The EMBO Journal vol.10 no.5 pp.1127 - 1134, 1991

The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins

C.Wasylyk, A.Gutman, R.Nicholson[1] and B.Wasylyk[2]

CNRS-LGME/INSERM U184, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

[1]Present address: Carcinogenesis Research Unit, School of Pathology, Faculty of Medicine, The University of New South Wales, PO Box 1 (Kensington), Sydney, NSW 2033, Australia
[2]Corresponding author

Communicated by P.Chambon

The c-*ets* protooncogenes have recently been shown to code for transcription factors that activate the oncogene responsive unit of the polyoma virus enhancer. We show that transcription of the stromelysin gene, which is highly expressed in transformed cells and tumours, is efficiently activated by c-Ets-1 and -2 through two DNA elements. The distal element is a highly conserved palindrome composed of two strong binding sites for c-Ets-1. The proximal element does not bind c-Ets-1, but may be activated indirectly by increased synthesis of c-Jun and c-Fos. Both *ets* responsive elements mediate activation by the oncoproteins Ha-Ras,v-Src and v-Mos. These results suggest that c-Ets participates in the mechanisms by which stromelysin gene expression is deregulated in transformed cells and tumours.

Key words: AP-1/c-Ets/oncogenes/stromelysin/transcription

Introduction

Transformation of cells by non-nuclear oncoproteins leads to deregulated transcription in the nucleus. Transcription factors that are oncogene products appear to be intermediates in these processes (reviewed by Gutman and Wasylyk, 1991). It is important to understand how these oncotranscription factors regulate the expression of genes that contribute to the transformed phenotype. We have previously shown that several non-nuclear oncogenes activate transcription through the oncogene responsive unit of the polyoma virus enhancer (Wasylyk *et al.*, 1988). Three oncotranscription factors mediate this activation, c-Ets, c-Jun and c-Fos (Wasylyk *et al.*, 1990). We have investigated here how c-Ets, together with c-Jun and c-Fos, regulates the expression of the stromelysin gene that is highly expressed in transformed cells and contributes to the transformed phenotype.

v-Ets was originally discovered as a fusion protein with v-Myb produced by the avian leukaemia virus E26 (LePrince *et al.*, 1983; Nunn *et al.*, 1983). The v-*ets* oncogene transforms erythroblasts and fibroblasts and affects myeloid transformation by v-*myb* (Nunn and Hunter, 1989; Golay *et al.*, 1988; Seth *et al.*, 1989). It belongs to a gene family that includes c-*ets-1* (p68), its progenitor, c-*ets-2*, *erg*, *elk-1* and -*2*, *PU1* (*Spi1*) and *E74*. A conserved domain is required for specific DNA binding, whereas the activation domain is less conserved (Gutman and Wasylyk, 1990; Karim *et al.*, 1990; J.Schneikert and B.Wasylyk, unpublished results). The activity of c-Ets-1 and c-Ets-2 may be regulated by both pre- and post-translational mechanisms, since their synthesis is induced upon growth stimulation of mouse fibroblasts by serum (Bhat *et al.*, 1987) and they are phosphorylated in response to mitogenic signals (Pognonec *et al.*, 1990; Fujiwara *et al.*, 1990).

Stromelysin is one of the matrix metalloproteinases that degrade extracellular matrix and basement membrane components (Nicholson *et al.*, 1989). They play a role in normal processes that involve matrix remodelling, such as development, uterine involution and wound healing, and in pathological conditions that involve uncontrolled degradation of the extracellular matrix, such as in rheumatoid arthritis, tumour invasion and metastasis (reviewed by Matrisian, 1990). The matrix metalloproteinases are frequently overexpressed in transformed cells (Matrisian *et al.*, 1985) and in tumours with metastatic potential (Goldfarb and Liotta, 1986; Matrisian *et al.*, 1986; Ostrowski *et al.*, 1988). Transcription of their genes is tightly regulated by growth factors, tumour promoters, oncogene products and hormone receptors. The positive and negative effects of these factors are mediated to some extent by the AP-1 motif, that is present in the promoters of rat, rabbit and human stromelysin and the human interstitial collagenase genes (Schönthal *et al.*, 1988; Kerr *et al.*, 1990; Jonat *et al.*, 1989; Nicholson *et al.*, 1990).

We show that c-Ets-1 and -2 efficiently activate the rat stromelysin promoter through two responsive elements. The distal element is a palindrome composed of two high affinity c-Ets-1 binding sites, that mediate synergistic activation by a mechanism that does not involve cooperative DNA binding. The proximal element does not bind c-Ets-1, but does bind c-Jun+c-Fos. The response of the proximal element may involve increased synthesis of AP-1, by c-Ets-1 activation of the c-*jun* and c-*fos* gene promoters. The stromelysin promoter responds efficiently to c-Ets and c-Jun+c-Fos independently, and their combined effects are not synergistic, in contrast to the oncogene responsive unit of the polyoma virus enhancer (Wasylyk *et al.*, 1990). Finally, we show that both *ets* responsive elements mediate promoter activation by the non-nuclear oncoproteins Ha-Ras, v-Src and v-Mos. We will discuss how c-Ets may participate in coordinate regulation of stromelysin and collagenase genes in both normal and transformed cells.

Results

The rat stromelysin promoter is efficiently activated by Ets expression

Stromelysin promoter activity was studied in HeLa cells by co-transfecting 1100-CAT (Figure 1), a control recombinant (to correct for variations in transfection efficiency) and *ets*

