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PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes

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We have found that the activity of the transcription factor PEA3 is regulated by the expression of non-nuclear oncogenes. This factor, although distinct from PEA1 (AP1), is activated by the same oncogenes (v-*src*, polyoma (Py) middle T, c-Ha-*ras*, v-*mos*, v-*raf*), by tetradecanoyl phorbol-acetate (TPA) and by serum components. We present evidence that PEA3 and PEA1 co-operate in the response of the polyoma virus (Py) α domain to oncogene expression. However, in contrast to PEA1, c-*fos* does not appear to be necessary for activation of PEA3, suggesting that PEA3 is a *fos* independent target for regulation of transcription by non-nuclear oncogenes.

Key words: AP1/*jun*/*fos*/oncogenes/PEA3

Introduction

In order to understand how many different oncogenes can transform cells, it is important to identify common regulatory events. Recent studies have linked the effects of non-nuclear oncogenes to the activity of the transcription factor AP1 or PEA1 (reviewed in Herrlich and Ponta, 1989; Imler and Wasylyk, 1989). AP1 is a composite factor, consisting of heterodimers between c-*fos* and c-*jun* gene family members (Curran and Franza, 1988). The canonical members of these families are themselves proto-oncogenes (Vingron et al., 1988; Vogt and Tjian, 1988) suggesting that an important event in cell transformation by non-nuclear oncogenes could be altered AP1 activity. However, to understand the diverse effects that non-nuclear oncogenes can have on gene expression, it is important to identify other nuclear targets. We show here that the oncogenes that alter PEA1 (AP1) activity also regulate the activity of PEA3. PEA1 and PEA3 are distinct factors, which interact with different motifs of the α domain of the Py enhancer (Martin *et al.*, 1988; see below). The potential role of PEA3 in the regulation of cellular gene transcription is discussed.

Results

Mutation of the PEA3 motif strongly inhibits α domain activation by the expression of v-src, Py-mt, Ha-ras, v-mos and v-raf, but not by c-fos or several other oncogenes

The *in vivo* activities of the transcription factors PEA1 and PEA3 were measured using the ability of oligonucleotides containing their specific recognition sequences (motifs) to stimulate transcription from the β-globin promoter (see reporter recombinants in Figure 1A). The oligonucleotide sequences are related to the α domain of the Py enhancer. The 'wild-type' PB oligonucleotides contain PEA1 and PEA3 motifs (see Figure 1B) [the PEA2 motif is disrupted by a 2 bp mutation (Piette and Yaniv, 1987; Figure 1B)]. The M1 and M5 oligonucleotides contain mutations in the extremities of the PEA3 and PEA1 binding sites, respectively. The reporter recombinants, together with an internal control plasmid, were transfected into LMTK$^-$ fibroblasts and, after 48 h of culture in low serum (0.05% foetal calf serum, FCS), total cellular RNA was extracted. Quantitative S1 nuclease mapping was used to measure the amount of specific RNA initiated from the reporter and control recombinant promoters (see Reporter and Control bands in Figures 2–4). The transfections were repeated at least three times, with two different preparations of DNA, to ensure reproducibility of the results. Although transcription from the internal control was in some cases affected by expression of oncogenes or transcription factors, this control was useful for comparisons between transfections containing the same expression vectors.

We have previously shown that α domain activity is increased by expression of the transforming oncogenes v-*src*, Py-mt, Ha-*ras*, v-*mos*, v-*raf* and c-*fos* (Imler *et al.*, 1988; Wasylyk *et al.*, 1988a). We investigated the effect of mutating the PEA motifs on activation of the α domain by oncogene expression. The activity of the M5 multimer, with the mutated PEA1 motif, was still strongly inducible by expression of all of these oncogenes except for c-*fos* (compare lanes 15–21 and 1–7, Figure 2 and Table I). In sharp contrast, the M1 multimer, with the mutated PEA3 motif, was much less inducible by oncogene expression (compare lanes 8–13 and 1–6, Figure 2 and Table I). However, c-*fos* expression did stimulate M1 activity (compare lanes 8 and 14, and 1 and 3, see Table I). (It should be noted that expression of certain oncogenes had a small inhibitory effect on transcription of the parent reporter pG1. Multimer activity is measured as the ratio of transcription from the reporter recombinants containing or lacking the multimer in the same conditions of oncogene expression, thereby compensating for this effect.) We have previously shown that treating cells with serum components, or the tumor promoter TPA, induces the α domain (Imler *et al.*, 1988; Wasylyk *et al.*, 1988a). We found that both M1 and M5 multimer activities were increased by both serum and TPA (Table I).

We have previously reported that expression of various immortalizing oncogenes did not affect α domain activity (Wasylyk *et al.*, 1988b). We studied the effects on M1 and M5 activity of expression of the SV40-early region, Py-LT, v-*myc*, Ad EIA, BPV-E2 and BPV-E5. These oncogenes had little effect on M1 and M5 activity (see Figure 3). The only effects we consider to be significant compared to the

