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Transforming but not immortalizing oncogenes activate the transcription factor PEA1

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The transcription factor PEA1 (a homologue of AP1 and c-*jun*) is highly active in several fibroblast cell lines, compared to its low activity in a myeloma and an embryocarcinoma (EC) cell line. Serum components are essential to attain these high levels of PEA1 activity in fibroblasts. This serum requirement is abbrogated by transformation with the oncogenes c-Ha-*ras*, v-*src* and polyoma middle T (Py-MT) but not by immortalization with polyoma large T (Py-LT), v-*myc*, c-*myc* or SV40 large T (SV40T). Expression in myeloma cells of the same transforming oncogenes, as well as v-*mos* and c-*fos*, activates PEA1, whereas expression of the same immortalizing oncogenes and EIA does not. These results suggest that a common target for transforming oncogenes is PEA1. Serum components have no effect on PEA1 activity in the myeloma and EC cell lines. In contrast, retinoic acid treatment of F9 EC cells augments PEA1 activity. These results suggest that transforming oncogene expression compensates for the absence of cell type-specific factors which are required to activate PEA1. Activation of PEA1 may lead to altered transcription of a set of transformation-related genes.

Key words: AP1/*jun*/differentiation/oncogenes/transformation

Introduction

Tumours are generated by the accumulation of several changes which affect the control of cell growth (for reviews, see Bishop, 1987; Montarras and Pinset, 1987; Ohlsson and Pfeifer-Ohlsson, 1987; Klein and Klein, 1985; Weinberg, 1985). These changes are thought to arise from the expression of oncogenes. The identification of a large number of oncogenes has led to attempts to classify them into functional groups with common properties. One such classification is into immortalizing and transforming oncogenes. Immortalization is the ability to convert cell lines of limited replicative potential into cell lines that can be passaged indefinitely. Transformation confers anchorage-independent growth. Transforming oncogenes are generally weak in their ability to immortalize cells and vice versa. The collaboration of both types of oncogene is required for malignant transformation (Rassoulzadegan *et al.*, 1982; Land *et al.*, 1983; Ruley, 1983).

We have recently shown that expression of the c-Ha-*ras* transforming oncogene stimlates the activity of the transcription enhancer factor PEA1 (Imler *et al.*, 1988a). PEA1 most probably belongs to a closely-related family of transcription factors which includes human AP1 (Angel *et al.*, 1987; Lee *et al.*, 1987; Piette and Yaniv, 1987), avian sarcoma virus v-*jun* oncogene (Bohman *et al.*, 1987; Angel *et al.*, 1988; Imler *et al.*, 1988b) and yeast GCN4 (Struhl, 1987). We have now found that expression of four other transforming oncogenes [v-*src*, polyoma middle T (Py-MT), v-*mos*, and c-*fos*] activate PEA1, whereas expression of immortalizing oncogenes [polyoma large T (Py-LT), *myc*, SV40 large T (SV40T) and EIA] does not, suggesting that a critical event in transformation is constitutive activation of PEA1.

Results

Large differences in the level of activity of PEA1 in various cell lines

In order to study the level of transcription stimulatory activity due to PEA1 *in vivo*, we constructed the wild-type (WT) reporter recombinant pG1PB4 (Figure 1A, lane 1) which has four head-to-tail copies of oligonucleotides containing the PEA1-specific binding site (see WT oligos, Figure 1A) directly upstream from the β-globin promoter. The activity of PEA1 was measured by comparing transcription from the β-globin promoter of pG1PB4 with two control mutated reporter recombinants, pG1PAB4 and pG1 (Figure 1A, lane 1). The oligonucleotides in pG1PAB4 contain two point mutations which inhibit PEA1 binding (see MUT oligos, Figure 1B and Piette and Yaniv, 1987). The other control, pG1, lacks inserted oligonucleotides. The comparison of transcription from the WT reporter recombinant (pG1PB4) with both control mutated recombinants in all experiments ensures that variations in activity are due to a factor which requires the PEA1-specific binding site for activity, and that no other factor (binding to other parts of the oligonucleotides) affects β-globin transcription.

We have previously shown, using reporter recombinants different from those described here, that there are low levels of PEA1 activity in MPC11BU4 myeloma cells and higher levels in LMTK$^-$ fibroblast cells (Imler *et al.*, 1988a). The new reporter recombinants were transfected into these cells, together with an internal control recombinant (pΔRCM in myeloma cells, pΔRCBx2 in all other cell lines, see lane 3 in Figure 1) to monitor for variations in transfection efficiency, and after 48 h total RNA was extracted. The amounts of specific RNA initiated from the β-globin promoter of the reporter recombinants and conalbumin promoter of the internal controls were measured by quantitative S1 nuclease mapping [see REPORTER (GLOB) and ONCOGENE VECTOR or CONTROL (CONP) bands respectively in Figure 2]. The autoradiograms shown in Figures 2, 5 and 6 have been cut to simplify illustration of bands of different mobility and intensity. The experiments were repeated at least three times with a minimum of two