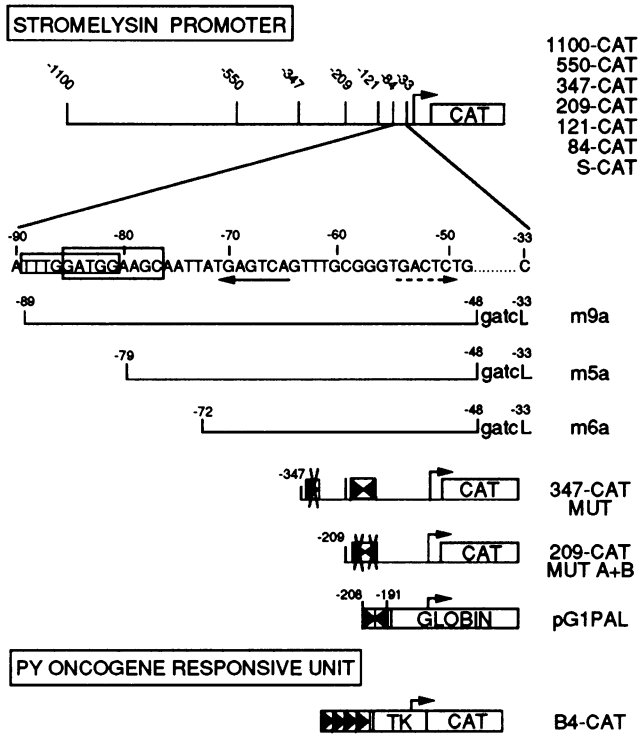

Fig. 1. Structure of reporters. Stromelysin promoter: 1100-CAT (STR-CAT) contains the −1100 to +8 region of the rat stromelysin promoter in pCAT (Nicholson *et al.*, 1990). 550-CAT, 347-CAT, 209-CAT, 121-CAT, 84-CAT and S-CAT contain promoter sequences from +8 to −550, −347, −209, −121, −84 and −33, respectively. m9a, m5a and m6a have upstream sequences linked to S-CAT (−89/−48, −79/−48 and −72/−48, respectively). 347-CAT MUT has a point mutation in an *ets* motif (−345 TCTGGAAGT −337 to TCTaGAAGT). 209-CAT MUT A+B has point mutations in two *ets* motifs (−209 GCAGGAAGCATTTCCTGGG −191 to GCAaGAAGCATTTCtTGG). pG1PAl has the stromelysin −208/−191 sequence upstream from the β globin promoter (at −110) in pG1. On the sequence the boxes enclose *ets*-like motifs, the arrow indicates an AP1 motif and the broken arrow a similar sequence. PY ONCOGENE RESPONSIVE UNIT: B4-CAT has four copies of the polyoma virus enhancer oncogene responsive unit, upstream from the thymidine kinase promoter in pBLCAT4.

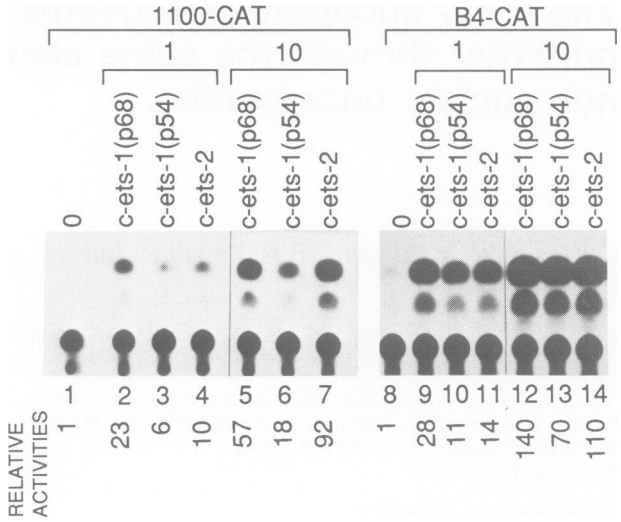

Fig. 2. The stromelysin promoter is activated by c-Ets-1 and c-Ets-2. HeLa cells were co-transfected with 5 μg reporter recombinants (1100-CAT, B4-CAT), 5 μg internal control (CH110) and 1 or 10 μg of expression vectors for either c-Ets-1 (p68), c-Ets-1 (p54) or c-Ets-2. The total amount of expression vector was made up to 10 μg with the control vector pSG5. CAT activities, corrected for variations in the internal control, were expressed relative to the 0 expression vector controls.

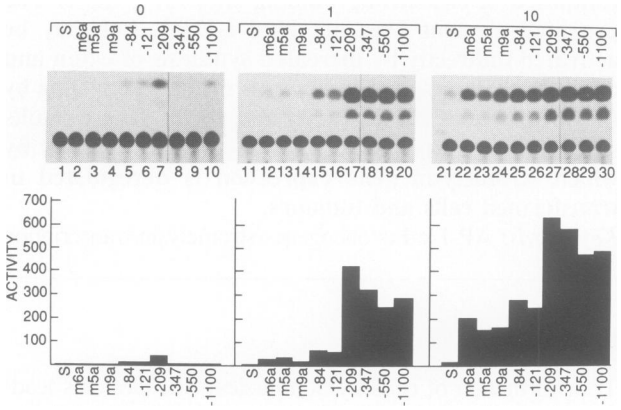

Fig. 3. There are two *ets* responsive regions in the stromelysin promoter. HeLa cells were co-transfected with 5 μg reporter recombinants containing deletions of the stromelysin promoter (Figure 1), 5 μg internal control (CH110) and either 10 μg of the control expression vector (pSG5, lanes 1−10), or 1 μg or 10 μg of the c-*ets*-1 (p68) expression vector (lanes 11−20 and 21−30, respectively; the 1 μg samples also contained 9 μg pSG5). CAT activities, corrected for variations in the internal control, are expressed in arbitrary units (c.p.m./OD).

expression vectors (Wasylyk *et al.*, 1990). c-Ets-1 (p68 or p54, different products resulting from alternative splicing) and c-Ets-2 activated the stromelysin promoter between 6- and 23-fold (lanes 1−4, Figure 2). With more co-transfected expression vector (10 μg instead of 1 μg), activation was almost 100-fold (lanes 5−7). c-Ets activated the stromelysin promoter almost as efficiently as four copies of the oncogene responsive unit from the polyoma virus enhancer (lanes 8−14 and Figure 1). Minimal promoter elements [S-CAT (see Figure 1) and pBLCAT4] did not respond significantly to Ets (see below and results not shown). p68 c-Ets-1 stimulated both reporters better than p54 (Figure 2), suggesting that this is not a promoter specific effect. Our results show that the stromelysin promoter is efficiently activated by c-Ets.

There are two ets responsive elements in the stromelysin promoter

The promoter was deleted from −1100 to −33 (Figure 1) to localize the *ets* responsive elements. The promoter had a low basal activity, that increased when sequences between −347 and −209 were deleted (lanes 1−10, Figure 3), suggesting that there is a negative element in this region. The response elicited with 1 μg of c-Ets-1 (p68) expression vector was decreased by deleting sequences between −209 and −121 (lanes 11−20). With 10 μg of expression vector, a second element became apparent, between −48 and −27 (lanes 21−20, see Figure 1 for m6a, m5a, m9a). The two elements may respond by different mechanisms, since they are activated at different levels of *ets* expression vector.