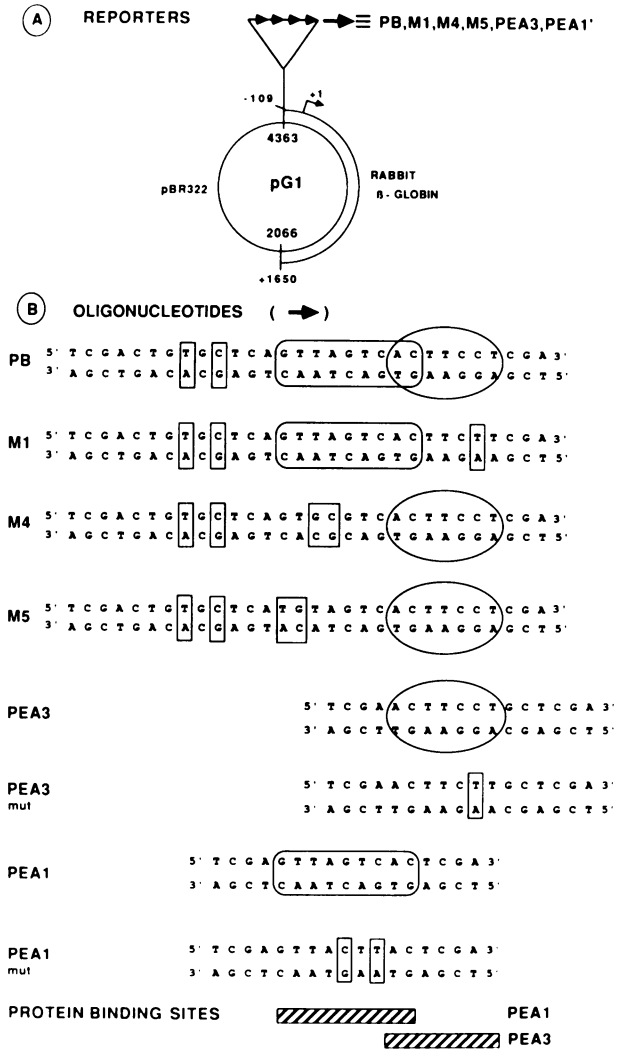

Fig. 1. Structure of reporter recombinants (**A**) and sequence of oligonucleotides. (**B**) The reporters contain four head to tail copies of oligonucleotides (→) upstream from the β-globin promoter of pG1. The oligonucleotides BP, M1, M4 and M5 all contain a double base pair mutation in the Py enhancer α domain sequence which disrupts the PEA2 motif (see common boxed base pairs). M1, M4 and M5 contain additional mutations in either the PEA3 or PEA1 motifs respectively (see boxed base pairs). The PEA3 and PEA1 oligonucleotides contain only the corresponding motifs, and the PEA3 and PEA1 mutant oligonucleotides have sequence alterations which inhibit binding of the cognate factors (see Piette and Yaniv, 1987; Martin *et al.*, 1988; Results; and our unpublished observations). PEA1' contains an additional nucleotide relative to the PEA1 oligonucleotides to allow directional cloning (5'-TCGAGTTAGTCACGTCGA-3'). The PEA1 and PEA3 binding sites are circled and are also indicated at the bottom of the figure.

experimental error are those due to Ad-EIA on both M1 and M5 and Py-LT on M5 (see lanes 1−5, 12, 19 and 3, 13, 17 respectively). The mechanism of inhibiton by EIA of α domain activity is unknown at present, but does not appear to involve decreased transcription of the c-*fos* or c-*jun* genes (our unpublished results). Stimulation of α domain activity by Py-LT expression is small (2.5-fold) compared to the effect of several other oncogenes (e.g. Py-mt stimulates M5 multimer activity 33-fold). Further investigations are required to establish the significance of this relatively smaller effect.

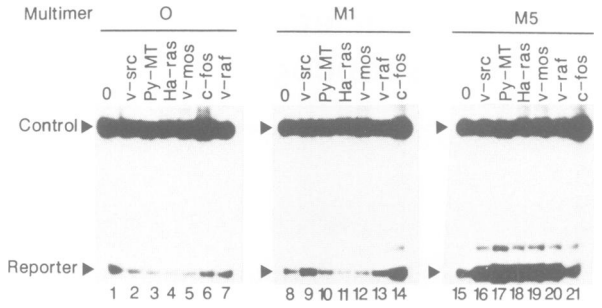

Fig. 2. Effect of transforming oncogene expression on M1 and M5 multimer activity. LMTK⁻ fibroblasts (9 cm plates) were transfected with 1 μg of the indicated reporters, 1 μg of the internal control plasmid (pβCB×2) and 5 μg of expression vectors for the indicated oncogenes. After 40 h in culture in low serum (0.05% FCS), total RNA was analysed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporters (see Reporter bands) and the conalbumin promoter of the control recombinants (see Control bands). The ratio of specific transcription from the reporters with multimers to that of the parent reporter is shown in Table I.

Table I. Stimulation of multimer activity by oncogene expression

Oncogene or Treatment	Control pG1	Ratio transcription from reporter/control					
		PB type multimer				Separate motifs	
		PB	M1	M4	M5	PEA3	PEA1
0	1	14	1	1	1	1	4
v-*src*	1	70	4	6	12	2	9
Py-mt	1	140	3	10	33	2	19
ras	1	182	2	11	38	3	14
v-*mos*	1	112	2	10	41	4	13
v-*raf*	1	112	2	4	23	3	6
TPA	1	70	6	2	10	2	10
serum	1	70	4	2	4	3	6
c-*fos*	1	–	11	1	1	1	17
c-*jun*	1	–	14	1	3	1	11
c-*fos*+c-*jun*	1	100	60	5	8	1	44
jun-B	1	–	3	–	1	–	–
c-*fos*+*jun*B	1	50	45	–	3	–	–

LMTK⁻ fibroblasts (9 cm plates) were transfected with 1 μg of either pG1 or the indicated multimer containing reporter, 1 μg internal control plast (pβCB×2) and either 5 μg of the expression vectors for the indicated oncogenes or 2.5 μg pcFOS5, pcJUN5 or pJUND5. After 40 h in culture in 0.05% foetal calf serum, total RNA was extracted and analysed by quantitative S1 nuclease mapping. The values presented are the average of three different experiments, with two different preparations of DNA, and the SD was on average ± 10%.

These results show that mutating the PEA3 motif strongly inhibits activation of α domain activity by expression of v-*src*, Py-mt, *ras*, v-*mos* and v-*raf*, but not by c-*fos*. They strongly suggest that PEA3 is required for efficient induction of α domain activity.