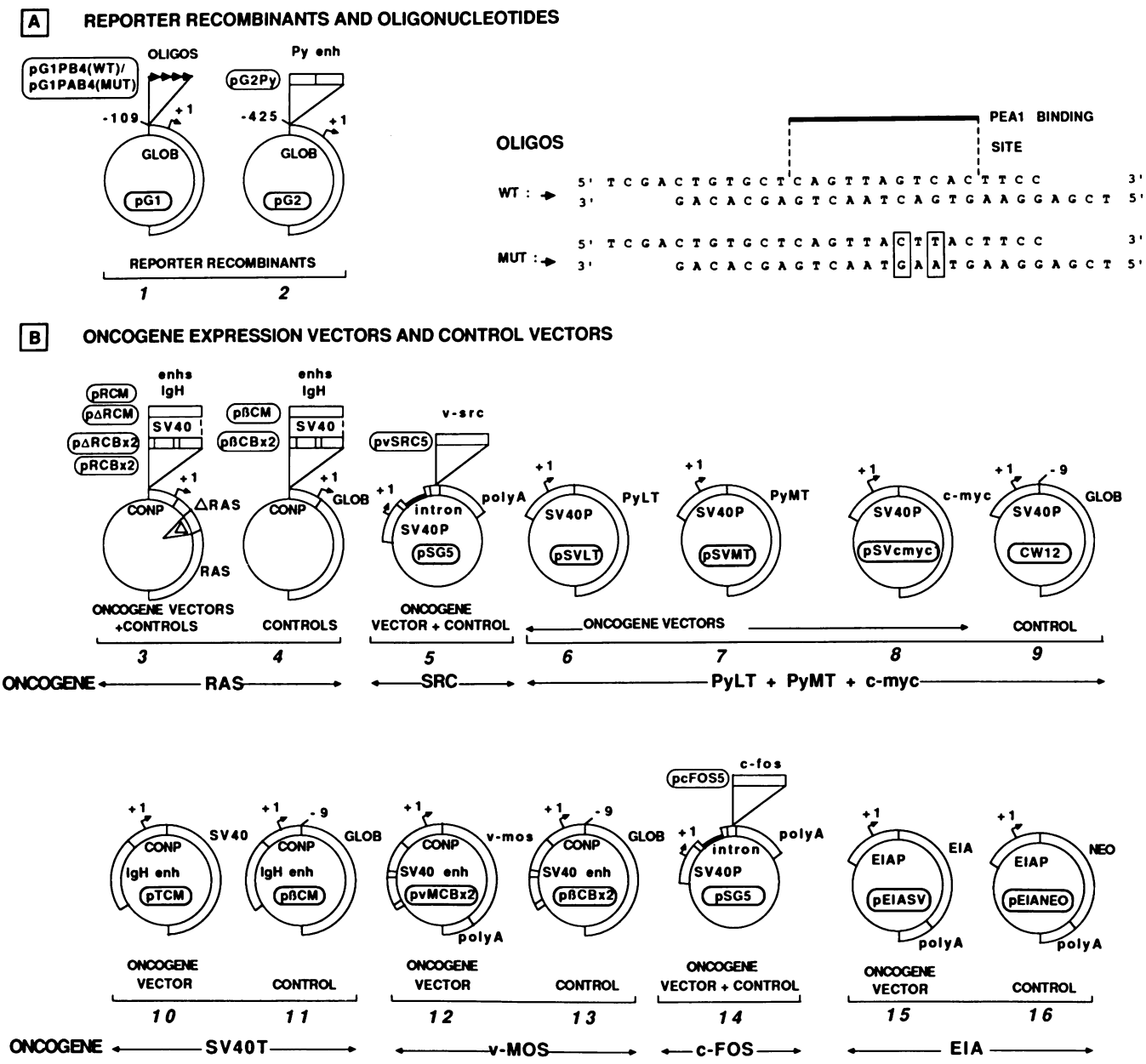

Fig. 1. (A) Structures of reporter recombinants and oligonucleotides The reporter recombinants were used to measure the stimulatory activity of either four head-to-tail copies of oligonucleotides (oligos) or the Py enhancer on transcription from the promoter of the rabbit β-globin gene. The specific binding site for the transcription factor PEA1 (Piette and Yaniv, 1987; Imler *et al.*, 1988a) is indicated on the sequence of the oligonucleotides. WT: wild-type, MUT: mutant, OLIGOS: oligonucleotides, enh: enhancer. (B) Structures of oncogene expression vectors and control vectors. The oncogene vectors are expression vectors for the different oncogenes indicated under the lanes (*ras*: activated c-Ha-*ras*, *src*: v-*src* from RSV, PyLT: Py large T, PyMT: Py middle T, c-*myc*: mouse c-*myc*, SV40: SV40 early region, v-*mos*: v-*mos* from MoMLV, c-*fos*: mouse c-*fos*, EIA: adenovirus 2 EIA). These vectors contain heterologous promoter sequences (CONP: chicken conalbumin promoter, SV40P: SV40 early promoter, EIAP: adenovirus 2 EIA promoter). The control expression vectors (controls) contain either c-Ha-*ras* structural gene sequences with a deletion which prevents expression of *ras* (ΔRAS), rabbit β globin structural gene sequences (GLOB) or kanamycin resistance gene sequences (NEO) downstream from promoter sequences identical to those in the corresponding expression vectors. +1: specific RNA initiation site for corresponding promoter. −109, −425, −9: upstream limit of β-globin sequences. polyA: polyadenylation signal. The thin lines represent pBR322 prokaryotic vector sequences. IgH: immunoglobulin heavy chain.

different preparations of DNA and representative autoradiograms are shown. The presence of the PEA1 binding sites in pG1PB4 (WT, Figure 2) stimulated β-globin transcription <2-fold in the myeloma cells (see lanes 1 and 3, Figure 2) and ~50-fold in LMTK$^-$ fibroblasts (see lanes 14, 17). pG1PAB4, in which the PEA1 binding sites are mutated (M, Figure 2), is essentially inactive compared to pG1 in both cell lines (compare lanes 1, 5, 14 and 20). These results show that there are low levels of transcription stimulatory activity due to the PEA1 factor in the myeloma cells compared to the high levels in the fibroblast cell lines. The comparison with the mutated reporters also shows that other unknown factors do not contribute to oligonucleotide activity.

The reporter recombinants were transfected into F9 embryocarcinoma (EC) and NIH3T3 fibroblast cells. Transcription from the wild-type reporter recombinant was

