Net, a new ets transcription factor that is activated by Ras

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Ras signaling appears to be mediated in part by transcription factors that belong to the ets gene family. To identify downstream targets for the Ras signal transduction pathway, we have used Ras-transformed mouse fibroblasts to isolate a new member of the ets gene family, net. Net has sequence similarity in three regions with the ets factors Elk1 and SAP1, which have been implicated in the serum response of the fos promoter. Net shares various properties with these proteins, including the ability to bind to ets DNA motifs through the Ets domain of the protein and form ternary complexes with the serum response factor SRF on the fos serum response element, SRE. However, Net differs from Elk1 and SAP1 in a number of ways. The pattern of net RNA expression in adult mouse tissues is different. Net has negative effects on transcription in a number of assays, unlike Elk1. Strikingly, Ras, Src, and Mos expression switch Net activity to positive. The study of Net should help in understanding the interplay between Net and other members of the Elk subfamily and their contribution to signal transduction through Ras to the nucleus.

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The ets gene family codes for transcription factors that are involved in the generation of human cancers, cell transformation, and development (for review, see Seth et al. 1992; Macleod et al. 1992; Janknecht and Nordheim 1993; Wasylyk 1994; Wasyllyk et al. 1993; Treisman 1994). The Ets proteins contain a conserved Ets domain that binds specifically to DNA sequences with a core GGA element (ets motifs). They can be grouped into subfamilies on the basis of different criteria such as homology in the Ets domain, position of the Ets domain in the protein, and the presence of other similar sequence elements with comparable functions. The subfamilies appear to have arisen through duplication of an ancestral gene. The Elk subfamily proteins Elk1 and SAP1 have three regions with similar sequences [A, B, and C; Rao et al. 1989; Dalton and Treisman 1992; Fig. 1B]. They are unusual in the Ets family as the Ets domain (region A) is amino terminal. The B region is required to form a ternary complex with the serum response factor (SRF) and the ets and SRF motifs of the fos serum response element (SRE) [Dalton and Treisman 1992; Janknecht and Nordheim 1992; Hippskind et al. 1991; Rao and Reddy 1992a; Treisman 1992]. The Elk1 C region is an activation domain that is stimulated by phosphorylation by MAP kinase (MAPK), and directly imparts regulation on the SRF [Janknecht et al. 1993; Marais et al. 1993]. This model provides an important conceptual framework for understanding the Ras signaling pathway, of which MAPK is a component.

The ras oncogene is frequently mutated in human tumors. It is a component of a highly conserved signal transduction pathway that is present from yeast to man (for review, see Blenis 1993; Khosravi and Der 1994; Moodie and Wolfman 1994) and links the exterior of the cell to nuclear effectors of cell growth and differentiation (Chambers and Tuck 1993). Signals flow through receptor tyrosine kinases, intermediary linking proteins, Ras, and then a cascade of kinases composed of Raf, MAP kinase kinase kinase (MAPKKK) MAPKK, and MAPK to downstream effectors. Ets factors mediate transformation and regulation of development by Ras. Trans-dominant mutants of Ets inhibit Ras transformation [Langer et al. 1992] and revert Ras-transformed cells [A. Giovane et al., unpubl.]. Genetic studies in Drosophila show that the Ets-protein Yan/Pok is a negative regulator of photoreceptor development that acts antagonistically to the proneural signal mediated by Ras [Lai and Rubin 1992, Tei et al. 1992].

We have identified a new Ets protein, Net, which is another member of the Elk subfamily. It has three regions of similarity with Elk1 and SAP1 (A, B, and C) and interacts with SRF to form a ternary complex. Interestingly, Net inhibits transcription, in contrast to Elk1. Ras
expression leads to transcription activation by Net. The properties of Net are reminiscent of the negative Yan/Pok factor that participates in Ras signaling.

Results

RNA coding for a new Ets protein is present in Ras-transformed fibroblasts

Ets domain-coding sequences were amplified from Ras-transformed NIH-3T3 fibroblast cDNA with degenerate primers [oligonucleotides 1–3, Materials and methods, sequence complexity 30,000–100,000]. The PCR product of the expected size was subcloned, and the sequences of individual clones were found to code for the Ets domains of GABPα (two clones), Ets2 (three clones), and an unknown protein that we named Net (four clones). Net Ets domain-coding sequences were found to be present in cDNA libraries of mouse 10-day embryos, embryonic stem (ES) cells, F9 EC cells, retinoic acid-differentiated F9, and P19 cells and heart, by use of PCR with specific oligonucleotides derived from the cloned sequence [4 and 5, Materials and methods]. Seven net cDNA clones ranging in size up to 2.3 kb were isolated from the embryonic stem cell libraries by screening with an ets domain probe and sequenced entirely on both strands (Fig. 1A, see Materials and methods). The compiled cDNA sequence was estimated to lack no more than 50 bp at the 5’ end and up to 450 bp at the 3’ end sequences by rapid amplification of cDNA ends (RACE-PCR, Frohman 1988). The longest open reading frame (ORF) is preceded by an in-phase termination codon and has a near optimum sequence for translation initiation (purine at −3, G at +4; Kozak 1991). The 3’ end is AT rich (60.5%), has an uninterrupted stretch of 26 A-T residues, and contains four ATTTA motifs reminiscent of unstable mammalian RNAs that code for proto-oncogenes (Sachs 1993).