Mutation of the PEA3 binding site does not decrease transcription activation by PEA1 (AP1) in vivo

We investigated the effects of the mutations in the PEA3 and PEA1 motifs on activation of transcription by PEA1, a composite transcription factor, consisting of heterodimers of members of *fos* and *jun* gene families (Curran and Franza, 1988). The canonical c-*fos* + c-*jun* component of PEA1 was

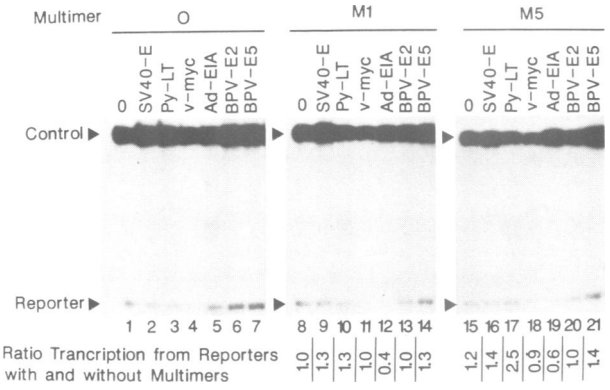

Fig. 3. Effect of immortalizing oncogene expression on M1 and M5 multimer activity. LMTK$^-$ fibroblasts were transfected with 1 μg of the reporters, 1 μg of the internal control plasmid (pβCB$\times$2) and 5 μg of the expression vectors for indicated oncogenes. After 40 h in culture in low serum (0.05% FCS) total RNA was analysed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporter (see Reporter bands) and conalbumin promoter of the control (see Control bands). The ratio of specific transcription from the reporters with multimers to that from the parent reporter is shown.

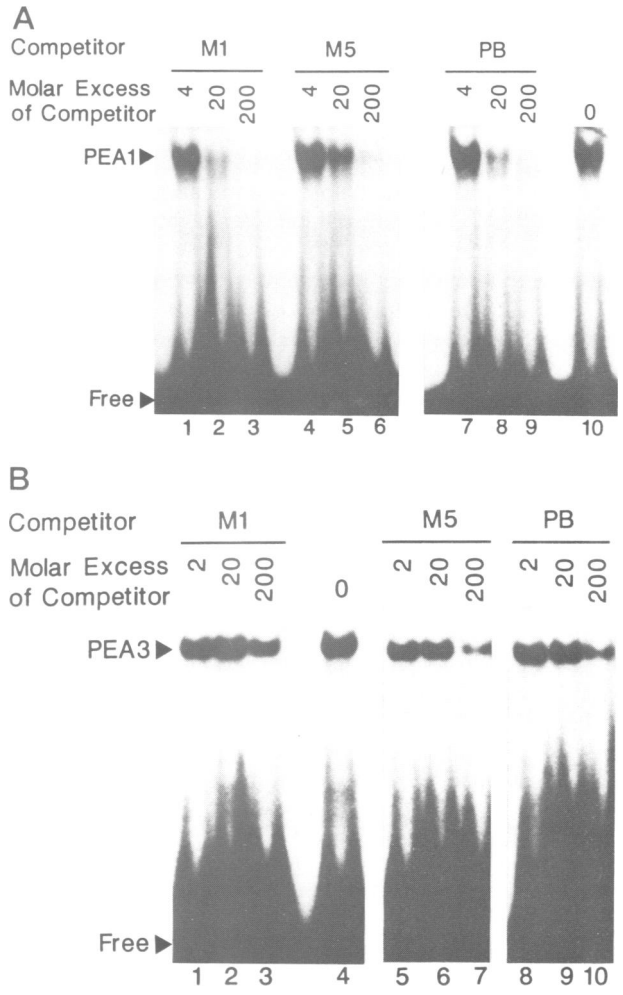

Fig. 4. Competition by α domain oligonucleotides for binding of PEA1 and PEA3 to their cognate motifs. Nuclear extract (10 μg) from induced NIH3T3-hMTIIA-*fos* cells (see legend to Figure 7), were pre-incubated with the indicated competitor oligonucleotides for 15 min at 4°C before the addition of labelled PEA1 or PEA3 probes. After a further 10 min at 25°C, the factor–DNA complexes were resolved in 6% acrylamide gels. PEA1 and PEA3 indicate the corresponding specific complexes, and Free, excess unretarded PEA1 or PEA3 probes.

introduced into cells by co-transfecting expression vectors for both proteins. Co-expression of c-*fos* and c-*jun* stimulated transcription from the reporter containing the PB oligonucleotides (compare 0 and c-*fos* + c-*jun* for PB, Table I). Mutating the PEA3 motif did not greatly affect activation (compare 0 and c-*fos* + c-*jun* for M1 and PB, Table I), whereas mutating the PEA1 motif decreased transcription activation >10-fold (compare 0 and c-*fos* + c-*jun* for M5 and PB, Table I). A second member of the *jun* gene family is *jun*-B. Transcription activation by *jun*-B + c-*fos* was not diminished by the mutation in the PEA3 motif (compare 0 and c-*fos* + *jun*-B for M1 and PB, Table I), but was decreased >15-fold by the PEA1 motif mutation (compare 0 and c-*fos* + *jun*-B for M5 and PB, Table I). Transfection of the individual expression vectors had a much smaller effect on transcription than co-transfecting the *fos* + *jun* expression vectors (see M1 in Table I), in agreement with other evidence for complementation between *fos* and *jun* for activation of transcription (see Introduction). These results show that the mutation in the PEA3 binding site, in contrast to the mutation in the PEA1 motif, does not affect transcription activation by the c-*fos* + c-*jun* or c-*fos* + *jun*-B transcription complexes.