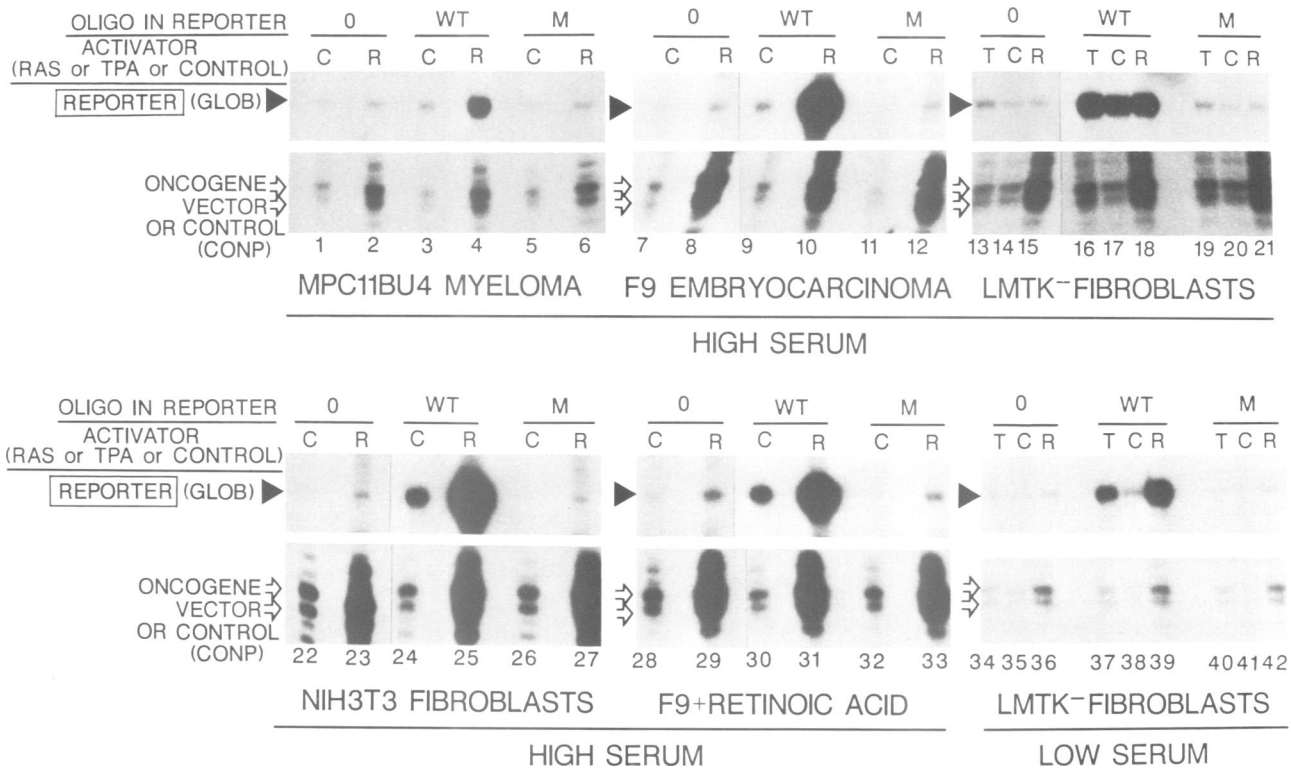

Fig. 2. Different levels of activity of PEA1 in various cell lines and effect of serum, TPA, differentiation and *ras* expression. The reporter recombinants pG1, pG1PB4 or pG1PAB4, respectively, containing no (O), wild-type (WT) or mutant (M) oligonucleotides (OLIGOS) were co-transfected with an equal molar amount of either the control vector (CONTROL, pΔRCM or ΔRCBx2) or the *ras* expression vector (pRCM or pRCBx2). After 48 h total RNA was analysed by quantitative S1 nuclease mapping for specific RNA initiated from the β-globin promoter of the reporter recombinants [REPORTER (GLOB)] or conalbumin promoter of either the control vector [CONTROL (CONP) bands in lanes labelled C] or the *ras* expression vector (ONCOGENE VECTOR bands in R lanes). The quantities of transfected DNA were: 2 μg test DNA, 4.0 μg pRCM, pΔRCM in MPC11BU4; 5 μg test DNA, 9.4 μg pRCBx2, pΔRCBx2 in F9EC and NIH3T3 cells; 2.5 μg test DNA, 4.7 μg pRCBx2, pΔRCBx2 in LMTK⁻ cells; 2 μg test DNA, 3.7 μg pRCBx2 pΔRCBx2 in F9 + retinoic acid cells. F9 cells were incubated with 10^{-7} M retinoic acid for 4 days before transfection in the presence of retinoic acid. The cell medium contained 10% fetal calf serum (HIGH SERUM) except where indicated for LMTK⁻ cells, the serum was decreased to 0.05% for the last 24 h of incubation (LOW SERUM). The lanes labelled T (TPA) correspond to transfections in LMTK⁻ fibroblasts with the reporter recombinants and the control vectors, which were treated with 100 ng/ml TPA for the last 24 h of transfection.

stimulated ~4-fold in the EC cells and 15-fold in the fibroblasts [see REPORTER (GLOB) and CONTROL (CONP) bands in lanes 7, 9, 11, 22, 24 and 26]. These results show that different cell lines contain different levels of PEA1 activity, which range from low in MPC11BU4 myeloma and F9 EC cells, intermediate in NIH3T3 fibroblasts, to high in LMTK⁻ fibroblasts. These variations in activity are qualitatively similar to those observed for activity of the polyoma virus (Py) enhancer in the same cells (Wasylyk *et al.*, 1987), suggesting that variations in PEA1 activity account, to a significant extent, for variations in the activity of the Py enhancer.

PEA1 activity is increased in LMTK⁻ cells by serum components and in F9 cells by treatment with retinoic acid

We have identified factors which affect PEA1 activity in F9 EC cells and LMTK⁻ fibroblasts. With LMTK⁻ fibroblasts, we found that decreasing the serum concentration decreased PEA1 activity ~25-fold (compare lanes 35, 38, 41 and 14, 17, 20 in Figure 2). It is striking that PEA1 activity remains low in F9 EC cells (as well as in MPC11BU4 myeloma cells) in the presence of high serum concentrations, showing that serum components are insufficient to achieve high levels of PEA1 activity in these cells.

In attempts to identify other factors that could activate PEA1, we differentiated F9 cells with retinoic acid for 4 days, and then transfected them in the presence of retinoic acid. Transcription of the WT reporter was stimulated by ~15-fold (lanes 28, 30, 32, Figure 2). This is a 4-fold increase in PEA1 activity compared to untreated cells. A comparable increase in PEA1 activity was also observed when F9 cells were differentiated with retinoic acid and cAMP (results not shown). These results are in agreement with those of Kryszke *et al.* (1987) who showed, using cell-free extracts, that differentiation of F9 cells led to an increase in PEA1-specific DNA binding activity.

The ras oncogene stimulates PEA1 activity in F9, LMTK⁻ and other cell lines

We next studied the effect on PEA1 activity of introducing the c-Ha-*ras* oncoprotein into cells. This was achieved by co-transfecting a *ras* expression vector (pRCM for myeloma cells, pRCBx2 for other cell lines, see lane 3, Figure 1 and Wasylyk *et al.* 1987) with the three reporter recombinants. Two types of control vector were generally used in comparative transfections. pΔRCM and pΔRCBx2 (Figure 1, lane 3) are identical to the *ras* oncogene vectors pRCM and pRCBx2 respectively, except for a small deletion between the first exon and the first intron of the *ras* structural gene

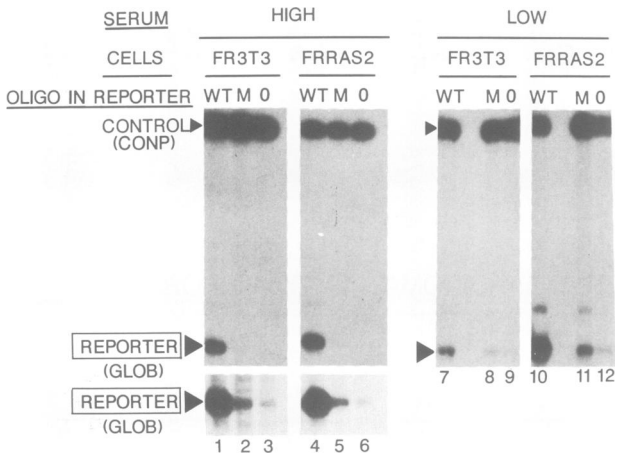

Fig. 3. Effect of serum on PEA1 activity in FR3T3 and FRRAS2 cells. The cells were transfected with 2 μg reporter recombinants containing either no (O), mutant (M) or wild-type (WT) oligonucleotides (OLIGOS) and 2 μg control recombinant [CONTROL (CONP)]. For the last 36 h of the 48 h transfection period the medium contained either 10% or 0.05% fetal calf serum (HIGH and LOW SERUM, respectively). Total RNA was analysed by quantitative S1 nuclease mapping for specific RNA initiated from the globin promoter of the reporter DNA [REPORTER (GLOB)] and conalbumin promoter of the control DNA [CONTROL (CONP)]. A longer exposure of the GLOB bands in **lanes 1–6** is shown.