The ORF of mouse net codes for an ets domain that is very similar to human Elk1 and SAP1 (Fig. 1B, ~80% similarity) and is located at the amino terminus, unlike other Ets proteins. Mouse Net also resembles human Elk1 and SAP1a in regions B and C (~70% and 60%, respectively). To ensure that Net is a third member of the Elk subfamily, we isolated mouse elk1 and SAP1a cDNAs from Ras-transformed NIH-3T3 fibroblast RNA as well as several cDNA libraries (ES cell and 10-day embryo), and human net cDNA from a HeLa cell cDNA library (see Materials and methods). The nucleotide and deduced protein sequences of each member of the subfamily are very similar between species and clearly distinct from each other in each species (Fig. 1B), showing that they are distinct members of the elk subfamily.
net RNA is abundant in fibroblast cell lines and has a different expression pattern from elk1 and SAP1

Two net RNAs migrating at ~2.5 and 4.5 kb were detected on RNA blots [Fig. 2]. The shorter RNA corresponds closely in size to the cloned cDNA [Fig. 1A]. Both RNAs hybridize to full-length net probes and fragments containing either related (the ets domain, positions 201–711, Fig. 1A) or dissimilar (B to C region, positions 712 and 1326) sequences. They do not cross-hybridize to mouse elk1, SAP1, and ets2 probes. The nature of the longer RNA is under investigation.

Table 1. net mRNA expression pattern in murine and rat cell lines

| net mRNA level* | Cell lines | Description |
|-----------------|------------|-------------|
| ++ ++ ++ S–3T3  | fibroblast, Swiss 3T3 |
| ++ ++ ++ NIH-3T3 | fibroblast, NIH-3T3, C11 [FDH] |
| ++ ++ ++ NIH–3T3 ras | Ki-ras-transformed C11, DT U1 [FDH] |
| ++ ++ ++ NIH–3T3 ras | v-ras-transformed NIH-3T3, 3611 |
| ++ ++ TM3 | testis, Leydig cells, BALB/c mouse |
| ++ ++ WEHI3 | myelomonocytic leukemia |
| ++ ++ 70Z/3 | pre-B lymphocyte |
| ++ ++ BW5147 | T-lymphoma |
| + P19 | embryonal carcinoma |
| + F9 | embryonal carcinoma |
| + + + + F745 | erythroleukemia |
| + + + + MPC11 BU4 | plasmacytoma, Ig secreting |
| + + + + X63Ag8 | nonsecreting myeloma |
| + + + + X63Ag8WS | secreting myeloma |
| 0/- ES | embryonal stem, D3, SV129 mouse |
| ++ + NG108.15 | neuroblastoma/glioma |
| ++ + FR 3T3 | rat fibroblasts |
| ++ + * FR PyMT | Py mT-transformed FR 3T3, MTT4 |
| ++ + + * FR ras | ras-transformed FR3T3 |

Relative expression levels of net RNA are shown in arbitrary units, where ++ ++ ++ was the highest level [100%], ++ ++ intermediate (~70%), ++ low (~40%), and + very low (~10%), as observed on Northern blots. Semiquantitative comparisons of net-specific mRNA bands were aided by PhosphorImager analysis. Results were adjusted for the amount of loaded mRNA by comparison to ethidium bromide-stained gels and the internal control α-actin.

Specific DNA binding by Net is inhibited by carboxy-terminal sequences

Equimolar amounts of Net and SAP1b [synthesized in rabbit reticulocyte and quantitated by SDS-PAGE of [35S]methionine-labeled proteins; not shown] were analyzed by mobility shift assay with the PEA3* ets motif as a probe. Net and SAP1b formed one major and several minor complexes [Fig. 3A; C points to the major complex, c.f. lanes 1 and 4 with the lysate control, lane 7]. Complex formation was inhibited by excess cold wild-type competitor [lanes 1–9] but not by a mutated competitor altered in the ets motif [lanes 10–18, Materials and methods]. The Net complexes are supershifted with Net antibodies [not shown]. Carboxy-terminal deletion mutants of Net were synthesized and quantitated by SDS-PAGE of labeled proteins and PhosphorImager analysis [Fig. 3B]. Deletion up to the Ets domain progressively increased the affinity for the PEA3* probe ~20-fold [Fig. 3C; the amounts were estimated by PhosphorImager analysis and corrected for protein levels]. These results show that the Ets domain of Net is sufficient for DNA binding and that carboxy-terminal sequences are inhibitory.