The mutations in the PEA1 and PEA3 motifs decrease the affinity of the α domain only for the corresponding factor in vitro

We have investigated the effects of the mutations in the α domain on its affinity for PEA1 and PEA3 *in vitro*. PEA1 and PEA3 DNA binding activities in cell extracts were measured using gel retardation assays with oligonucleotides containing exclusively the PEA1 and PEA3 motifs as probes (see Figure 1B). We initially confirmed published results that these oligonucleotides only bind the corresponding factor (Martin *et al.*, 1988, results not shown), and thus that PEA1 and PEA3 are separate factors that bind to different motifs and do not appear to form a tight complex in solution. Using competition assays we found that the mutation introduced in the PEA3 motif of the α domain did not significantly affect its affinity for the PEA1 factor (compare lanes 1–3, 7–9 and 10, Figure 4A). In contrast, the mutation in the PEA1 motif did decrease the affinity for the PEA1 factor (compare lanes 4–6, 7–9 and 10). Conversely, the mutation in the PEA3 motif decreased the affinity of the α domain for the PEA3 factor (compare especially lanes 3, 4 and 10, Figure 4B), whereas the mutation in the PEA1 motif did not affect the affinity of the competitor for PEA3 (compare especially lanes 4, 7 and 10). The affinity of the α domain was apparently higher for PEA1 than for PEA3 (compare molar excess of competitor required for efficient competition in Figures 4A and B). This most probably results both from the localizations of the PEA3 and PEA1 motifs relative to the ends of the oligonucleotides, and from the presence of two additional nucleotides from the Py enhancer in the PEA3 oligonucleotides. These results show that the PEA1 and PEA3 motif mutations in the α domain decrease its affinity only for the corresponding factor.

Co-operation between PEA3 and PEA1 in the induction of Py α domain activity by oncogene expression

In order to investigate the possibility that PEA1 and PEA3 co-operate in the induction of Py enhancer α domain activity by oncogenes, we compared induction of the wild type PB domain with mutated domains, with mutations in either the PEA3 or PEA1 motif. In addition, we sought a more drastic PEA1 motif mutation than M5, since the M5 multimer was inducible to a relatively low extent by co-expression of *fos* + *jun* (compare 0 and *fos* + *jun* for PB, M1 and M5, Table I). It should be noted that, due to co-operativity, the effect of a mutation in the PEA1 motif would be greater in the absence than in the presence of PEA3. We chose to study the M4 mutation since it had a greater inhibitory effect than M5 on *trans*-activation by c-*fos* + c-*jun* (compare c-*fos* + c-*jun* for M4, M5 and PB in Table I), and the mutated nucleotides still lie outside the DNA sequence required for efficient PEA3 binding (Martin *et al.*, 1988; this study). In the absence of oncogene expression, mutations in both the PEA3 and PEA1 motif had drastic effects on multimer activity (compare 0 line for PB, M1, M4 and M5, Table I), suggesting that both factors were absolutely required for basal activity. Oncogene expression stimulated the activities of both the PB wild-type and the mutated α domains. However, the extent of induction of the PB multimer was much greater than that expected from the individual activities of mutated domains [compare PB, M1 and M4 activities with and without oncogene (v-*src*, Py-mt, *ras*, v-*mos*, v-*raf*) expression Table I]. These results suggest that co-operativity between PEA1 and PEA3 is required for full induction of α domain activity by oncogene expression. Further evidence for co-operativity comes from the observations that: (i) the M5 mutation has a less drastic effect on oncogene induction of PB multimer activity than would be expected from its effect on *trans*-activation by c-*fos* + c-*jun* (see Table I) and (ii) the relatively small effect of oncogene expression on the activities of isolated PEA3 and PEA1 motif multimers (see below).

The activities of isolated PEA3 and PEA1 motif multimers are increased by oncogene expression

To investigate whether the PEA3 and PEA1 motifs alone are sufficient for oncogene induction of transcription, we used oligonucleotides which contain only the corresponding motif (see Figure 1B) and which, *in vitro*, efficiently bind only the corresponding factor (Martin *et al.*, 1988; our unpublished results). Both PEA3 and PEA1 multimer activities were significantly and reproducibly stimulated by expression of v-*src*, Py-mt, *ras*, v-*mos* and v-*raf*, and treatment of cells with TPA or serum (see Table I). In contrast, expression of c-*jun*, c-*fos* and c-*jun* + c-*fos* only stimulated PEA1 multimer activity (see Table I). For the PEA3 motif multimer, similar but quantitatively larger results were also obtained in F9 embryo carcinoma cells (data not shown). These data show that both the PEA3 and the PEA1 motifs alone, isolated from one another, can mediate activation of transcription by oncogenes. The results with the PEA1 multimer are similar to those for the related collagenase TRE multimer described by Schöntal *et al.* (1988). The extent of induction of PEA1 and PEA3 multimer activity by oncogene expression is less than that of the combined motifs in the PB multimers, which is in agreement

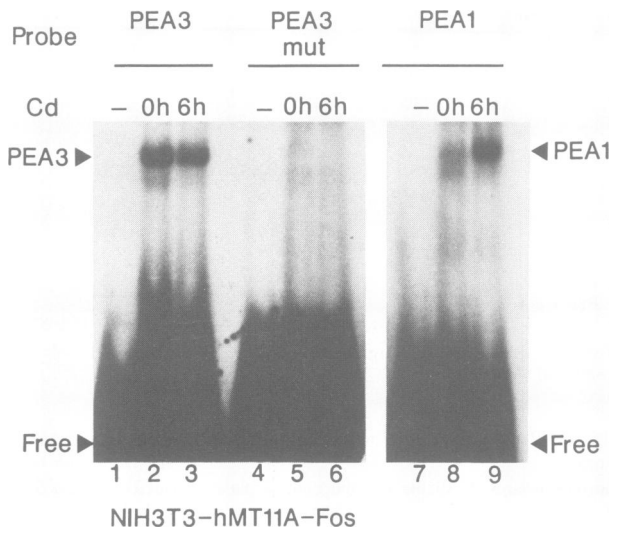

Fig. 5. Induction of c-*fos* synthesis increases the DNA binding activity of PEA1 but not of PEA3. Nuclear extracts (10 μg protein) from NIH3T3-hMTIIA-fos cells, both before and after a 6 h incubation with 40 μM cadmium chloride (to induce *fos* synthesis), were analysed by gel retardation with the indicated probes. PEA1 and PEA3 indicate the position of the corresponding specific complexes, and Free, excess non-retarded probe. **Lanes 1, 4, 7** without extract.