sequences (see Materials and methods) which prevents expression of the *ras* protein (see Imler *et al.*, 1988a). The other control expression vectors contained β-globin structural gene sequences in the place of *ras* sequences (see pβCM and pβCBx2; lane 4 Figure 1). Both previous (Wasylyk *et al.*, 1987; Imler *et al.*, 1988a) and unpublished results show that such control vectors do not affect the activity of the reporter recombinant pG1PB4 (WT). *ras* expression resulted in a large increase in PEA1 activity in the cell lines with low or intermediate levels of PEA1 activity [10- to 50-fold depending on cell line, see REPORTER (GLOB) bands in lanes 1–12, 22–27, 35, 36, 38, 39, 41, 42 of Figure 2). *ras* expression also gave, in some cases, a much smaller stimulation of β-globin transcription in the absence of a good PEA1 binding site (see REPORTERS O and M in lanes 1, 2, 7, 8, 14, 15, 22, 23, 28, 29 and 35, 36). It should be noted that the quantity of stable specific RNA transcribed from the oncogene and control vectors are different [compare ONCOGENE VECTOR and CONTROL (CONP) bands, Figure 2] most probably due to differences in RNA stability (Wasylyk *et al.*, 1987). The controls used here (pΔRCM and pΔRCBx2) consistently gave less stable RNA than the wild-type oncogene vector, whereas the pβCM and pβCBx2 controls used in our previous studies gave more stable RNA (unpublished results; Wasylyk *et al.*, 1987). The expression

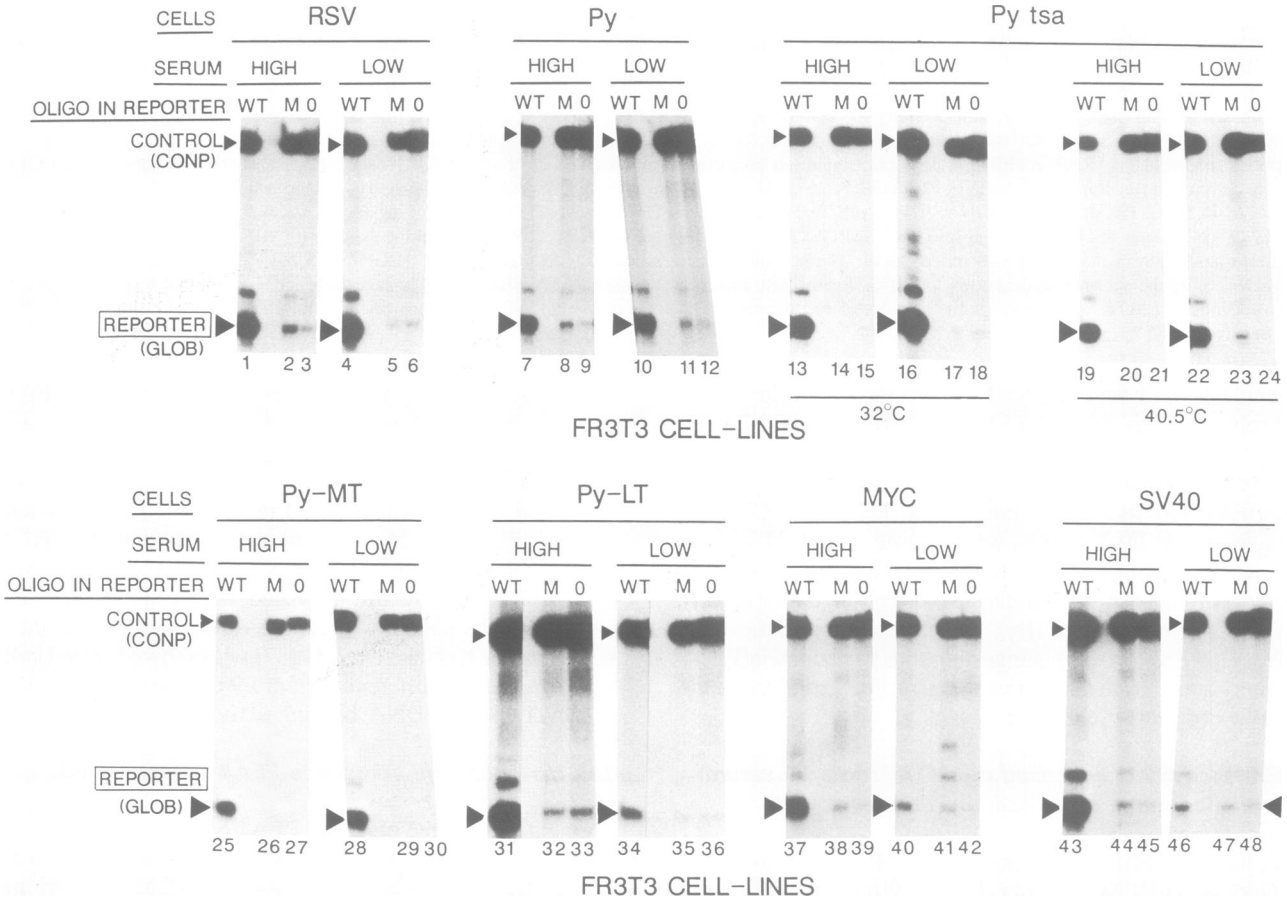

Fig. 4. Effect of serum on PEA1 activity in transformed or immortalized FR3T3 cell lines. The cell lines transformed with RSV, Py early region (Py), Py early region with a temperature-sensitive mutation in large T (Py tsa) and with Py middle T (Py-MT), or immortalized with Py large T (Py-LT), v-*myc* (MYC) or SV40 were transfected with 2 μg reporter recombinants containing either no (O), mutant (M) or wild-type (WT) oligonucleotides (OLIGO) and 2 μg control recombinant (CONTROL). For the last 36 h of the 48 h transfection period the medium contained either 10% or 0.05% fetal calf serum (HIGH or LOW SERUM, respectively). For the Py tsa cell line, transfection was at 32°C, the cells were then divided by trypsinization, and incubated either at 32 or 40.5°C, in high or low serum as described above. Total RNA was analysed by quantitative S1 nuclease mapping for RNA initiated from the globin promoter of the reporter recombinants [REPORTER (GLOB)] or the conalbumin promoter of the control recombinant [CONTROL (CONP)].

vector bands are useful controls for transfection efficiency, when the oncogene vector lanes, or the control vector lanes, are compared amongst themselves. In addition, for a constant transfection efficiency, the ratio in band intensities between the two types of vectors is constant (unpublished results). The control vectors did not affect transcription from the reporter recombinants (data not shown). These results show that *ras* expression leads to an increase in PEA1 activity in several cell lines in which PEA1 activity is either at a low or intermediate level.