Net forms a ternary complex with SRF and DNA containing motifs for both proteins

The Net-related proteins Elk1 and SAP1 form ternary complexes with SRF and DNA containing ets and SRF motifs, such as G wt-wt [Treisman et al. 1992]. Net bound very weakly to G wt-wt [Fig. 4A, lanes 1,2, visible on longer exposure; Fig. 4B, lanes 10,11], whereas SRF
Ras activates Net

Figure 3. Specific binding of Net to the ets motif is inhibited by carboxy-terminal sequences. (A) Net binds specifically to the PEA3\(^*\) motif. Equal molar amounts (quantitated by SDS page of [\(\text{S}^3\text{S}\)]-labeled proteins) of SAP1b (lanes 1–3, 10–12) or Net (lanes 4–6, 13–15), or an equal volume of reticulocyte lysate incubated without specific RNA (Lys, lanes 7–9, 16–18) were analyzed by mobility-shift assay with the \(\text{P}^{32}\)P-labeled PEA3\(^*\) probe. The indicated molar excess of unlabeled wild-type (WT COMP, lanes 1–9) or mutant (MUT COMP, lanes 10–18) competitors was used. (B,C) Carboxy-terminal sequences inhibit DNA binding by the Ets domain of Net. The amounts of the indicated carboxy-terminal deletion mutants were estimated by SDS-PAGE of [\(\text{S}^3\text{S}\)] methionine-labeled proteins (B), and their affinity for the PEA3\(^*\) probe analyzed by mobility shift (C). Radioactivity was measured with a PhosphorImager. (O) Lysate control; (P) proteins; (C) specific complexes; (F) excess free probe; (M) radioactive protein size markers.

formed a prominent specific complex (C1, lane 3) only when its motif was intact (G wt-mut, lane 11). Net displaced the SRF complex to a slower migrating form when present with SRF (C2, lane 4) but only when both the ets and SRF motifs were intact (G mut-wt and G wt-mut, respectively; lanes 8,12). The change in mobility was more dramatic when a shorter form of SRF was used, SRF[122–265], that contains the DNA-binding domain and the Elkl interaction domain (Fig. 4B, lanes 1–3,10,11; the ternary complex is indicated by a small arrowhead). Elkl and SAP1 require the B domain for ternary complex formation. Net mutants retaining the B domain formed ternary complexes efficiently (lanes 3–7; see Fig. 3 for structures). There was a large increase in binary Net–DNA complex formation when the B domain was deleted (Fig. 4B, see bands labeled with a dot and c.f. especially lanes 7, 8, 15, and 16; see above), which was greater than expected from the increased binding affinity caused by deletion of the B domain. Similar results were obtained with a natural c-fos SRE containing a nonconsensus ets motif (not shown). We conclude that the B domain of Net mediates interactions with SRF, similar to Elkl and SAP1.

Net expression represses transcription specifically through ets motifs

Net expression in NIH-3T3 cells specifically and reproducibly inhibited the low basal activity of a reporter containing ets motifs (Fig. 5A, lanes 1,2,7–9; data not shown), in contrast to cEts1p[68] (Fig. 5C, lanes 1,2,5,6,11). Net also efficiently inhibited cEts1p[68] activation (Fig. 5C, 6–8,11–13). These effects were specific to the reporter with the response element (lanes 1–3) showing that it was not attributable to nonspecific competition for general transcription factors. Elkl expression stimulated transcription (Fig. 5A, lanes 1,3,7,10,11) and increased activation by cEts (p68) (Fig. 5C, lanes 1,2,4,6,9–11,14,15). Protein blots indicated that similar amounts of Net and Elkl were expressed (Fig. 5B, lanes 7–11; data not shown). These results suggest that Net could be a negatively acting factor, in contrast to Elkl.

Ha-Ras activates Net

Ha-Ras oncogene expression did not affect the constitutive activity of the ets motif (Fig. 5A, lanes 1,4,7,12), but converted the effects of coexpressed Net from negative to positive (lanes 2,5,7–9,12–14). Ras expression did not affect the activity of cEts1p[68] or GAL4–VP16 (data not shown; Webster et al. 1988) and had only a small effect on Elkl (at most 1.5-fold; Fig. 5A, lanes 1,3,4,6,7,10–12,15,16). Protein blots showed that Ras expression did not alter the levels of Net or Elkl (Fig. 5B, lanes 7–16),
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**Figure 4.** Net-SRF ternary complex formation requires the B domain. (A) Net forms a ternary complex with SRF and specific DNA. Mobility shifts of equimolar amounts of Net and SRF (separately translated) either alone (lanes 2, 6, 10, and 3, 7, 11, respectively) or after mixing (lanes 4, 8, 12), compared with an equal volume of unprimed incubated lysate (lanes 1, 5, 9) with probes containing motifs that are wild type for ets and SRF (G wt-wt, lanes 1-4), mutant for ets (G mut-wt, lanes 5-8) or mutant for SRF (G wt-mut, lanes 9-12). (C1) SRF-specific complex; (C2) ternary complex; (F) free probe. (B) The B domain is required for complex formation between Net and SRF (122-265). Equimolar amounts of SRF (amino acids 122-265, lanes 2-9), Net and various deletion mutants (lanes 3-9, 11-17; see Fig. 3B for structures) that were synthesized in reticulocyte lysates were analyzed by mobility shift either separately or in combinations as indicated with the G wt-wt probe. Ternary complexes are indicated by small arrowheads and Net binary complexes with a dot. (SRF) SRF-specific complex; (F) excess free probe.

suggesting that Ras activates Net by post-translational modification.