The sequence 5′-GCA/CGGAAGT-3′ is required for c-Ets-1 (p68) binding

We delimited the c-Ets-1 DNA binding site by gel retardation with c-Ets-1 (p68) synthesized in reticulocyte lysates. Probes

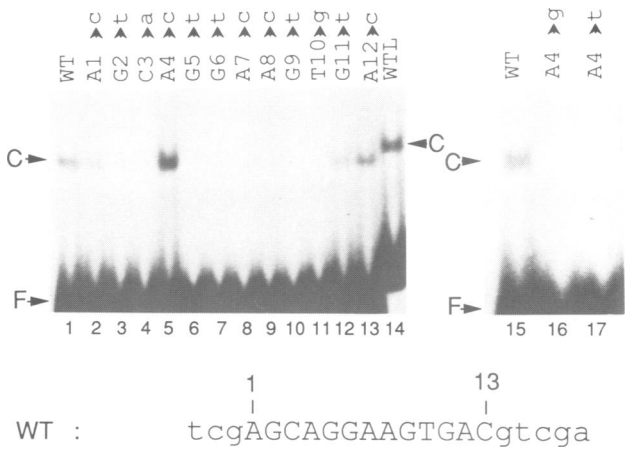

Fig. 4. The DNA sequence 5′-GCA/CGGAAGT-3′ is required for c-Ets-1 (p68) to bind specifically to DNA. c-Ets-1 (p68) was synthesized in rabbit reticulocyte lysates and specific complexes (C) formed with oligonucleotide probes were resolved by gel retardation. The sequences of the wild-type probes (WT and WTL, top strand, 5′ to 3′) are shown below the figure and the mutations in WT above the appropriate lanes. Capital letters indicate nucleotides present in the polyoma virus enhancer. F: free probe.

with either 13 or 25 bp from the polyoma virus enhancer (WT and WTL, lanes 1, 14, Figure 4) efficiently bound c-Ets-1 (p68). Using 13 bp probes that were systematically mutated, the critical sequences were shown to extend from G2 to T10 (lanes 1−13). Interestingly, at A4, c but not g or t permitted binding (lanes 5 and 15−17). Therefore, the sequence 5′-GCA/CGGAAGT-3′ is required for c-Ets-1 (p68) binding.

The distal c-ets responsive element lies between −208 and −191

The three best homologies in the stromelysin promoter to the *ets* motif (−345 to −337, tCtGGAAGY; −208 to −200, GCAGGAAGc; −191 to −199, cCAGGAAaT) were mutated by converting G4 to A (this mutation abolishes binding by c-Ets-1, see Wasylyk *et al.*, 1990 and Figure 11 for numbering). The mutation in the −345/−337 sequence (347-CAT MUT, Figure 1) did not affect activation by c-Ets-1 (p68) (lanes 1−8, Figure 5A), in agreement with the deletion analysis (347-CAT and 209-CAT, Figure 3). The two other motifs form an imperfect palindrome (see Figure 6, bottom). A double mutation in the palindrome (209-CAT MUTA+B, Figure 1) decreased activation by c-Ets-1 (p68) (lanes 3, 4, 9, 10, Figure 5B), as well as by c-Ets-1 (p54) and c-Ets-2 (lanes 5−8 and 11−14, Figure 5B). Two other less homologous sequences (−85 to −77, GatGGAAGc and −89 to −81, tttGGAtGG) did not mediate c-Ets-1 (p68) stimulation (121-CAT, 84-CAT, m9a,m5a and m6a, Figures 1 and 3). Our results show that the distal c-*ets* responsive element lies within the sequences between −208 and −191.

The two c-ets motifs of the distal element mediate synergistic activation by c-Ets-1 (p68)

The distal responsive sequence was introduced upstream from the β globin promoter in the reporter pG1 (Figure 1).

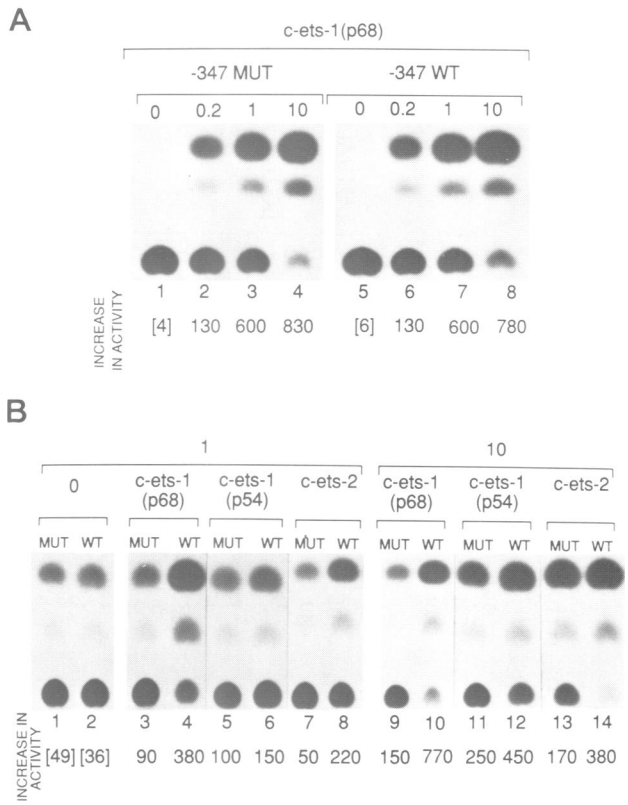

Fig. 5. The upstream *ets* responsive element is located between −208 and −191. (**A**) The −345 to −337 element does not mediate activation by c-Ets-1 (p68). HeLa cells were co-transfected with 5 μg 347-CAT (−347 WT) or 347-CAT MUT (−347 MUT), 5 μg internal control (CH110), and the c-*ets*-1 (p68) expression vector (0, 0.2, 1, 10 μg made up to 10 μg with pSG5). (**B**) The −208/−191 sequence mediates activation by c-Ets-1 (p68 and p54) and c-Ets-2. HeLa cells were co-transfected with 5 μg 209-CAT (WT) or 209-CAT MUTA+B (MUT), 5 μg internal control (CH110) and expression vectors for c-Ets-1 (p68 or p54) or c-Ets-2 (0, 1 or 10 μg made up to 10 μg with pSG5). The increase in CAT activity, corrected for variations in the internal control, is given in arbitrary units (c.p.m./OD). Background activity, in the absence of expressed ets-1, is given in parentheses. Lanes 9−14 are less exposed than lanes 1−8, to facilitate comparison.