If *ras* stimulates PEA1 activity through a similar pathway to that of serum in LMTK$^-$ cells, or retinoic acid in F9 EC cells, it might be expected that PEA1 in these cells is less sensitive to further stimulation by *ras* expression. Indeed, in LMTK$^-$ cells in high serum, PEA1 activity was insensitive to *ras* expression (see lanes 14, 15, 17, 18, 20 and 21). It is interesting that another activator of PEA1, the tumour promoter TPA, also only increased PEA1 activity in the low serum conditions (compare lanes 13, 14, 16, 17, 19, 20 and 34, 35, 37, 38, 40, 41). In retinoic acid-differentiated F9 cells, the increase in PEA1 activity due to *ras* expression was smaller than in the undifferentiated cells [~5-fold compared to ~20-fold, see lanes 30, 31 and 9, 10 and also lanes 28, 29 and 7 and 8 to take into account the effect on the control reporter recombinant pG1 (0)]. These results suggest that *ras* expression can replace serum or other unknown factors, to achieve high levels of PEA1 activity. They also suggest that *ras* transformation of fibroblasts will lead to a constitutive high level of PEA1 activity which may be serum independent.

Transformation by ras uncouples PEA1 activity from its requirement for serum components in FR3T3 fibroblasts

We compared PEA1 activity in FR3T3 fibroblasts and in low passage FRRAS2 cells (Matrisian *et al.*, 1985), which are transformed with the c-Ha-*ras* oncogene. The reporter recombinants were co-transfected with a control recombinant pβCBx2 (Figure 2, lane 4), to correct for variations in transfection efficiency. There were essentially similar levels of PEA1 activity in both cell lines in high serum conditions [compare the REPORTER (GLOB) bands with the CONTROL bands in lanes 1–6, Figure 3]. [It should be noted that there is a small residual activity of the mutated oligonucleotides in these cells (~3-fold, compare reporters M and O in lanes 2, 3, 5, 6).] Decreasing the serum concentration resulted in a large decrease in PEA1 activity in FR3T3 cells as judged by the decrease in transcription of the wild-type reporter recombinant in comparison with the internal control (~10-fold, compare REPORTER and CONTROL bands in lanes 1 and 7). Strikingly, in the low serum conditions, transcription from the wild-type reporter recombinant was only ~2-fold higher than from the mutated reporters (compare lanes 7–9). With the *ras*-transformed cells decreasing the serum concentration had little or no effect on PEA1 activity (compare lanes 4–6 and 10–12). In contrast, high levels of transcription were observed from the control recombinant in both cell lines in high and low conditions (see control bands), indicating that some other transcription factors remain active in low serum conditions in the non-transformed cells. Similar results were obtained in another *ras*-transformed cell line (not shown). These data show that in *ras*-transformed FR3T3 cells, high PEA1 activity is no longer dependent on serum components.

High PEA1 activity is serum independent in transformed, but not in immortalized FR3T3 fibroblasts

FR3T3 fibroblast can be immortalized by Py-LT, *myc*, SV40T and transformed by *ras*, Rous sarcoma virus (RSV) *src* and Py-MT (see Connan *et al.*, 1985). We have tested the serum requirements for PEA1 activity in low passage transformed and immortalized FR3T3 cell lines. In a RSV-transformed cell line, we found that high PEA1 activity was serum independent (lanes 1–6, Figure 4). A similar result was obtained with an independent RSV-transformed cell-line (results not shown). A cell line transformed with the complete early region of Py also had serum-independent high levels of PEA1 activity (see lanes 7–12). Two experiments showed that this was due to the presence of Py-MT and not Py-LT. In cells transformed with Py DNA with a temperature-sensitive mutation in Py-LT (Py tsa, Perbal and Rassoulzadegan, 1980), PEA1 activity was serum indepen-

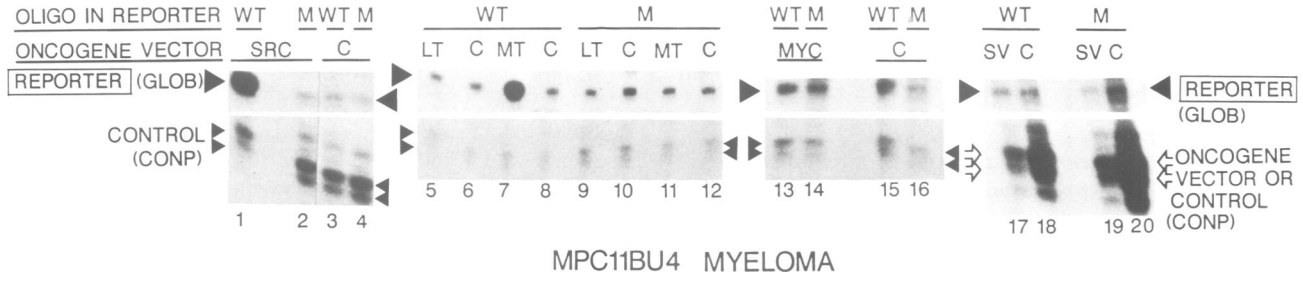

Fig. 5. Effect of oncogene expression on PEA1 activity in MPC11BU4 myeloma cells. The cells were transfected with 2 μg of the test recombinants containing either wild-type (WT) or mutant (M) oligonucleotides (OLIGO) together with either oncogene expression vectors for v-*src* (SRC, **lanes 1, 2**) polyoma virus large T (LT, **lanes 5, 9**) polyoma virus middle T (MT, **lanes 7, 11**), c-*myc* (MYC, **lanes 13,14**) or SV40 large T (SV40, **lanes 17, 19**) or their respective control vectors (**C lanes**, see Figure 1). The quantities of oncogene expression vectors or control vectors were in lanes: **1, 2**: 2.8 μg pvSRC5 (lane 5, Figure 1); **3, 4**: 2.0 μg pSG5 (lane 5, Figure 1); **5, 9**: 4.3 μg pSVLT (lane 6, Figure 1); **7, 11**: 4.5 μg pSVMT (lane 7, Figure 1); **6, 8, 10, 12**: 4.4 μg CW12 (lane 9, Figure 1); **13, 14**: 17.4 μg pSVcmyc1; **15, 16**: 12 μg CW12 (lane 9, Figure 1); **17, 19**: 17.5 μg pTCM (lane 10, Figure 1); **18, 20**: 12.5 μg pβCM (lane 11, Figure 1). In some transfections a third recombinant was included, whose transcription did not vary with oncogene expression, and thus served as a more precise control for transfection efficiency than transcription from the expression vector and the control vector. The third recombinant was 2 μg pβCM for **lanes 1–4** and **13–16**, and 2 μg pΔRCM for **lanes 5–12**. 48 h after transfection total RNA was extracted and analysed by quantitative S1 nuclease mapping for RNA initiated from the reporter recombinant globin promoter [bands labelled (REPORTER (GLOB)], and the conalbumin promoter of either the third control recombinant [bands labelled CONTROL (CONP) and indicated with a filled arrowhead, **lanes 1–16**] or the oncogene vector or control vector [bands labelled ONCOGENE VECTOR or CONTROL (CONP) and indicated with an open arrow, **lanes 17, 20**].