**v-Src and v-Mos stimulate Net activity**

v-Src specifically stimulated coexpressed Net [Fig. 6, lanes 1,2, 6-8; data not shown] less efficiently than Ras [lanes 9-11]. v-Mos also reproducibly activated Net [lanes 1-5]. v-Raf expression from several vectors had no detectable effect on Net. However, the effects on control recombinants were relatively small in NIH-3T3 compared with other cell lines [results not shown; Wasylyk et al. 1989], precluding definitive conclusions about activation of Net by Raf. MAPK (p42) expression had no significant effect on Net activity and little effect on Elk1 [less than twofold]. Activation of MAPK by coexpression with Ras [see Janknecht et al. 1993] led to a small activation of Net [less than twofold] and only somewhat more on Elk1 [two- to threefold; data not shown]. The effects were even smaller when MAPK was activated with Raf1 [not shown; several Raf vectors were used]. Elk1 is efficiently activated in RK13 cells by coexpressing MAPK (p44) and active Raf1 compared with either MAPK or Raf1 alone [Janknecht et al. 1993], suggesting that MAPK is limiting in these cells. Our results suggest that MAPK is not limiting in NIH-3T3 cells and that inhibition by Net does not result from titration of a limiting quantity of MAPK.

**Net expression inhibits the fos SRE**

Net expression inhibited the constitutive activity of the SRE but not of the basal promoter [Fig. 7, lanes 1,2,4-6; m in SREm signifies that the FAP motif is mutated]. Different reporters and expression vectors gave similar results [DSE-CAT+pBl-CAT2, Robin et al. 1991; pSG5-Net; pJ7fl-Net; p601D-Net; data not shown]. In contrast, Elk1 specifically increased SRE activity (~2.5-fold; lanes 1,3,4,7,8). Protein blots showed that comparable and increasing levels of Net and Elk1 were expressed [data not shown].

Decreasing endogenous Net levels with antisense net RNA specifically increased SRE activity (up to fourfold; Fig. 8A, lanes 1-10; note that the cells were serum starved for longer than above). Serum stimulated the SRE [about sixfold; Fig. 8A, lanes 1,2,6,7; Fig. 8B, lanes 11,12,16,17]. Anti-net RNA also specifically stimulated SRE in high serum [up to 10-fold, lanes 11-20]. Similar results were obtained in seven different experiments with various amounts of antisense vector and several different plasmid preparations [range between 3- and 10-fold activation]. The empty vector p601D, lacking net sequences, had no effect when it was used either in the place of the anti-net vector or to fill up the transfected DNA to the fixed amount applied to cells. Similar results were obtained whether pSG5 or pEMBL were used to fill up the transfected DNA [data not shown]. To ensure that Net levels were effectively decreased by antisense expression, we performed several controls. Only ~10% of NIH-3T3 cells are transfected and the decrease in Net expression would go undetected because of the untransfected cells. In COS cells we found that antisense net RNA decreased Net expression but had no effect on Elk1 [results not shown]. Specifically in transfected NIH-3T3 cells, net antisense decreased the negative effects of exogenously expressed Net on either constitutive or cEts1-induced PALx4 activity. In contrast, it had no effect on cEts1 activity alone, indicating that it did not have a general nonspecific effect [data not shown]. These observations indicate that net antisense RNA decreases Net protein levels. The results from both overexpression and
Ras activates Net

Figure 5. Regulation of transcription by Net, Elk1, and Ha-Ras through ets motifs. (A) Ras activates Net and Elk1. The transfections contained pBL-CAT4 (lanes 1–6), PALx4 (lanes 7–16), pTL2-Net (1 μg, lanes 8 and 13; 5 μg, lanes 2, 5, 9, and 14), pTL1-Elk1 (1 μg, lanes 10 and 15; 5 μg, lanes 3, 6, 11, and 16), pSG5 (5 μg, lanes 1, 4, 7, and 12), pRCHx2 (5 μg, lanes 4–6 and 12–16), pΔRCHx2 (5 μg, lanes 1–3 and 7–11). (B) Protein blot corresponding to CAT assay in (A). Blots prepared with total cell extracts (100 μg/lane) were probed with Net-specific (top) or Elk1-specific (bottom) antibodies. [C] Net, but not Elk1, inhibits cEts1[p68]. Transfections contained pBL-CAT4 (lanes 1–4), PALx4 (lanes 5–15), pTL2-Net (1 μg, lanes 7 and 12; 5 μg, lanes 3, 8, and 13), pTL1-Elk1 (1 μg, lanes 9 and 14; 5 μg, lanes 4, 10, and 15) and pSG5-cEts1[p68] (1 μg, lanes 6–10; 2 μg, lanes 2–4 and 11–15), pSG5 (7 μg, lanes 1 and 5).

down-regulation of endogenous Net suggest that Net could be a negative regulator of transcription.