HeLa cells were co-transfected with the reporters, an internal control (pβCBx2) and expression vectors, and total RNA was analysed by quantitative S1 nuclease mapping. c-Ets-1 p(68) stimulated transcription through the palindromic element (~10-fold, lanes 1−6, Figure 6; similar results were obtained with p54 and c-Ets-2, data not shown). The distal element responded maximally with 1 μg of expression vector (lanes 1−6), as expected from the results described above (see 209- and 121-CAT, Figure 3). Strikingly, mutating either motif, as well as both motifs, completely abolished activation (compare pG1, mutA, mutB and mutA+B, lanes 1−3, 7−15). These results show that both elements of the palindrome are essential for activation.

c-Ets-1 binds non-cooperatively to the two motifs of the palindrome

We compared the affinity of c-Ets-1 (p68) for various DNA sequences by gel retardation assays. Probes containing either the two palindromic motifs or the *ets* motif from the polyoma enhancer (PEA3) formed complexes with similar mobility and intensity (lanes 2 and 5, Figure 7A). G4 to A mutations, in both arms of the palindrome and in the PEA3 probe,

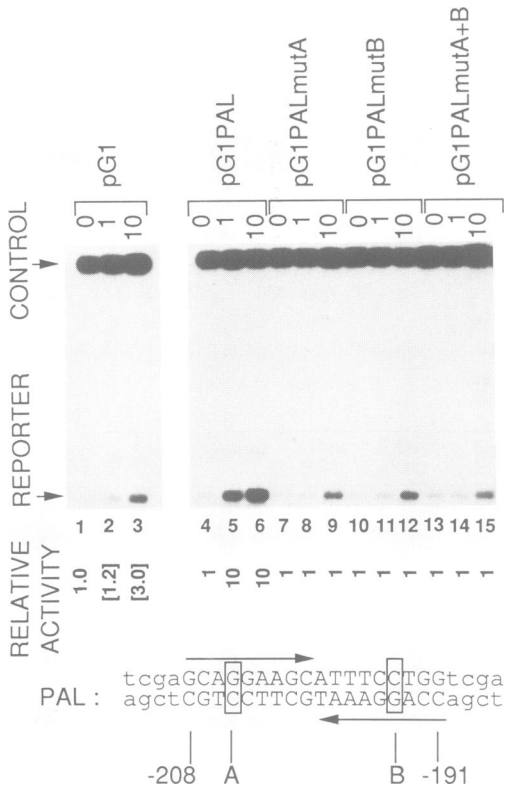

Fig. 6. Synergistic activation by c-Ets-1 (p68) through the two *ets* motifs of the −208/−191 responsive element. HeLa cells were co-transfected with 2 μg reporters (pG1, pG1PALmutA, pG1PALmutB, pG1PALmut A+B), 1 μg internal control (pβCBx2) and the c-*ets*-1 (p68) expression vector (0, 1 or 10 μg made up to 10 μg with pSG5). RNA specifically initiated from the reporter and the internal control (see corresponding bands) was measured by quantitative S1 nuclease mapping. The amounts are expressed relative to pG1 with the same amount of expression vector. Values in parentheses are relative to pG1 without the c-Ets-1 (p68) expression vector (lane 1). The stromelysin sequence in pG1PAl is shown in capital letters below the figure. The imperfect palindrome is indicated by arrows. The boxed base pairs were mutated (G:C to a:t) either individually (−205 in mutA and −194 in mutB) or together (mutA+B).

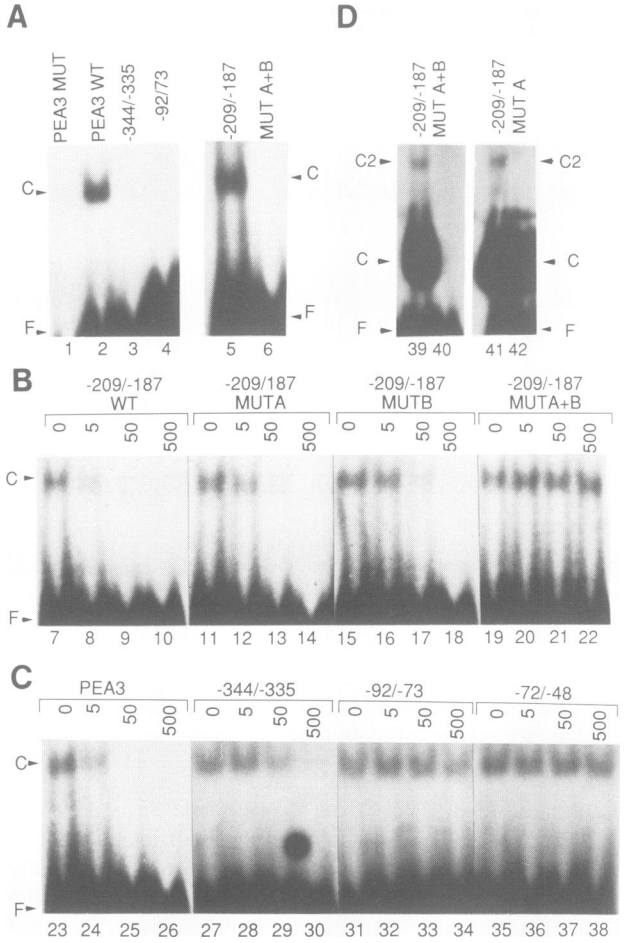

Fig. 7. The −208/−191 sequence binds c-Ets-1 (p68) with higher affinity than other similar sequences in the stromelysin promoter. **A.** (lanes 1−6) A specific retarded complex is detected only with the −208/−191 element. c-Ets-1 (p68) synthesized in reticulocyte lysates was incubated with the indicated probes [−344/−335, −92/−73, −209/−187 WT and MUT A+B from stromelysin, and PEA3 WT and MUT (G4 to a) from the polyoma virus enhancer (Wasylyk *et al.*, 1990)] and specific complexes (C) resolved by gel retardation. **B.** (lanes 7−22) Both motifs of the imperfect palindrome compete for c-Ets-1 (p68) complex formation. The indicated unlabelled sequences from the stromelysin promoter (5, 50, 500 molar excess) were used to compete with PEA3 probe for c-Ets-1 (p68) complex (C) formation, and analysed by gel retardation. **C.** (lanes 23−38) Only the −344/−335 sequence competes to a low extent for c-Ets-1 (p68) complex formation. Competitions were as in B. with the indicated competitors. **D.** (lanes 39−42) A minor complex (C2), with lower mobility, forms only a probe containing two intact motifs. Specific complexes (C, C2) formed between the indicated probes (−209/−187 MUT A+B, MUT A) and c-Ets-1 were resolved by gel retardation. C, C2: specific complexes; F: free probe.