with co-operativity between PEA3 and PEA1 contributing to induction of PB multimer activity. However, caution must be exercised in comparisons between different multimers. For example, the basal activity of the PEA1 motif is significantly higher in the isolated motif construction than in the M1 multimer, suggesting that other factors, such as relative spacing between motifs, could affect both basal and induced activity. Further experiments are required in order to establish the relative sensitivities of PEA1 and PEA3 to oncogene expression, when they are either alone or in the presence of the other co-operating factor.

Expression of fos increases the DNA binding activity of factors binding to the PEA1 motif but not to the PEA3 motif

The effect of *fos* expression on the formation of PEA1 and PEA3 specific complexes was studied in extracts from NIH3T3 cells which contain an exogenous integrated *fos* gene under the control of the human metallothionein IIA promoter (J5-1-5d2, Yu *et al.*, 1986). Gel retardation assays with oligonucleotides which exclusively bind PEA3 or PEA1 were used to measure specific DNA binding activity. In uninduced cells, one major retarded complex was detected with the PEA3 probe (compare lanes 1 and 2, Figure 5). The complex was not detected with the PEA3 mutant probe containing the same mutation as in the M1 oligonucleotide (compare lanes 5 and 2, Figure 5), showing that the complex was specific. Furthermore, this result supports our conclusions that the PEA3 motif mutation in M1 inhibits binding of PEA3. The PEA1 probe gave two retarded bands (see lane 8, Figure 5). Only the upper band was specific, since it was not observed with a PEA1 oligonucleotide carrying a mutation known to prevent PEA1 binding, and it was specifically competed with the PEA1 oligonucleotide (see Piette and Yaniv, 1987; Figure 1B PEA1 mut. oligonucleotides; and results not shown). In extracts from cells in which *fos* production had been induced by a 6 h

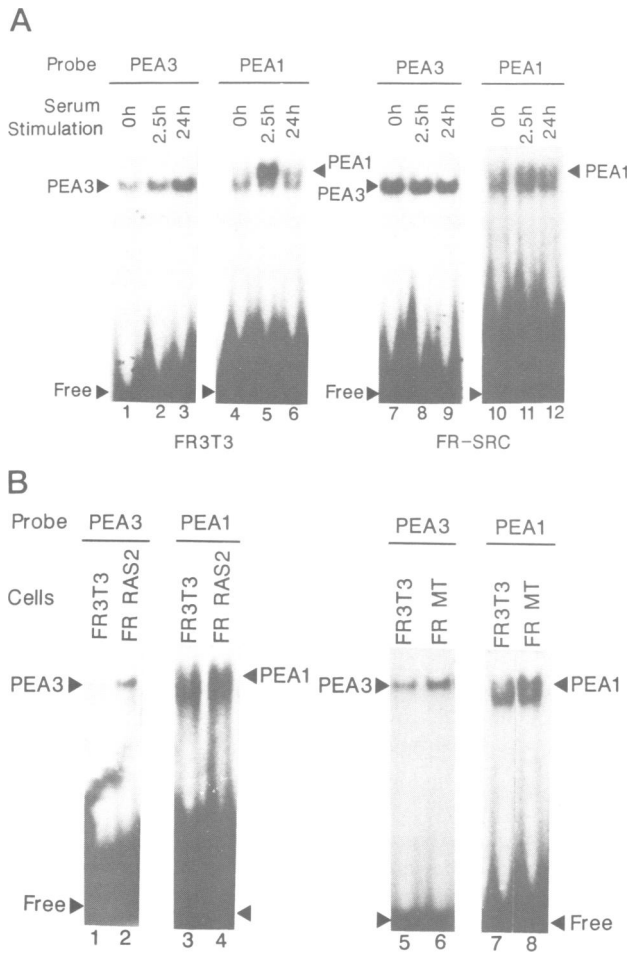

Fig. 6. PEA1 and PEA3 DNA binding activity in FR3T3 cells and derived cell lines transformed with v-*src*, *ras* and Py-mt. Subconfluent cell cultures were incubated for 24 h in low serum (0.5% FCS) before extract preparation. In addition, in (**A**), the cells were incubated with 15% FCS for either 2.5 or 24 h. Whole cell extracts (10 μg protein) were analysed by gel retardation using the PEA1 and PEA3 probes. PEA1 and PEA3 indicate the position of the corresponding specific factor–DNA complexes, and Free, excess unretarded probe.

incubation with cadmium, there was an increase in PEA1 specific DNA binding activity (compare lanes 8 and 9, Figure 5). In contrast, there was no change in the amount of PEA3-specific complex (compare lanes 2 and 3). (The quality of the extracts and reproducibility of the analysis was systematically verified using gel retardation assays with a probe for NFI, a ubiquitous DNA binding protein. The use of NFI as a standard for comparing normal and transformed cell extracts was described by Piette *et al.*, 1988.) These results show that induction of *fos* enhances the DNA binding properties of PEA1 but not of PEA3.