Discussion

Net is a new member of the Ets gene family that belongs to the Elk subfamily

Our objective is to study the role of ets family members in the Ras signal transduction pathway. We searched for new, previously unidentified Ets proteins that might be expressed in Ras-transformed fibroblasts by reverse transcriptase–polymerase chain reaction (RT–PCR) using a panel of degenerate primers against conserved sequences of the ets domain. We identified sequences coding for a new ets factor [Net] as well as GABPα and Ets2. However, this approach was not exhaustive because we also isolated by RT–PCR with specific primers RNAs coding for mouse Elk1 and SAP1. We subsequently cloned the mouse cDNAs. The protein sequences of Net, Elk1, and SAP1 are similar, with an amino-terminal ets domain and two further regions of homology, B and C [Fig. 1B]. Net is not the mouse homolog of human Elk1 or SAP1. RNAs for Elk1 and SAP1 from Ras-transformed NIH-3T3 cells code for proteins that are almost identical to their human counterparts and clearly distinct from mouse Net [Fig. 1B]. Human cells contain RNA coding for Net as well as SAP1 and Elk1. elk1 and net genomic sequences are different [data not shown]. elk1, net, and SAP1 mRNAs have different sizes and expression patterns [see above; Rao et al. 1989; Treisman 1994, note that an additional SAP2 protein is mentioned in Dalton and Treisman 1992]. Ets1 and Ets2, which belong to another ets subfamily, have distinct expression patterns and have opposite effects on gene expression [Bhat et al. 1989]. Similarly, the Elk1-like factors have different expression patterns and presumably have different functions.

DNA binding by Net is inhibited by sequences outside the Ets domain

Net binds specifically to ets motifs, and its Ets domain (amino acids 1–91) is sufficient for binding, similar to
other Ets proteins (for Elk1 and SAP1, see Dalton and Treisman 1992; Janknecht and Nordheim 1992; Rao and Reddy 1992a; Treisman et al. 1992; for others, see reviews by Seth et al. 1992; Macleod et al. 1993, Wasylyk 1994; Wasylyk et al. 1993). Sequences flanking the GGAA/T inner core of the ets motif affect its affinity for different members of the family, so that very divergent members of the family (e.g., Ets1 and PU1) will bind exclusively to certain motifs. The Ets domains of Elk1, SAP1, and Net are very similar (Elk1 to SAP1, 80%; Elk1 to Net, 77%; and SAP1 to Net, 79%). Net, Elk1, and SAP1 appear to have substantially similar sequence preferences [Treisman et al. 1992; see Results; data not shown], although we cannot exclude that there are differences, as has been observed for SAP1 and Elk1 [Treisman et al. 1992].

DNA binding by Net is inhibited by sequences carboxy-terminal to the Ets domain, similar to Elk1 and SAP1 [Janknecht and Nordheim 1992; Rao and Reddy 1992; Treisman et al. 1992]. Inhibition is relieved by deletions near the carboxyl terminus as well as in the middle of the protein [see Results]. DNA binding by Ets1 is similarly inhibited by sequences throughout the protein, although particular regions have more important effects [Lim et al. 1992; Wasylyk et al. 1992]. The inhibitory sequences may mask the DNA-binding domain by forming an intramolecular complex or may otherwise
Net interacts with SRF to form a ternary complex on the fos SRE

A potential cofactor for Net is SRF. Net requires SRF to bind to the fos SRE and form a ternary complex. SRE-related sequences with stronger ets motifs form ternary complexes more efficiently. The B region of Net is required for ternary complex formation, although we cannot exclude that other sequences may also contribute. The related factors Elk1 and SAP1 have similar properties ([Hipskind et al. 1991, Dalton and Treisman 1992, Janknecht and Nordheim 1992, Rao and Reddy 1992], consistent with the sequence similarity in the B region [Net to Elk1, 71%; Net to SAP1, 76%; Elk1 to SAP1, 67%]. However, Elk1 and SAP1 appear to form the ternary complex more efficiently, suggesting that Net may interact less efficiently with SRF, the SRE, or both. According to the grappling hook model [Treisman et al. 1992] the closed conformation of Elk1 and SAP1 is opened by SRF and DNA. In the ternary complex, essential interactions with SRF are mediated by box B and possibly carboxy-terminal sequences, whereas the Ets domain interacts with DNA and makes no contacts with SRF. The sequence between the Ets domain and region B acts as a flexible tether, allowing the location and orientation of the ets motif to vary with respect to the SRE-binding site. The Elk1 tether is dispensable for ternary complex formation [Janknecht and Nordheim 1992, Treisman et al. 1992]. The tethers of Net, SAP1, and Elk1 have different sequences and lengths, which could affect their functions, such as the ability to form complexes on different SREs.