abolished binding (lanes, 1, 2, 5 and 6, Figure 7). A minor, more slowly migrating complex was observed with the palindromic probe (C2, lanes 39 and 41). This complex probably contains two molecules of Ets bound to the palindrome since it was not observed with a probe containing only one intact motif (lane 42). Mutating either motif of the palindrome decreased the intensity of the major complex by about a half (results not shown). Most complexes under these conditions contain one c-Ets-1 molecule bound to the palindrome. In competition assays, the −209/−187 sequence competed about twice as efficiently as the PEA3 sequence for the formation of the c-Ets-1 (p68)−PEA3 probe complex (lanes 7−10, 23−26, Figure 7). G4 to A mutations in either motif of the palindrome only marginally affected competition, whereas mutating both motifs abolished competition (lanes 11−22). These results show that both motifs of the palindrome can bind c-Ets-1 (p68) independently, and that its affinity for c-Ets-1 is no greater than expected from the presence of two motifs on the same molecule.

The distal element binds c-Ets-1 with higher affinity than other similar sequences of the stromelysin promoter

The −92/−73 and the −344/−335 sequences did not form detectable complexes with c-Ets-1 (p68) (lanes 3 and 4, Figure 7) and competed at best with 10-fold lower affinity than either motif of the palindrome (lanes 27−30, 31−34, Figure 7). These results show that the sequences with the highest affinity for c-Ets-1 mediate trans-activation. However, high affinity alone is not sufficient, since both motifs of the palindrome are absolutely required for activation (see above).

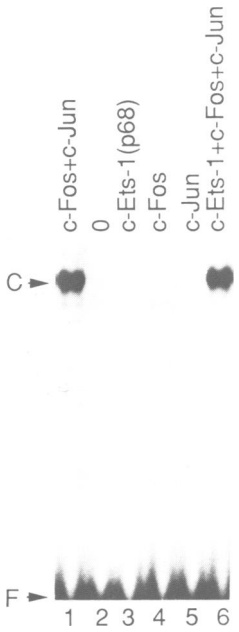

Fig. 8. c-Ets-1 (p68) does not bind to either a DNA fragment containing the AP1 motif or the AP1 factor bound to its cognate motif. c-Fos, c-Jun and c-Ets-1 (p68) synthesized in reticulocyte lysates were preincubated in various combinations for 30 min at 37°C before incubation with the −72/−48 stromelysin probe. Specific complexes (C) were resolved by gel retardation. Incubations contained a total of 6 μl of lysate, the final volume was made up with lysate incubated without added RNA. F: free probe.

The downstream responsive element does not bind c-Ets-1

The −72/−48 sequence is not homologous to the *ets* motif, but does have a consensus AP-1 motif (5′-TGACTCA-3′, see Figure 1). The −72/−48 DNA fragment did not bind c-Ets-1 (p68 or p54) in either competition assays (lanes 35−38, Figure 7) or gel retardation (lane 3, Figure 8 and results not shown). It did bind the c-Fos+c-Jun complex (compare lanes 1, 2, 4, 5, Figure 8). The mobility of the c-Jun+c-Fos−DNA complex was not altered by c-Ets-1 (p68 or p54) (lanes 1 and 6 and not shown), even when various c-Ets-1 specific antibodies were also added (not shown). Thus activation by c-Ets-1 through the proximal element does not involve either direct binding or protein−protein interactions with DNA bound c-Fos and c-Jun.

c-Ets-1 (p68) does not cooperate with c-Jun+c-Fos for activation of the stromelysin promoter

The combined activity of c-Ets-1 (p68), c-Jun and c-Fos is absolutely required for activation of the monomeric oncogene responsive unit from polyoma virus enhancer (Wasylyk *et al.*, 1990). In contrast, the stromelysin promoter was activated by either c-Ets-1 (p68) or c-Jun+c-Fos alone (Figure 9, lanes 1−3, 5, 6), and the effect of combining them was about additive (lanes 4 and 7). These results suggest that these factors do not cooperate in activation of the stromelysin promoter.

The distal ets responsive element mediates activation by c-Ha-Ras, v-Src and v-Mos

Expression of c-Ha-Ras, v-Mos and v-Src in HeLa cells stimulated both the stromelysin promoter and the Py oncogene responsive unit (Figure 10 and results not shown).

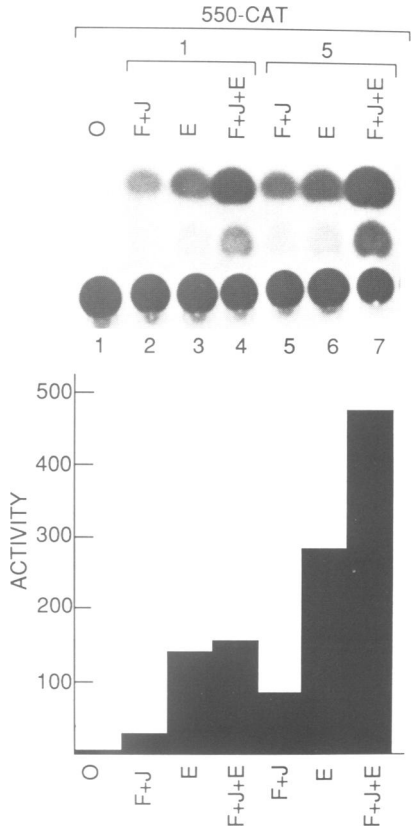

Fig. 9. Activation of the stromelysin promoter by the combination of c-Fos+c-Jun and c-Ets-1 (p68). HeLa cells were co-transfected with 550-CAT (5 μg), the internal control (5 μg CH110) and the indicated combinations (1 or 5 μg of each) of expression vectors for c-Fos (F), c-Jun (J) and c-Ets-1 (p68) (E). CAT activity, corrected for variations in the internal control, is given in arbitrary units (c.p.m./OD).

The double point mutation in the palindromic element decreased activation (Figure 10). The residual activation is probably mediated by the AP-1 motifs (compare m6a and S-CAT, Figure 10, and see Kerr *et al.*, 1988), but not by the sequences between −89 and −77 that resemble the *ets* motif (compare m9a, m5a and m6a, Figures 1 and 10). Polyoma middle T and v-Raf expression did not significantly activate 209-CAT or several other oncogene responsive elements including B4-CAT and a rel/NFKB multimer (results not shown). Polyoma middle T and v-Raf expression activated these reporters in several other cell lines, suggesting that this is a cell-specific property of the oncogenes. Our data show that the *ets* motifs of the distal element are required for stromelysin promoter activation by the cytoplasmic oncoproteins c-Ha-Ras, v-Src and v-Mos.