Transformed cells contain elevated PEA3 DNA binding activity

PEA3 and PEA1 DNA binding activity was measured by gel retardation assays in whole cell extracts from FR3T3 cels, and lines which had been transformed by v-*src*, Py-mt or *ras*. The cells were incubated in low serum conditions (0.5% FCS) for 24 h before preparing the extracts. There were higher levels of PEA3 in the transformed cell extracts compared to the parent cell line, which ranged from 4-fold for v-*src* (compare lanes 1 and 7, Figure 6A), 3-fold for *ras* (compare lanes 1 and 2, Figure 6B) and 2-fold for Py-mt (compare lanes 5 and 6, Figure 6B). In the same extracts NFI DNA binding activity was the same (results not shown) showing that not all DNA binding proteins were present in higher levels in transformed cell extracts. As described previously (Piette *et al.*, 1988) transformed cell extracts also contained detectably higher levels of PEA1 activity than the non-transformed parent cell line (compare in Figure 6A lanes 4 and 10 for v-*src*, and in Figure 6B lanes 3 and 4 for *ras* and 7 and 8 for Py-mt). However, the PEA1 complex was composed of several different closely migrating bands (see especially the *ras*-transformed cell extracts), making it difficult to measure the exact level of PEA1 in these extracts. In summary, in low serum condition, transformed cells contain higher levels of PEA3 compared to the parent non-transformed cell lines.

Serum induces PEA3 and PEA1 DNA binding activity in FR3T3 cells but not in transformed cells

FR3T3 fibroblasts were used to study serum induction of PEA1 and PEA3 DNA binding activity. They were grown in low serum (0.5% FCS) for 24 h and then incubated with 15% FCS, for either 2.5 or 24 h. There was a gradual 3-fold increase in PEA3 DNA binding activity during the 24 h period (compare lanes 1–3, Figure 6A). In contrast, PEA1 activity increased more rapidly and then declined (compare lanes 4–6). The results show that serum induces both PEA3 and PEA1 DNA binding activity, although the kinetics of induction of these factors are different. In contrast, in *src* transformed FR3T3 cells, a similar treatment with serum did not detectably alter the levels of either PEA3 or PEA1 (see lanes 7–12, Figure 6A). Similar results were also obtained in cell lines transformed by *ras* or Py-mt (data not shown). These results strongly suggest that one of the consequences of transformation is to abrogate the serum requirements for induction of both PEA3 and PEA1.

Increase in DNA binding activity of PEA1 and PEA3 due to expression of v-mos

We studied the effect of v-*mos* expression in LTR-*mos* cells (Jaggi *et al.*, 1986) on the DNA binding activity of PEA3 and PEA1. LTR-*mos* are derived from NIH3T3 cells by the integration of DNA containing v-*mos* coding sequences linked to the dexamethasone inducible MMTV-LTR promoter. Initially, three 'independent' NIH3T3 clones (cultivated in Bern, Paris and Strasbourg) were shown to contain very similar levels of PEA3 DNA binding activity (results not shown), suggesting that there was no significant clonal variation. NIH3T3 and LTR-MOS cells were grown in low serum conditions for 48 h (0.5% FCS) and then treated with dexamethasone. Whole cell extracts were prepared at different times and analysed by gel retardation assays. In control NIH3T3 cells, dexamethasone treatment had no effect on either PEA3 or PEA1 DNA binding activity (compare lanes 1–3 and lanes 4–6 respectively, Figure 7). In LTR-*mos* cells, dexamethasone treatment induced both PEA3 and PEA1 DNA binding activity to a similar extent, ~3-fold (compare lanes 7–9 and 10–12). These results show that oncogene expression alters the DNA binding characteristics of PEA3 and PEA1. In transfection experiments in the LTR-*mos* cells, *mos* expression stimulated both M1 and M5 multimer activity (results not shown), in keeping with an activation of both factors in these cells due

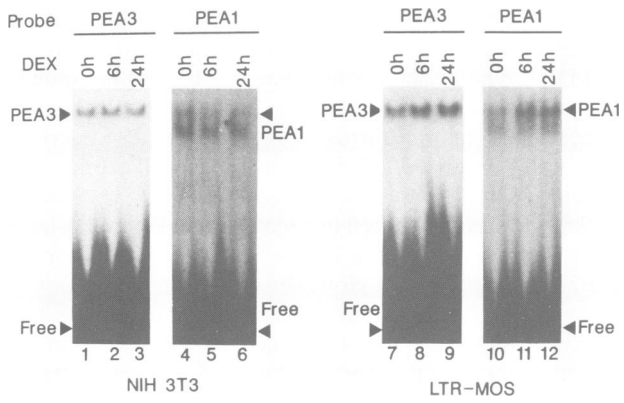

Fig. 7. Effect of *mos* expression on PEA1 and PEA3 DNA binding activity. Subconfluent NIH3T3 or the derived LTR-*mos* cells were pre-incubated for 48 h in low serum (0.5% FCS) before incubation with 10^{-7} M dexamethosone (DEX) for the indicated times. Whole cell extracts (10 μg protein) were analysed by gel retardation using PEA1 and PEA3 probes. PEA3 and PEA1 indicate the corresponding specific factor–DNA complexes, and Free, excess probe.

to oncogene expression. The results further suggest that PEA1 and PEA3 are different factors whose properties are altered by oncogene expression.