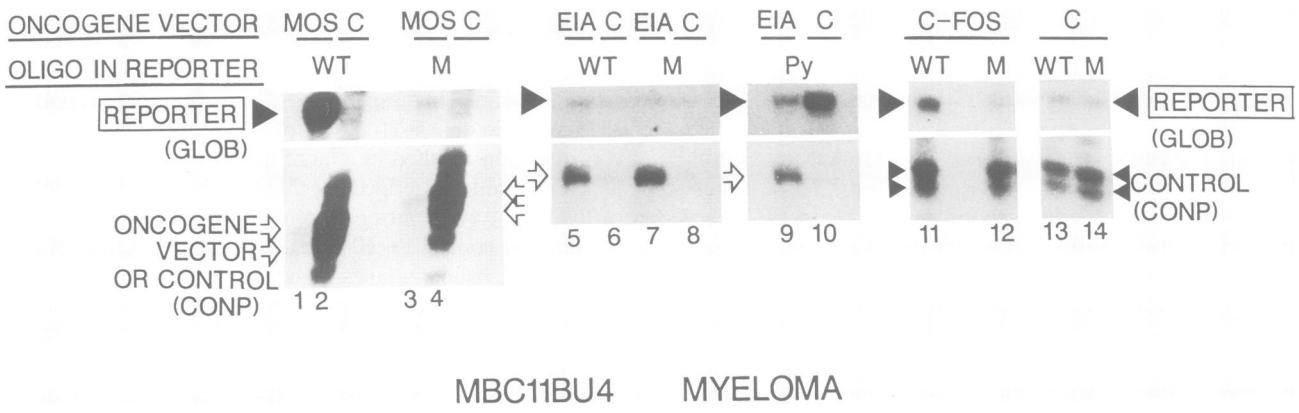

Fig. 6. Effect of v-*mos*, EIA and c-*fos* expression on PEA1 activity in MPC11BU4 myeloma cells. The cells were transfected with 2 μg of the reporter recombinants containing either wild-type (WT) or mutant (M) oligonucleotides (OLIGO) or the Py enhancer (Py) (REPORTER) together with either oncogene expression vectors for v-*mos* (**lanes 1, 3**), EIA (**lanes 5, 7, 9**), or c-*fos* (**lanes 11, 12**) or their respective control expression vectors (**C lanes**, see Figure 1). The quantities of expression vector or control expression vector were in lanes: **1, 3**: 13.5 μg pvMCBx2 (lane 12, Figure 1); **2, 4**: 10 μg pβCBx2 (lane 13, Figure 1); **5, 7, 9**: 2.2 μg pEIASV (lane 15, Figure 1); **6, 8, 10**: 2.0 μg pEIANEO (lane 16, Figure 1); **11, 12**: 3.3 μg pcFOS5 (lane 14, Figure 1) **13, 14**: 2.0 μg pSG5 (lane 14, Figure 1). The transfections in **lanes 11–14** contained 2 μg of a third recombinant, pβCM, whose transcription did not vary with oncogene expression and thus served as a more precise control for transfection efficiency. 48 h after transfection total RNA was extracted and analysed by quantitative S1 nuclease mapping for RNA initiated from the reporter recombinant globin promoter [bands labelled REPORTER (GLOB)] and either the conalbumin or EIA promoters of the expression or control expression vectors (bands indicated with an open arrow in **lanes 1–10**). Alternatively, RNA initiated from the conalbumin promoter of the third control recombinant was analysed (CONTROL bands indicated with a filled arrow head).

dent at both the permissive and non-permissive temperatures (lanes 13–24). In a cell line transformed by Py-MT alone, PEA1 activity was serum independent (lanes 25–30). In contrast, in a cell line expressing only Py-LT (lanes 31–36) PEA1 stimulated transcription ~30-fold in high serum (compare lanes 31–33) and only ~4-fold in low serum (compare lanes 34–36) showing that PEA1 activity was serum dependent. In a cell line expressing v-*myc*, transcription from the wild-type reporter recombinant compared to the mutated reporter recombinant was ~30-fold in high serum (37–39) and 2-fold in low serum (40–42). Similar results were obtained in a cell line immortalized with c-*myc* (data not shown). In a cell line expressing the SV40 early region T antigen, PEA1 activity was also serum dependent (compare the ~30-fold stimulation of reporter globin transcription in lanes 43–45, with the 2-fold stimulation in lanes 46–48). These results suggest that a common property of transforming oncogene (in this case *ras*, *src*, MT), not shared by immortalizing oncogenes (Py-LT, *myc* and SV40) is to render PEA1 activity essentially independent of serum factors.

Transforming, but not immortalizing, oncogenes transactivate PEA1 in short-term transfection assays

The above results might have been influenced by the selection procedures required to isolate cell clones, which could have affected the phenotype of the low passage cell lines used. We therefore tested whether oncogene expression in short-term transfections could also increase PEA1 activity. MPC11BU4 cells were chosen because they have low levels of PEA1 activity, even in high serum conditions, and PEA1 activity is increased by *ras* expression. The test recombinants were co-transfected either with expression vectors for the different oncogenes (oncogene vectors) or with control expression vectors. The control vectors always contained the same promoter transcription signals as the oncogene vector, but differed in that either other structural gene sequences replaced the oncogene coding sequences (Figure 1, compare expression and control vectors in lanes 6–8 with 9, 10 with 11, 12 with 13, 15 with 16) or oncogene cDNA sequences were deleted (see lanes 5 and 14). This strategy was guided by our previous experiments with either *ras* (Wasylyk *et al.*, 1987) or v-*jun* (Imler *et al.*, 1988b) expression vectors. The control vectors had no detectable effect on test recombinant activity (Wasylyk *et al.*, 1987; Imler *et al.*, 1988a,b and unpublished results). In some cases, where possible, a third recombinant was also co-transfected. This control gave the same level of transcription in all transfections which simplified comparison of transfection efficiencies (lanes labelled control with filled arrowheads, Figures 5 and 6). In other cases the expression vector and the control vector served as controls for transfection efficiency. We found that the ratio of transcription from the expression vectors and their controls was constant in each case, as shown in a large number of experiments (see lanes labelled exp. vector + control with an open arrow pointing to the bands, in Figures 5 and 6 and results not shown).