Discussion

We have shown that the oncoproteins c-Ets-1 and -2 efficiently activate the rat stromelysin gene promoter through two different elements. The distal sequence contains two high affinity c-Ets-1 binding sites. Both motifs resemble the c-Ets-1 binding site from the polyoma virus enhancer, and other sequences that were published during the preparation of this manuscript (Figure 11). They contain a key, centrally located core AGGAA and flanking sequences that contribute to affinity. The stromelysin, Py PEA3 and MSV sequences bind c-Ets-1 with similar affinity (C.Wasylyk, unpublished

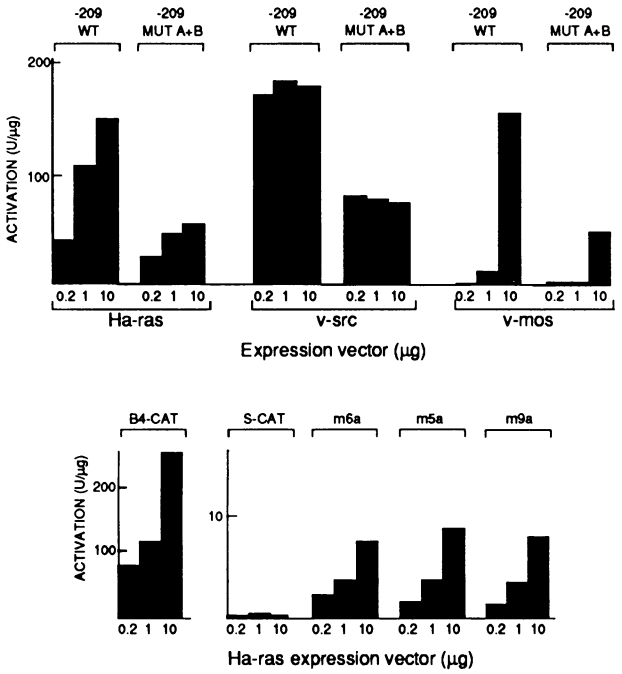

Fig. 10. Expression of Ha-Ras, v-Src and v-Mos activates the stromelysin promoter through the upstream ets responsive element. HeLa cells were co-transfected with the indicated reporters [1 μg 209-CAT (−209 WT) and 209-CAT MUT A+B (−209 MUT A+B); 1 μg B4-CAT; 5 μg S-CAT, m6a, m5a, m9a], 5 μg internal control (CH110) and vectors that express either activated Ha-Ras, v-Src or v-Mos (0.2, 1 or 10 μg). The increase in CAT activity, corrected for variations in the internal control, is given in arbitrary units (c.p.m./OD per μg of transfected reporter). The basal activities were: 209-CAT, 27; 209-CAT MUT A+B, 23; B4-CAT, 8; S-CAT, 0.8; m6a, 1.3; m5a, 1.7; m9a, 0.9.

1 9	
GCAGGAAGT	PY-PEA3
GCAGGAAGC	STROM RAT(-208/-200)
CCAGGAAAT	STROM RAT(-191/-199)
ACAGGAAGC	STROM HUMAN(-217/-209)
CCAGGAAGT	STROM HUMAN(-200/-208)
AGCGGAAGC	MSV
AGAGGATGT	TCR α
GGAGGAAAT	HTLV-1(-121/-135)
CCGGGAAGC	HTLV-1(-148/-163)
−C/G AGGAAG T/C	c-ETS-1 CONSENSUS
C C/T C/A GGAAGT	E74A
AGAGGAACT	PU.1

Fig. 11. Comparison of specific binding sites for Ets related transcription factors. The consensus sequence for c-Ets-1 is derived from the binding sites from the polyoma virus enhancer (Py PEA3), the stromelysin promoter from rat (STROM RAT) and man (STROM HUMAN, C.Wasylyk, unpublished results), the Moloney murine sarcoma virus (MSV) long terminal repeat (Gunther *et al.*, 1990), the T cell receptor alpha gene enhancer (TCRae, Ho *et al.*, 1990) and the human T cell leukaemia virus (HTLV-1) long terminal repeat (Bosselut *et al.*, 1990). The relative affinities of these sites are not known, and those for HTVL-1 were not precisely defined or investigated individually. E74A (Urness and Thummel, 1990) and PU.1 (Klemz *et al.*, 1990) are Ets-related proteins.

results), even though they differ in the base pairs that flank the core. Interestingly, for Py PEA3 and MSV some of these have opposite and compensating effects. The C to G at the second position decreases the affinity, whereas A to C in the third position has the opposite effect (see above and results not shown). Several sequences in the stromelysin promoter have one or two alterations in the core element (−345/−337, −89/−81 and −85/−77). They have a low affinity for c-Ets-1 and do not mediate c-Ets-1 activation. Interestingly, the PU.1 and Py motifs have the core motif but differ in their flanking sequences, and bind efficiently only to their cognate factors (C.Wasylyk, unpublished results).

Neither *ets* motif of the distal element is sufficient on its own for *ets* induction. Similarly, the single *ets* motif of the polyoma virus enhancer oncogene responsive unit does not respond to c-Ets-1 (Wasylyk *et al.*, 1990). The important feature appears to be the presence of more than one motif, rather than spacing or orientation, since various tetramers of the polyoma *ets* motif are inducible (see B4-CAT and Wasylyk *et al.*, 1990). The mechanism by which only two or more motifs respond is not known. Our results *in vitro* suggest that it does not involve enhanced binding of c-Ets-1 to a palindromic element, but we cannot exclude that there are additional factors which alter DNA binding *in vivo*.

The distal element is inducible by c-Ets-1, in the absence of other stromelysin sequences. This suggests that stromelysin promoter activity could be especially sensitive to variations in *ets* activity in the cell. The distal element is found in the stromelysin 1 promoters from rabbit and man (Quinones *et al.*, 1989). The two base pair changes in the human sequence (see Figure 11) do not affect either binding affinity or induction (C. Wasylyk, unpublished results). The HTLV-1 LTR contains two *ets*-like motifs on opposite strands separated by 15 bp (Bosselut *et al.*, 1990). However, it is not clear whether both sequences can bind c-Ets-1 and mediate activation. The *Drosophila E74* gene contains three binding sites for the *ets*-like protein E74A (Urness and Thummel, 1990). Some promoters contain one *ets* motif, suggesting that other factors are required in addition. c-Ets-1 will only induce a monomeric polyoma virus enhancer responsive unit in the presence of AP-1 (Wasylyk *et al.*, 1990). Similarly, the collagenase promoter may be regulated by a combination of c-Ets-1 and AP-1 (Gutman and Wasylyk, 1990). The Moloney murine leukaemia virus long terminal repeat and the T cell receptor alpha gene enhancer contain one *ets* motif, and may require other factors (Gunther *et al.*, 1990; Ho *et al.*, 1990).