Discussion

PEA3, a transcription factor distinct from PEA1 (AP1), has a similar pattern of induction by non-nuclear oncogenes, TPA and serum

There are three characterized, distinct transcription factors which bind to the α domain of the Py enhancer, PEA1, PEA2 and PEA3 (Piette and Yaniv, 1987; Martin *et al.*, 1988; Imler *et al.*, 1988; this work). Two of these, PEA1 and PEA3, share the property of being inducible by oncogene expression, serum and TPA (unlike PEA2; Satake *et al.*, 1988; Wasylyk *et al.*, 1988b). The evidence that PEA3 and PEA1 are different factors is: (i) they both bind only to their own, distinct motif *in vitro* (Martin *et al.*, 1988; this work); (ii) PEA1 expressed *in vivo* does not activate the PEA3 motif, even though this motif has an inducible activity; (iii) PEA1 and PEA3 have distinct patterns of induction by serum *in vitro*; (iv) they have different behaviours towards *fos* expression, both *in vivo* and *in vitro* (this work). Despite these differences they share the following properties: (i) their activity is inducible *in vivo* by non-nuclear oncogenes (v-*src*, Py-mt, Ha-*ras*, v-*mos*, v-*raf*), serum and TPA; (ii) their DNA binding properties are altered by serum ingredients, oncogene expression and cell transformation (see also Piette *et al.*, 1988 for PEA1); (iii) their activity *in vivo* is not induced by the oncogenes SV40-LT, Py-LT, *myc*, EIA, BPV E2 and E5. What is the significance of these common properties?

There is some evidence that the non-nuclear oncogenes may be linked in the process of signal transduction from the exterior of the cell to the nucleus, in the order serum → *src* + Py-mt → *ras* + protein kinase C → *mos* → *raf* → *jun* + *fos* (for discussions see Schönthal *et al.* 1988; Wasylyk *et al.*, 1988a; Herrlich and Ponta, 1989; Imler and Wasylyk, 1989). Our present observations are consistent with the existence of such a pathway, since a second distinct factor is activated by the same oncogenes. They also predict that the events in this pathway that regulate PEA3 activity are downstream from *raf*, and independent of c-*fos* and c-*jun*. Several lines of evidence suggest that PEA3 activity is independent from that of *fos* or *jun*: (i) *fos* or *jun* expression does not affect PEA3 motif activity *in vivo*; (ii) *fos* expression does not alter PEA3 specific DNA binding activity *in vitro*; and (iii) the same c-*fos* anti-sense RNA, which inhibits oncogene activity of the collagenase TRE (Schönthal *et al.*, 1988), does not appear to block oncogene induction of PEA3 activity (P.Flores, in preparation).

These results suggest that PEA3 is a primary target for signal transduction, which should therefore respond rapidly to extracellular signals. How can this prediction be reconciled with the slow induction *in vitro* of PEA3 DNA binding activity relative to PEA1. We have recently shown that PEA3 activity is maximally induced at the latest 1 h after serum or TPA stimulation of LMTK$^-$ cells, and that this activation is not prevented by blocking protein synthesis with cycloheximide (Wasylyk *et al.*, 1988b; P.Flores, in preparation). These results suggest that there are two aspects to the control of PEA3 activity, a rapid post-translational mechanism and a slow accumulation of protein (DNA binding activity). Similarly, bimodal mechanisms of activation appear to be shared by AP1 (PEA1, Brenner *et al.*, 1989; Angel *et al.*, 1988b; Schönthal *et al.*, 1988) and SRF (Norman *et al.*, 1988; Prywes *et al.*, 1988), and may account for short and long term responses to cellular signals.

Co-operation between different factors in the response to oncogene induction

The emerging evidence for multiple targets in the nucleus for growth factor/oncogene activation leads to the question of how these signals are integrated into coordinate regulation during the cell cycle. Growth factors, serum and oncogenes elicit distinct patterns of induction of c-*jun* and *jun*-B (Lamph *et al.*, 1988; Ryder and Nathans, 1988; Ryder *et al.*, 1988; Ryseck *et al.*, 1988; Quantin and Breathnach, 1988; Pertovaara *et al.*, 1989; Bartel *et al.*, 1989; Sistonen *et al.*, 1989) whereas the levels of the third known member of the gene family, *jun*-D, remains constant (Hirai *et al.*, 1989; Ryder *et al.*, 1989). At least three members of the *fos* gene family are induced with different kinetics during the cell cycle (Cohen and Curran, 1988; Gertz *et al.*, 1989; Zerial *et al.*, 1989). Several members of the *fos* and *jun* gene families can form heterodimers (see, for example, Cohen *et al.*, 1989; Gertz *et al.*, 1989; Neuberg *et al.*, 1989; Turner and Tjian, 1989; Zerial *et al.*, 1989), suggesting that at different times in the cell cycle different heterodimers can form. Presumably, the specificity for activation of transcription lies in the sequences that these composite transcription factors can bind to (e.g. Quinn *et al.*, 1989), and also in the interactions they may have with other transcription factors. The α domain of the Py enhancer may provide a useful model in understanding some aspects of these interactions.

Our results suggest that there are co-operative interactions between some component(s) of PEA1 and PEA3. (i) Mutations in either the PEA1 or the PEA3 motifs have very drastic effects on Py α domain activities. (ii) The PEA3 and PEA1 motifs alone are not as inducible as the associated motifs in the α domain, even though these sequences are sufficient for efficient binding of the corresponding factors. (iii) Several other PEA1-like motifs, in the absence of a PEA3 motif, are less inducible by oncogene expression than

the Py α domain (our unpublished results). Further studies are required to study the mechanisms of these interactions on the α domain of Py enhancer.

Potential role of PEA3 in cellular and viral gene transcription

We can expect that the Py virus has adopted a cellular regulatory mechanism for the control of its enhancer. As a preliminary approach to understanding the role of PEA3 in the regulation of cellular and viral promoters, we have searched through the EMBL databank for regions upstream from mRNA start sites with complete sequence homology to either 11 or 9 bp sequences from around the PEA3 motif (Figure 8). We also searched for related sequences, but only in promoters which are known to be inducible by oncogene expression (Figure 8). There is evidence that some of these homologies could be functionally significant.