Inhibition of transcription by Net

We found that Net inhibits transcription under a variety of conditions. Net expression decreases basal TK promoter activity through the inactive ets-responsive element from the stromelysin promoter [Wasylyk et al. 1991]. In contrast, cEts1[p68] stimulates through this element. Net inhibits cEts1[p68] activation, even though both proteins bind with similar although diminished affinity to the motif compared with a consensus motif [PEA3⁺; A. Pintzas, unpubl.]. The activity of the SRE motif is also specifically inhibited by Net. Coexpression of SRF did not overcome this inhibition with a variety of reporters, in different serum conditions, and in different cell types [results not shown]. This inhibition was not simply a consequence of overexpression of exogenous Net because decreasing endogenous Net levels with antisense net RNA specifically activates the SRE. Some negative transcription factors have negative domains that are alanine rich. Interestingly, there is a sequence similarity between Net and the inhibitory region of the Drosophila Krüppel factor [Licht et al. 1990, AAAASA-FXXX, Net amino acids 209–219 between domains B and C] that is absent in Elk1 and SAP1. It remains to be seen whether Net has a separable negative-acting function. Our results suggest that Net is a negative ets transcription factor but do not prove that Net is a true repressor. The transient expression assay is a deceptively simple system that masks an incredibly complex in-vivo environment in which the relative concentrations and specific activities of the proteins could be different.

Net and Elk1 behave differently in a number of assays. Rao and Reddy [1992b] reported that Elk1 acts as a transcriptional activator through a trimer of ets motifs [E74] in COS cells. Hill et al. [1993] found that Elk1 expression squelched transcription as a result of titration of limiting components or formation of nonfunctional transcription complexes. However, Elk1 activated transcription when fused to a heterologous DNA-binding domain [Hill et al. 1993, Rao and Reddy 1993a]. In agreement with these results, we found that Elk1 stimulated transcription in both NIH-3T3 [see above] and COS cells [not shown]. Furthermore, Elk1 enhances activation by cEts1[p68]. Repression by Net does not appear to involve titration of MAPK because expression of MAPK has little or no effect on Net and Elk1, in contrast to studies in other cell types [Janknecht et al. 1993]. These results suggest that Net has a different role from Elk1.

Net activates transcription when coexpressed with Ras or Src

Net activates transcription through the ets-responsive element when it is coexpressed with Ras, Src, and, to some extent, Mos. This suggests that Net participates in the Ras signal transduction pathway, perhaps as a direct target for one of its components. Elk1 is phosphorylated in the C domain by serum/growth factor stimulation of cells, and by MAPK [Marais et al. 1993, Rao and Reddy 1993b], a downstream component of the Ras pathway. The carboxyl terminus of Elk1 functions as a regulated transcription activation domain, whose activity in vivo is dependent on the integrity of the MAPK sites [Marais et al. 1993, Janknecht et al. 1993]. The C domains of Net and Elk1 are similar [Net to Elk1, 51%; Net to SAP1a, 77%; Elk1 to SAP1α, 60%], and the important MAPK sites are conserved between Net, Elk1, and SAP1α [positions 359–365, 383–389, and 381–387, respectively; Marais et al. 1993]. Ras activation of Net may involve mechanisms similar to serum activation of Elk1. However, serum stimulation did not activate Net, even though we used different reporters and conditions [data not shown]. Similarly, net antisense increased SRE activity even when it was stimulated by serum. This raises the intriguing possibility that serum stimulation of the SRE may have a Ras-independent component. Alternatively, expression of oncogenic Ha-Ras may activate pathways that are unaffected by serum and endogenous c-Ras, and Net, but not Elk1, may be a target for this pathway.
There are at least three *ets* factors that interact directly with SRF and the SRE, suggesting that they may mediate the effects of different signals to this important element. In addition, these factors can regulate transcription in the absence of SRF and thus may mediate signal transduction to different promoters independently of SRF. We have found that Net activity is modulated by Ras expression. The identification of this new factor should help in understanding the mechanisms by which Ras regulates signal transduction pathways.

**Materials and methods**

**Purification of RNA and cDNA synthesis**

Total RNA was extracted with hot phenol (Sambrook et al. 1989) and poly(A)+ mRNA with an mRNA purification kit (Pharmacia). For cDNA synthesis, 5 μg of total RNA (or 2 μg of poly(A)+ mRNA) was heated for 5 min at 65°C, cooled on ice, and immediately incubated with 1 μg of random hexanucleotide primers (or 0.5 μg of d(T)23]) in 50 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KC1, 10 mM DTT, 3 mM MgCl2, 1 mM each dNTP, and 400 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL) for 1.5 hr at 37°C.