The proximal *ets* responsive element of the stromelysin promoter does not contain an *ets* motif, and does not bind c-Ets-1. It contains a consensus AP-1 motif that is conserved in the rabbit and human stromelysin and collagenase promoters (Matrisian, 1990). c-Ets-1 does not interact with the c-Jun+c-Fos−DNA complex *in vitro*. However, *in vivo* c-Ets-1 may bind through protein−protein interactions with other members of the AP-1 family, or with unknown factors. An alternative possibility is that c-Ets-1 stimulates AP-1 activity indirectly, perhaps by increasing the synthesis of c-Fos and c-Jun. In experiments that are not shown we found that c-Ets-1 expressed from high levels of expression vector augments both c-*jun* and c-*fos* promoter activity. A deletion of the c-*fos* promoter that removes two *ets*-like motifs decreases activation. Interestingly, one of these motifs (−323/−315) binds $p62^{TCF}$ that is required with SRF for serum induction of the c-*fos* promoter (Shaw *et al.*, 1989). The short sequence of the c-*jun* promoter that has been

published (−132/+6, Angel *et al.*, 1988) does not contain the *ets* core sequence AGGAA.

The stromelysin promoter responds to AP-1 alone, and there is no synergy with the effects of c-Ets-1. This element contains a second AP-1-like motif (TGACTCt, 6/7), on the opposite strand and separated by 9 bp from the consensus sequence (Figure 1). However, the combination of AP-1 motifs does not appear to generate an AP-1 responsive unit, since Nicholson *et al.* (1990) did not detect cooperation between the two elements. The human and rabbit stromelysin promoters do not contain the second AP-1 motif (Quinones *et al.*, 1989).

The distal and proximal *ets* responsive elements of the stromelysin promoter mediate promoter activation by several non-nuclear oncoproteins, c-Ha-Ras, v-Src and v-Mos. Work from a number of laboratories has shown that AP-1 motifs can mediate the transcriptional effects of non-nuclear oncoproteins. Our results show that a different oncoprotein transcription factor, c-Ets-1, can also mediate some of these effects. Thus, c-Ets appears to be an important intermediate between non-nuclear oncoproteins and the genes that are overtranscribed in transformed cells, such as stromelysin. This deregulated function may have a counterpart in normal cells. It is intriguing that during development, c-Ets is expressed in migrating cells such as the mesencephalic neural crest (Vandenbunder *et al.*, 1989). Migration requires the matrix degrading properties of metalloproteinases such as stromelysin (Matrisian, 1990). Trophoblasts secrete metalloproteinases that are involved in attachment to the uterine epithelium (Brenner *et al.*, 1989). Trophoblasts contain large amounts of a factor that binds to the *ets* motif, presumably c-Ets (Asano *et al.*, 1990). Our studies suggest that Ets is a component of the signalling network that links oncoproteins outside the nucleus with transcription of a gene that has an important role in both pathological and normal processes.

Materials and methods

Construction of recombinants

347-CAT and MUTA+B: the *Rsa*I (−331)−*Sph*I (−550) stromelysin promoter fragment of 550-CAT was replaced with the oligonucleotides 5′-CTTCTGGAAGTTCTTTGT-3′ and 5′-ACAAAGAACTTCCAGAAG-CATG-3′ (WT) or 5′-CTTCTaGAAGTTCTTTGT-3′ and 5′-ACAAAG-AACTTCtAGAAGCATG-3′ (MUT). 209-CAT: the *Ase*I (−188)−*Sph*I (−550) region of 550-CAT was replaced with 5′-CGGCAGGAAGCATTT-CCTGGAGAT-3′ and 5′-TAATCTCCAGGAAATGCTTCCTGCCGC-ATG-3′ (WT) and 5′-CGGCAaGAAGCATTTCtTGGAGAT-3′ and 5′-TAATCTCCAaGAAATGCTTCtTGCCGCATG (MUTA+B). pG1PAL, pG1PALmutA, pG1PALmutB, pG1PALmutA+B: oligonucleotides containing the −208 to −191 region of the stromelysin promoter were inserted in the *Xho*I site of pG1 in the same orientation relative to the globin start site as the stromelysin promoter. mutA, mutB and mutA+B signify that, respectively, either the left arm, or the right arm or both arms of the imperfect palindrome contain a mutation in the *ets* motif (G:C to a:t of the boxed base pair of the sequence in Figure 6). The recombinants were verified by DNA sequencing of the inserts. Other recombinants are described elsewhere (stromelysin promoter-CAT: Nicholson *et al.*, 1990; JUN-CAT: Angel *et al.*, 1988; pFC4, pFC8: Weisz and Rosales, 1990; pβCBx2, *ets* expression vectors: Wasylyk *et al.*, 1990; c-Ha-*ras*, v-*src*, v-*mos* expression vectors: Wasylyk *et al.*, 1988).

Transfection of cells and analysis

HeLa cells (10^6) in Dulbecco's medium containing 2.5% fetal calf serum and 2.5% calf serum were transfected by the calcium phosphate technique with 20 μg of DNA composed of 1−5 μg reporter (CAT or pG1 based), 1−5 μg internal control (CH110, a β-galactosidase expression vector from Pharmacia, or pβCBx2) and expression vectors for various oncogenes, made up to 20 μg with pEMBL18. After 20 h the cells were washed and incubated in new medium for an additional 24 h. Total RNA was extracted by the hot phenol technique and analysed by quantitative S1 nuclease mapping as described previously (Wasylyk *et al.*, 1990). Alternatively, cellular extracts were prepared and normalized for β-galactosidase activity (Petkovich *et al.*, 1987). CAT activity was quantified by counting the amount of [C^{14}]chloramphenicol converted to the mono-acetylated forms. Transfections were repeated at the very least twice with two preparations of DNA.