Some of the promoters with PEA3 motifs can be grouped according to the types of inducers which activate their transcription: (i) acute phase response (α and γ fibrinogen; Fowlkes *et al.*, 1984); (ii) γ interferon (2-5A synthetase and IFI-54 K; Wathelet *et al.*, 1988) and (iii) mitogens and oncogenes (transferrin receptor, Ad EIA enhancer, interleukin-2, metallothionein-2, EBV early cytoplasmic, collagenase, stromolysin, c-*fos*). The role of the PEA3 motif in the first two responses is unknown, but there is some evidence for a role in mediating the effect of mitogens and oncogenes on the other promoters. The transferrin receptor promoter is activated by mitogens, and the PEA3-like sequence is located in the minimum promoter element required for both transcription activity and specific binding of proteins in extracts (Miskimins *et al.*, 1986). The Ad EIA enhancer is repressed by EIA and activated by EIB (Yoshida *et al.*, 1987), which is reminiscent of the opposing effect of EIA and non-nuclear oncogenes (which share transforming properties with EIB) on PEA3 activity. Activation of the interleukin-2 promoter with TPA and phytohaemagglutinin in T cells leads to changes in the pattern of binding of proteins to several parts of the promoter, one of which encompasses the PEA3 motif (B′ site, Nabel *et al.*, 1988). The EBV early cytoplasmic antigen promoter (BHRF1) is inducible by TPA, and subsequent transcription of BHRF1 is associated with entry into the lytic cycle in B cells (Hardwick *et al.*, 1988). We have observed that PEA3 activity is low in myeloma cells, and is activated by TPA and oncogene expression (our unpublished results), showing that the PEA3 motif can be a responsive element in B cells. The BHRF1 promoter also has a PEA1/AP1-like motif, and is activated by the Z *trans*-activator which has sequence similarity with c-*fos*, c-*jun* and GN-4 (Farrell *et al.*, 1989). AP1/PEA1 motifs are associated with PEA3 motifs in the collagenase, stromelysin (transin) and c-*fos* promoters, suggesting that the association could have functional significance. The collagenase PEA3-like motif specifically binds proteins present in cell extracts (Angel *et al.*, 1987a), and it has been noted that there are unidentified elements in this promoter besides the TRE (AP1-motif) which mediates TPA induction of promoter activity (Angel *et al.*, 1987b). The stromelysin PEA3 like motif is present as a palindrome (see Figure 8), in a region of the promoter which is completely conserved between rabbits and rats (Frisch and Ruley, 1987). The PEA3-like motif in the c-*fos* promoter is required for the response of the c-*fos* promoter to serum, and is the binding site for p62, which participates in the formation of a ternary complex over the serum response element (Shaw *et al.*, 1989). The enticing possibility is that p62 is related to PEA3, and that this factor(s) is involved in early events in signal transduction leading to induction of c-*fos* transcription.

5'-CAGGAAGTGAC-3'	
Human transferrin receptor	(HSTR5,35)
Human factor IX	(HSFIXG,2770)
Rat fibrinogen gamma chain	(RNFIBG1,419)
Human Adenovirus 5 EIA enhancer	(ADEE1AED,4)
Human Spumaretrovirus LTR	(RESPULTR,1271)
Polyomavirus enhancer	(POLLAT,164)
5'-CAGGAAGTG-3'	
Human interleukin 2	(HSIL05,938)
Human 2-5A synthetase	(HS25ASYP,468)
Human interferon inducible IFI-54K	(HSINIFI,113)
Mouse nerve growth factor α-subunit	(MMNGFA1,76)
Rat fibrinogen alpha chain	(RNFBAG,1540)
Rat metallothionein-2	(RNMT12C,338)
Rat thyroglobulin	(RNTHYRP,181)
Rat U2 small nuclear RNA	(RNUG2A,333)
Chicken δ-2 crystallin	(GGCRY,324)
Drosophila alcohol dehydrogenase	(DOADHG,463)
Yeast LTE1	(SCLTE1,110)
Yeast MRS3	(SCMRS3,250)
EBV early cytoplasmic antigen	(EBV,53502)
5'-CAGGATGT-3'	
Human/mouse c-fos	(MMCFOS,236)
5'-GAGGATGT-3'	
Human/rabbit collagenase	(MSCN2A,436)
5'-CAGGAAGCATTTCCTG-3'	
Rat/rabbit stromelysin	(RNTRAN,900)

Fig. 8. Promoters with sequence homology to the PEA3 motif. The EMBL nucleotide sequence databank was searched for perfect homologies to either an 11 or 9 bp sequence from around the PEA3 motif of the Py enhancer α domain. Only homologies within sequences upstream from RNA start sites are shown. In addition, several closely related sequences in the c-*fos*, collagenase and stromelysin−transin promoters are shown. The sequence name in the databank and the position of the first nucleotide are in parentheses.

Materials and methods

Standard molecular biological techniques were used. Construction and structure of recombinants, transfections and quantitative S1 nuclease analysis were as described previously (Imler *et al.*, 1988; Wasylyk *et al.*, 1987, 1988a). pJUNB5 contains the *Eco*RI (26−1552) *jun*-B cDNA fragment (Ryder *et al.*, 1988) in the *Eco*RI sites of pSG5 (Green *et al.*, 1988). pcJUN5 contains the *Eco*RI (39)−*Bam*HI(2100) c-*jun* cDNA fragment (Angel *et al.*, 1988a) between the *Eco*RI and *Bam*HI sites of pSG5. Transfections were repeated several times with at least two independent DNA preparations. Nuclear extracts were prepared as described by Rosales *et al.* (1987), and whole cell extracts according to Kumar and Chambon (1988). Protein concentrations were determined by the Bradford method (Pierce). Protein−DNA complexes were formed using 10 μg protein and excess 5′ ^{32}P-labelled oligonucleotides and electrophoresed on 6% acrylamide gels in 0.25 M TBE as described by Martin *et al.* (1988). At least two independent extracts were analysed for each experiment, and the quality of the extracts

verified using a probe for the ubiquitous abundant factor NFI. Occasionally an SP1 probe was also used.

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