rabbit conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used for rabbit polyclonals. GAM.PO: goat-anti-mouse conjugated with horseradish peroxidase (Jackson Immuno-Research Laboratories) was used for mouse monoclonals.

In vitro transcription and translation

RNA was synthesized with 500 ng of *Xba*I linearized pSG5-based vectors in 100 μl reactions containing 20 μl of T7 buffer ×5 (200 mM Tris–HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 10 μl 100 mM DTT, 10 μl NTP ×10 (5 mM each), 2 μl 10 mM m7GpppG (Cap, Pharmacia), 3 μl RNAsin (35 U/μl, Promega), 1.5 μl of T7 RNA polymerase (20 U/μl, Promega) for 90 min at 37°C. RNA was acid–phenol extracted (phenol equilibrated in 10 mM sodium acetate, pH 5.2) to remove plasmid DNA that is soluble in the phenol phase, precipitated and resuspended in 50 μl of TE. For translation, 1–5 μl of RNA was incubated with 5 μl of [^{35}S]methionine (15 μCi/μl), 2 μl RNAsin (35 U/μl), 1 μl amino acid mix (1 mM, without Met, Promega) and 35 μl of rabbit reticulocyte lysate (Promega) in a final volume of 50 μl for 90 min at 30°C. 2 μl aliquots were analysed by SDS–PAGE. Dried gels were exposed for 1 h with a Fuji-Phosphor-Imager plate. Proteins were quantitated from the incorporated radioactivity, corrected for the number of methionines.

Electrophoretic mobility shift assays

In vitro-translated proteins (1–5 μl) brought to a constant volume with mock reticulocyte lysate (incubated without RNA) were incubated for 30 min at 25°C in 20 μl reactions [5 mM HEPES, pH 7.9, 2.5 mM $MgCl_2$, 2.5 mM EDTA, 5 mM NaCl, 2 mM spermidine, 2.5 mM DTT, 1 μg poly d(I-C), 2 μg/μl BSA] with excess *c-fos* SRE or IgH (μE2-π) probes. Samples were electrophoresed immediately on pre-run (60 min, 15 mA, 75 V) 4% polyacrylamide gels at 30 mA (150V) and 20°C for 2 h with recirculating buffer. Gels contained 0.25× TBE and 2.5% glycerol (all figures except for Figure 4, panels 1 and 2). Gels were dried and exposed to X-ray films.

Probes

cFos SRE. TCGACAGGATGTCCATATTAGGACATCTGCGTCAGCTCGA. The Ets and SRF binding sites are underlined.

IgH (μE2-π). GAACACCTGCAGCTGGCAGGAAGCAGGT. The E box μE2 and Ets π sites are underlined.

Single-stranded oligonucleotides (40 ng) were end-labelled with T4 polynucleotide kinase and [α-^{32}P]ATP (5 pmol/μl) and reassociated by heating the mixtures for 5 min at 95°C and letting the temperature decrease overnight. Double-stranded probes were purified on 10% polyacrylamide gels and eluted with 400 μl of TE. The specific activities were ~ 500 000 c.p.m./ng.

In vivo protein–protein interactions

The newly described eukaryotic GST vector pBC (Chatton *et al.*, 1995) was used to produce glutathione-*S*-transferase (GST) fusion proteins in mammalian cells. The GST-tagged proteins and potential interacting factors were co-expressed in COS-7 cells transfected by the BBS-calcium phosphate technique (see above) and grown in 5% calf serum-supplemented DMEM. For 100 mm dishes, the cells were scraped, washed with PBS and lysed in 100 μl of lysis buffer (0.4 mM KCl, 20 mM Tris–HCl, pH 7.5, 20% glycerol, 5 mM DTT, 0.4 mM PMSF) by freezing and thawing twice. Cell lysates were cleared by centrifugation (10 min at 10 000 *g*), and 80 μl aliquots were incubated for 2 h at 4°C

with 40 µl of a 50% suspension (in 0.1% gelatin, 0.01% sodium azide) of glutathione–agarose beads (Sigma chemicals) in 1 ml of low-stringency buffer Ls (50 mM Tris–HCl, pH 7.8, 0.1% NP-40, 250 mM NaCl). The beads were washed twice with 500 µl of Ls buffer, resuspended in 20 µl of SDS-loading buffer and boiled for 5 min. Proteins were fractionated by 10% SDS–PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting (see above).

Acknowledgements

We thank Dino Moras for invaluable support for the modelling, Bruno Tocqué for continuing encouragement and support, Richard Baer for his cooperation and friendly help with obtaining recombinants, Bruno Chatton for help with the eukaryotic *in vivo* GST system, Yves Lutz for generously providing the anti-Gal and anti-GST antibodies, Richard Treisman for pT7–SAP1a, Xiao-Hong Sun for the pRcCMV.E47 vector, Michael Walker for the anti-E47 antibody, Irwin Davidson and Antoine Giovane for critical reading of the manuscript, members of the laboratory for useful discussions and encouragement, the staff of the IGBMC facilities for their invaluable help, the Ligue Nationale Française contre le Cancer for a fellowship for S.-M.M, and various funding agencies including: the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ligue Nationale Française contre le Cancer, the Ligue Régionale (Haut-Rhin) contre le Cancer, the Ligue Régionale (Bas-Rhin) contre le Cancer (the Legs Meyer) and the Bioavenir Program (Ministère de la Recherche et Ministère de l'Industrie).

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Received on February 16, 1996; revised on June 25, 1996