Expression of the oncogenes v-*src* (from RSV) and Py-MT in MPC11BU4 cells stimulated PEA1 activity to a similar extent as *ras* expression (~20-fold in both cases, see lanes 1–4 and 7, 8, 11, 12 for v-*src* and MT respectively, in Figure 5 and compare with lanes 3–6, Figure 2). In contrast Py-LT, c-*myc* and SV40T antigen expression had no effect on PEA1 activity (see lanes 5, 6, 9, 10 for LT, 13–16 for c-*myc* and 17–20 for SV40T). Several observations suggest that this lack of stimulation of PEA1 activity was not due to non-functional expression vectors. (i) Transcription from the expression vectors was readily detectable (see lanes 17, 19, and results not shown). (ii) The expression vectors for Py-LT and c-*myc* were the same as those used to immortalize the FR3T3 cell lines used here, and other cell lines (Land *et al.*, 1983). The Py-LT expression vector is similar to the Py-MT expression vector, which did stimulate PEA1 activity, except for one restriction fragment which leads to the production of Py-LT mRNA instead of Py-MT mRNA. We also did not observe an effect of the

c-*myc* expression vector in short-term transfections of FR3T3 cells, even though this vector immortalizes these cells. (iii) SV40T antigen was readily detectable by indirect immunofluorescence in the nucleus of cells transfected with the pTCM expression vector (data not shown). These results show that expression of v-*src*, MT and *ras* increases PEA1 activity. In contrast, expression of LT, c-*myc* and the SV40T antigen does not affect PEA1 activity.

v-mos and c-fos, in contrast to EIA, trans-activate PEA1

The short-term transfection assay was also used to study *trans*-activation by oncogenes which had not been studied in transformed or immortalized FR3T3 cell lines. The expression of v-*mos* (from MoMLV) increased PEA1 activity (lanes 1−4, Figure 6). In contrast, EIA expression had no effect (lanes 5−8). This lack of activity was not due to inefficient production of EIA because, as described previously (Borrelli *et al.*, 1984), EIA expression inhibited Py enhancer activity (see lanes 9, 10). In addition, transcription from the EIA expression vector was readily detectable (see bands labelled with an open arrow). c-*fos* expression resulted in a significant increase in PEA1 activity (~5-fold, lanes 11−14), which was however smaller than that of *ras*, v-*src*, MT and v-*mos* (~20-fold) but greater than that of LT, *myc*, SV40T or E1A (<2-fold). This was most probably not due to inefficient expression of c-*fos* from its particular expression vector. The c-*fos* expression vector lacked the 3′-terminal sequences that destabilize its mRNA (Rüther *et al.*, 1985). Transfection of the same expression vector in F9 cells led to very efficient activation of PEA1 activity (in preparation), even though similar amounts of stable RNA were transcribed from the expression vector in both cell lines. These results show that v-*mos* like *ras*, v-*src* and MT has PEA1 activatory properties, whilst EIA, like LT, *myc* and SV40T, does not stimulate PEA1. In contrast c-*fos* activates PEA1, but to a lesser extent than the other transforming oncogenes in this cell line.

Discussion

What is the mechanism of regulation of the transcription enhancer factor PEA1?

We have found that the activity of the transcription enhancer factor PEA1 is stimulated by serum components, a tumour promoter TPA, the oncoproteins v-*src*, Py-MT, c-Ha-*ras*, v-*mos*, v-*raf*, c-*fos* and by retinoic acid, but not by the oncoproteins Py-LT, *myc*, SV40T or EIA (see above, results not shown for c-*fos* in some cell lines and U.Rapp *et al.* in preparation for v-*raf*). How might the mechanism of activation by these factors be linked?

The effects of growth factors in serum are transduced from cell membrane receptors to the final site of action by multicomponent signalling pathways. Several oncoproteins are known to be deregulated counterparts of cellular proteins which participate in signal transduction. Transformation probably results in constitutive activation of these pathways resulting in decreased requirements for growth factors (Bishop, 1987; Montarras and Pinset, 1987; Ohlsson and Pfeifer-Ohlsson, 1987). In support of this hypothesis, we report here that the expression of some oncogenes decreases the serum requirements for activation of PEA1, showing that they may activate one or several pathway(s) targeted towards PEA1. One of these pathways may involve protein kinase C, since TPA, which is known to activate this kinase (Nishizuka, 1984; Blumberg, 1988) stimulates the activity of PEA1. The effects of the *src*, MT, *ras* and *fos* oncogenes are probably linked to this pathway, as shown by several different observations. Transformation by *src*, Py-MT or *ras* alters turnover of phosphatidylinositol (PI; Sugimoto *et al.*, 1984; Kaplan *et al.*, 1986; Wakelam *et al.*, 1986; Marshall, 1987), a second messenger which regulates protein kinase C activity (Nishizuka, 1984). *src*, in association with Py-MT, forms a complex with and phosphorylates a PI kinase. This may account for the effect of these oncogenes on PI turnover (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987). Inhibition of endogenous *ras*, by micro-injection of anti-*ras* antibodies into cells, results in the inhibition of protein kinase C activity, as shown by a decrease in the induction of proliferation by either phorbol esters or phosphatidic acid (Yu *et al.*, 1988). c-*fos* transcription is induced by TPA, and inhibition of protein kinase C depresses this induction (Mitchell *et al.*, 1986; Bravo *et al.*, 1987).