**PCR with degenerate oligonucleotides**

Degenerate oligonucleotides 1–3 [below] were chosen on the basis of the most conserved amino acid sequences of the *Ets* domain. One hundred nanograms of DT cell cDNA and 100 ng of oligonucleotides 1 and 2 or 1 and 3 were incubated in 50 μl containing 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus), 10 mM Tris-HCl (pH 8.3), 50 mM KC1, 1.5 mM MgCl2, 0.001% gelatin, and 200 μM dNTP for 10 min at 94°C, and 30 cycles for 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. Oligonucleotides with human sequence were used to amplify murine Elk [12-13] and SAP [14–15] cDNAs from DT cell cDNA (100 and 110 bp, respectively). The PCR products were digested with EcoRI and BamH1, or EcoRI alone, cloned into the corresponding sites of pSG5 (Green et al. 1988), and sequenced. Sequences for oligonucleotides 1–3 and 12–15 are [1] 5'-cgcggatcc-CTTTTCTGC(A/G)TC(A/G)A(A/G)TA(A/G)G(A/G)CTA-3'; [2] 5'-cgcggatccG(T/G)NC(T/G)NTGTGT(A/G)GA(A/G)CCACCA(A/G)TA-3'; [3] 5'-cgcggatcc(A/G)T(A/G)TA(A/G)TA(A/G)TA(A/G)CA(A/G)TA-3'; [4] 5'-cgcggatcc(A/G)T(A/G)TA(A/G)CA(A/G)TA(A/G)TA(A/G)CA-3'; [5] 5'-cgcggatccG(T/G)NC(T/G)NTGTGT(A/G)GA(A/G)CCACCA(A/G)TA-3'; and [15] 5'-cgcggatcc(T/G)NC(T/G)NTGTGT(A/G)GA(A/G)CCACCA(A/G)TA-3'. These sequences are based on the Elk and SAP murine sequences cloned from DT cell cDNA libraries by PCR. Two oligonucleotides that were based on a ZapII sequence around the cloning site of the cDNA, and nested primers from murine *elk* and *SAP* were used to amplify murine *elk* and *SAP* cDNAs. The cDNA fragments were cloned and sequenced. A HeLa cell random-primed cDNA *ζ* ZapII library was screened with a murine net cDNA probe under low stringency conditions.

**Construction of recombinants**

-pTL2-Net: net cDNA, with a consensus Kozak sequence GCCCACTCCC replacing sequences 5' to the ATG (nucleotide 295, Fig. 1) in pTL2. An *XmnI*-BglII fragment generated by PCR [as above] with oligonucleotides 6 and 7 was ligated with a BglII-EcoRI fragment from Net-Bluescript [cDNA from clone 26] between the *XmnI* and *EcoRI* sites of pTL2 (pSG5 with an extended polynucleotide between *EcoRI* and *BglII*). The PCR-derived insert was sequenced. Sequences for oligonucleotides 6 and 7 are [6] 5'-aattcccgggcACCATGGGAGGATAAATTCCGAG-ATTTGAGCA-3'; and [7] 5'-ACACAGAGAAGCGTGGCAATGGGCAGCA-TGAGC-3'.

-pSG5-Net: The complete cDNA [clone 26] in the *EcoRI* site of pSG5.

-SRm: Double-stranded sequence of the SRE mutated in the FAP site TCGACAGGATTCATATTAGGACATCAGGCCTCTGTAAC was inserted in the *SalI* site of the pBL-CAT4 polynucleotide in the sense orientation and sequenced.

-p601D-Net and p601D-anti-Net: Net cDNA in either orientation in the *EcoRI* site of p601D.

-p601D: The 345-bp rat β-actin promoter region in the place of the SV40 promoter region of pTL2 (Beddington et al. 1989; Lukin et al. 1992).

-pTL1-elm: Human HindIII-BamHI elk1 cDNA from p77-elm1 (Morais et al. 1993) between the HindIII and BamHI sites of pTL1.
ate, 2 mM MnCl₂, 1 mM dATP, and 15 units of terminal deoxy-
nucleotidyl transferase (Boehringer) for 1 hr at 37°C, extracted
with phenol and chloroform, ethanol precipitated, and resus-
pended in 20 μl of TE. Tail dCNA (2 μl) was amplified by two
rounds of PCR. First round: oligonucleotides 8 and 5, 10 min at
94°C, 20 cycles of 1 min at 94°C, 2 min at 40°C and 3 min at
72°C. Second round: oligonucleotide 9 and internal oligonu-
cleotide 9 with 5 μl of the first round, 10 min at 94°C, 30 cycle of
1 min at 94°C, 2 min at 60°C, 3 min at 72°C.