In vitro analysis

RNA was synthesized from linearized pSG5 derived expression vectors with T7 RNA polymerase. The amount of RNA was measured by incorporating radioactive precursors, and its integrity was verified by gel electrophoresis. Optimized amounts of RNA were used to synthesize proteins in rabbit reticulocyte lysates according to the manufacturers' instructions (Amersham or Promega). Efficient synthesis of full-length protein was assured by measuring both incorporation of radioactive precursors ([^{35}S]methionine) and SDS−PAGE. Gel retardation and competition experiments were as described previously (Wasylyk *et al.*, 1990).

Acknowledgements

We thank M.Karin and A.Weiss for the gift of recombinants, F.Ruffenach and A.Staub for the synthesis of oligonucleotides, the tissue culture staff for cells and medium, B.Boulais, B.Fournaise and C.Werlé for the illustrations, and CNRS, INSERM, ARC and FNCLCC for financial assistance.

References

Angel,P., Hattori,K., Smeal,T. and Karin,M. (1988) *Cell*, **55**, 875−885.
Asano,M., Murakami,Y., Furukawa,K., Yamaguchi-Iwai,Y., Sataki,M. and Ito,Y. (1990) *J. Virol.*, **64**, 5927−5938.
Bhat,N.K., Fisher,R.J., Fujiwara,S., Ascione,R. and Papas,T. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3161−3165.
Bosselut,R., Duvall,J.F., Gégonne,A., Bailly,M., Hémar,A., Brady,J. and Ghysdael,J. (1990) *EMBO J.*, **9**, 3137−3144.
Brenner,C.A., Adler,R.R., Rappolee,D.A., Pedersen,R.A. and Werb,Z. (1989) *Genes Dev.*, **3**, 848−859.
Fujiwara,S., Koizmi,S., Fisher,R.J., Bhat,N.K. and Papas,T. (1990) *Mol. Cell. Biol.*, **10**, 1249−1253.
Golay,J., Introna,M. and Graf,T. (1988) *Cell*, **55**, 1147−1158.
Goldfarb,R.H. and Liotta,L.A. (1986) *Sem. Thromb. Hemostasis*, **12**, 294−307.
Gunther,C.V., Nye,J.A., Bryner,R.S. and Graves,B.J. (1990) *Genes Dev.*, **4**, 667−679.
Gutman,A. and Wasylyk,B. (1990) *EMBO J.*, **9**, 2241−2246.
Gutman,A. and Wasylyk,B. (1991) *Trends Genet.*, **7**, 49−54.
Ho,I.-C., Bhat,N.K., Gottschalk,L.R., Lindsten,T., Thompson,C.B., Papas,T.S. and Leiden,J.M. (1990) *Science*, **250**, 814−818.
Jonat,C., Stein,B., Ponta,H., Herrlich,P. and Rahmsdorf,H.J. (1989) In Birkedal-Hansen,H. (ed.), *Proceedings of the Matrix Metalloproteinase Conference, Destin, Florida*. Gustav Fischer Verlag, in press.
Karim,F.D., Urness,L.D., Thummel,C.S., Klemsz,M.J., McKercher, S.R., Celada,A., Van Beveren,C., Maki,R.A., Gunther,C.V., Nye,J.A. and Graves,B.J. (1990) *Genes Dev.*, **4**, 1451−1453.
Kerr,L.D., Holt,J.T. and Matrisian,L.M. (1988) *Science*, **242**, 1424−1427.
Kerr,L.D., Miller,D.B. and Matrisian,L.M. (1990) *Cell*, **61**, 267−278.
Klemsz,M.J., McKercher,S.R., Celada,A., Van Beveren,C. and Maki,R.A. (1990) *Cell*, **61**, 113−124.
LePrince,D. Gégonne,A., Coll,J., De Taisne,C., Schneeberger,A., Lagrou,C. and Stehelin,D. (1983) *Nature*, **306**, 395−397.
Matrisian,L.M. (1990) *Trends Genet.*, **6**, 121−125.
Matrisian,L.M., Glaichenhaus,N., Gesnel,M.C. and Breathnach,R. (1985) *EMBO J.*, **4**, 1435−1440.
Matrisian,L.M., Bowden,G.T., Krieg,P., Fürstenberger,G., Briand,J.P., LeRoy,P. and Breathnach,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9413−9417.
Nicholson,R., Murphy,G. and Breathnach,R. (1989) *Biochemistry*, **28**, 5195−5203.
Nicholson,R.C., Mader,S., Nagpal,S., Leid,M., Rochette-Egly,C. and Chambon,P. (1990) *EMBO J.*, **9**, 4443−4454.
Nunn,M.F. and Hunter,T. (1989) *J. Virol.*, **63**, 398−402.
Nunn,M.F., Seeburg,P.M., Moscovici,C. and Duesberg,P.H. (1983) *Nature*, **306**, 391−395.
Ostrowski,L.E., Finch,J., Krieg,P., Matrisian,L.M., Patskan,G.,

O'Connell,J.F., Phillips,J., Slaga,T.J., Breathnach,R. and Bowden,T.G. (1988) *Mol. Carcin.*, **1**, 13–19.
Petkovich,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature*, **330**, 444–450.
Pognonec,P., Boulukos,K.E., Bosselut,R., Boyer,C., Schmitt-Verhlulst,A.M. and Ghysdael,J. (1990) *Oncogene*, **5**, 603–610.
Quinones,S., Saus,J., Otani,Y., Harris,E.D., Jr and Kurkinen,M. (1989) *J. Biol. Chem.*, **264**, 8339–8344.
Schönthal,A., Herrlich,P., Rahmsdorf,H.J. and Ponta,H. (1988) *Cell*, **54**, 325–334.
Seth,A., Watson,D.K., Blair,D.G. and Papas,T.S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7833–7837.
Shaw,P.E., Schroter,H. and Nordheim,A. (1989) *Cell*, **56**, 563–572.
Urness,L.D. and Thummel,C.S. (1990) *Cell*, **63**, 47–61.
Vandenbunder,B., Pardanaud,L., Jaffredo,T., Mirabel,M.A. and Stehelin,D. (1989) *Development*, **106**, 265–274.
Wasylyk,C., Imler,J.L. and Wasylyk,B. (1988) *EMBO J.*, **7**, 2475–2483.
Wasylyk,B., Wasylyk,C., Flores,P., Begue,A., LePrince,D. and Stehelin,D. (1990) *Nature*, **346**, 191–193.
Weisz,A. and Rosales,R. (1990) *Nucleic Acids Res.*, **18**, 5097–5106.

Received on December 13, 1990; revised on February 7, 1991