For 3’-end amplification [the conditions were the same as for
5’-end amplification with 100 ng cDNA and oligonucleotide 8
with 10 for the first round, and with the internal oligonucle-
tide 11 for the second round. Sequences for oligonucleotides
8–11 are (8) 5’-cgggagaattctccgagTTTTTTTTTTTTTTTTTT-
TGAATTCCAGGATATCCGGGAAAGAG. 1-344 NET:

**Mobility shift assays**

Proteins (1–5 μl) brought to a constant volume with mock
reticulocyte lysate (incubated without added RNA) were incu-
bated for 45 min at 25°C in 20 μl of either 20 mM HEPS [pH
7.9], 20% glycerol, 0.1 mM EDTA, 2 mM DTT, 1 μg/20 μl of poly
[di[C]-Cl], 50 mM KCl with excess PEA3* probe, or 5 μM
PEA3* [pH 7.9], 2.5 mM MgCl₂, 2.5 mM EDTA, 5 mM NaCl, 2
mM spermidine, 2.5 mM DTT, 1 μg/20 μl of poly[di[C]-Cl], 2 μg/μl
of BSA with excess SRE probes. The samples were loaded im-
mediately on prerun [30 min at 15 mA [75 V] 4% or 6% poly-
acylamide (bis:acrylamide, 1:29] gels in 0.25× TBE and run for
60 min at 30 mA (150 V) with recirculating buffer.

Oligonucleotides used for mobility shifts: PEA3*, 5’-TC-
GAGGCGGAAGTGGCTCA-3’; PEA3* mut, 5’-TCAG-
CATGAAGTGACGTCGA-3’; SRE, 5’-TACACAGGATGC-
TAGATAGGACA-3’ spanning the human e-fos promoter from
324 to 300 (Janknecht and Nordheim 1992); G oligo, 5’-
GCCCAATGCCGGAAATTGCCCATATAAGGACTCTA-
GA-3’ selected oligonucleotide with Elk1 and SRF motifs (Tre-
isman et al. 1992); G mut-wt, 5’-GCCCAATGACTGAAAT-
TGCCCATATAAGGACTCTA-3’ (Treisman et al. 1992); wt-
mot, 5’-GCCCAATGCCGGAAATTGCCCATATAAGGAC-
TCTAGA-3’ (Treisman et al. 1993).

Only one strand is shown. The complementary strands form
complete double strands with blunt ends. Oligonucleotides
were 5’-labeled by T4 polynucleotide kinase and purified on
native 10% polyacrylamide gels.

**Antibodies and protein (Western) blots**

Net and Elk1 antisera were raised in rabbits against the ovalbu-
m-min-MBS-coupled peptides [200 μg/injection, cysteine (C)] was
added for coupling: PB263, 385-[CHMVPHPSLDRAPSPPVLL-
SPSSQKS-409 [Net], PC11, 411-[C]SVDSLSTRVLSPPQK-
427 [Elk1].

For Western blots, total cell extracts from transiently trans-
fected COS [20 μg] or NIH-3T3 [100 μg] cells were electropho-
sed on 10% SDS–polyacrylamide gels, blotted to nitrocel-
llose filters, and revealed with NET[37S] or Elk[512]–specific
antibodies and the ECL detection kit (Amersham’s protocol).

Specific DNA-binding affinities of the ΔNET mutants for the
PEA3* probe were estimated from the amount of radioactive
probe that formed a complex in mobility shifts with a known
amount of protein estimated by SDS-PAGE of proteins labeled
with [35S]methionine in reticulocyte lysates [with the number
of methionines taken into account]. Equimolar amounts of pro-
teins were used for mobility shifts.

**Cell culture and transfections**

NIH-3T3 C11 cells [the NIH-3T3 subline was used originally to
derive the Ras-transformed line DT] maintained in Dulbecco’s
modified Eagle medium (DMEM) with 10% fetal calf serum
were transfected by the calcium phosphate method with 20 μg/100-mm dish of plasmid DNA containing 2 μg of pCH110 (transfection efficiency control), 1 μg of reporters, expression vectors (see figure legends), and the appropriate amounts of control vectors (pSG5, pARCBx2). The cells were split, incubated with precipitated DNA for 24 hr, washed twice with DMEM, incubated with DMEM plus 0.05% fetal calf serum for 24 or 48 hr (see Fig. 8), scraped, freeze-thawed three times in solution A (15 mM Tris-HCl) at pH 7.9, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM DTT, 0.4 mM PMSF, and centrifuged. β-Galactosidase activity was measured first to correct for transfection efficiency. For CAT assays, samples were heated to 65°C for 10 min, acetylated chloramphenicol was separated by thin-layer chromatography with a 95% chloroform/5% methanol solvent, and radioactivity was quantitated with a Fuji PhosphorImager. Experiments were repeated at least three times. In the figures, one representative CAT assay is shown, and error bars on the graph indicate one standard deviation.

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Note added in proof

Mouse net was cloned independently by T. Liberman and colleagues (ERP, Lopez, M., P. Oettgen, Y. Akharalii, U. Dendorfer, and T.A. Liberman. 1994. ERP, a new member of the ets transcription factor/oncoprotein family: Cloning, characterization, and differential expression during B-cell development. Mol. Cell. Biol. 14: 3292–3309) and human Net by R. Treisman and colleagues (SAP2, Treisman, R., pers. comm.).

The nucleotide sequence data for mouse Elk1, mouse SAP1a, and human Net have been submitted to the EMBL, GenBank, and DDBJ data libraries. The sequence data for mouse net have been submitted under accession number Z32815.

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