Other evidence would place the PEA1 activating oncogenes on a common pathway, in the order *src* + MT → *ras* → *mos* → *raf* → *fos* → PEA1. Firstly, *src*, MT and *ras* associate with the inner surface of the plasma membrane, whereas *mos* and *raf* are found in the cytoplasm, and *fos* and PEA1 in the nucleus. Secondly, cell lines have been selected which are resistant to transformation by *fos* or *ras*. The block to *fos* prevents transformation by *mos* and *ras*, (Zarbl *et al.*, 1987), whilst the block to *ras* prevents transformation by v-*src* (Noda *et al.*, 1983; Norton *et al.*, 1984). Thirdly, injection of antibodies to *ras* blocks transformation by v-*src*, but not by *mos* or *raf* (Smith *et al.*, 1986). The affected oncogenes are assumed to be on the same pathway and upstream from the block. However, all the results may not fit this model. For example, the block to *fos* transformation does not prevent re-transformation by Py-MT (Zarbl *et al.*, 1987). How might the biochemical activities of the oncogenes be interrelated? An intriguing possibility is that they are linked as a cascade of kinases (Hunter, 1987), culminating in the phosphorylation of PEA1 or *fos*. c-*src* is a tyrosine kinase, and association with MT stimulates its kinase activity. Tyrosine phosphorylation is essential for transformation by *src* and Py-MT (Hunter and Cooper, 1986). *ras* somehow transduces the proliferative signals mediated by the serine−threonine-specific protein kinase C (Yu *et al.*, 1988). *mos* and *raf* are serine−threonine kinases (Bishop, 1987). A final event in the cascade may be phosphorylation of PEA1 or of *fos*. *fos* does not bind to DNA directly, but stimulates transcription indirectly by binding to other transcription factors (Distel *et al.*, 1987; Lech *et al.*, 1988). Recent evidence shows that *fos* protein can complex with PEA1 (Rauscher *et al.*, 1988). We have found that *fos* expression *trans*-activates PEA1, suggesting that *fos*−PEA1 complex formation leads to activation of transcription *in vivo*.

Our results show that serum growth factors are insufficient to stimulate PEA1 activity to high levels in MPC11BU4 myeloma or F9 EC cells. This does not appear to be due to a defect in the signalling pathway because oncogene expression stimulates PEA1 activity. Different types of cells require different factors for growth (Evans, 1986) suggesting that PEA1 activation in myeloma and EC cells is linked to receptors whose growth factors are absent in the culture

medium used here. Oncogene expression may either abrogate the need for such factors, or may stimulate their synthesis, thereby creating an autocrine loop which activates PEA1. Alternatively, oncogene expression may sensitize the cells to the components present in the medium by stimulating the synthesis of appropriate receptors. We found that treating F9 EC cells with retinoic acid increases PEA1 activity. Retinoic acid can stimulate protein kinase C *in vitro* (Ohkubo *et al.*, 1984), and retinoic acid treatment of F9 cells increases the level of epidermal growth factor receptor (Griep and DeLuca, 1986) which could render PEA1 activity sensitive to serum components. However, our preliminary experiments suggest that PEA1 activity is independent of serum components in both F9 and retinoic acid-treated F9 cells suggesting that another mechanism is involved in this case.

We found that c-*fos* expression is less efficient in stimulating PEA1 activity in myeloma cells in comparison with the other transforming oncogenes, whereas it is highly and equally as efficient in F9 EC cells (in preparation). We have reported a similar cell specificity for v-*jun* transcription stimulatory activity (Imler *et al.*, 1988b). These cell type-specific effects could help explain why certain oncogenes transform only some cell types and why some naturally occurring tumours are associated predominantly with a particular oncogene (Bishop, 1987).

The role of PEA1 in transformation

Complete transformation of primary cells requires the collaboration of an immortalizing and a transforming oncogene (Rassoulzadegan *et al.*, 1982; Land *et al.*, 1983; Ruley, 1983; for reviews, see Klein and Klein, 1985; Weinberg, 1985; Knudson, 1986). All the transforming oncogenes that we have tested stimulate the activity of PEA1, whereas the immortalizing oncogenes do not. This suggests that distinct targets and pathways could exist for immortalizing compared to transforming oncogenes, and that PEA1 mediates at least some of the events associated with transformation. It should be pointed out that the classification of SV40T antigen as an immortalizing oncogene stems from the observation that, by a number of criteria, SV40 is much less efficient than polyoma in transforming rat fibroblasts (Perbal and Rassoulzadegan, 1980), and that SV40T collaborates with *ras* in malignant transformation (Weinberg, 1985). However, we recently learnt that Piette *et al.*, (1988) have shown that in SV40-transformed NIH3T3 cells there is a high level of AP1 DNA binding activity which is serum independent, suggesting that SV40T antigen might be able to activate AP1 (PEA1) transcription stimulatory activity. We do not detect such an activity in either of our *in vivo* assays. However, we cannot exclude that cell-specific events lead to manifestations of different activities of this multifunctional protein (Weinberg, 1985).

Transformation leads to altered transcription, amongst other events (Weinberg, 1985). PEA1 may be one of several transcription factors whose activity is altered by transformation. Other potential candidates are the TPA-inducible transcription factors NFκB (Nabel and Baltimore, 1987), AP2 and AP3 (Chiu *et al.*, 1987) and DSE (Treisman, 1987; Prywes and Roeder, 1987; Greenberg *et al.*, 1987). PEA1 may control the transcription of a set of transformation-related genes. Indeed, several transformation-induced genes have PEA1 binding sites in their promoters. For example, the v-Ha-*ras*-induced VL30 gene promoter (Owen and Ostrowski, 1987) contains multiple PEA1 binding sites (Saragosti and Piette, personal communication). A transformation-induced gene, transin, which may have a role in metastasis, contains a consensus PEA1 binding site in its promoter (Matrisian *et al.*, 1985). The promoter of the IL2 growth hormone gene contains multiple AP1 binding sites (Fujita *et al.*, 1986). It would be interesting to study how the activity of these and other genes are regulated by transforming and immortalizing oncogenes, and to study the role of PEA1 in this regulation. An important question is, how critical is the regulation of PEA1 activity in transformation?

Vogt and his colleagues have isolated a new oncogene, v-*jun*, from an avian sarcoma virus (Maki *et al.*, 1987). v-*jun* has protein sequence homology to human AP1 (Bohmann *et al.*, 1987) and yeast GCN4 (Vogt *et al.*, 1987) transcription enhancer factors. v-*jun*, AP1, GCN4 and mouse PEA1 have a similar DNA binding specificity (Struhl, 1987; Bohmann *et al.*, 1987; Angel *et al.*, 1988; Piette and Yaniv, 1987; Imler *et al.*, 1988b). v-*jun*, like PEA1 and AP1, requires serum components for activity (Imler *et al.*, 1988b and unpublished results). Thus, v-*jun*, PEA1, AP1 and GCN4 are most probably related members of a multi-gene family of transcription enhancer factors. The observations that v-*jun* induces fibrosarcomas and transforms chicken embryo fibroblasts (Cavalieri *et al.*, 1985; Maki *et al.*, 1987) and our present study demonstrating that the activity of its cellular homologue is increased by the expression of at least six transforming oncogenes, suggests that altering expression of genes whose transcription is regulated by the level of PEA1 activity may have a critical and general role in transformation.

Materials and methods

Standard molecular biological techniques were used. Transfections and quantitative S1 nuclease mapping were as described previously (Wasylyk *et al.*, 1987; Imler *et al.*, 1988a). The transfections were repeated a minimum of three times with two DNA preparations, and the results quantitated by scanning densitometry. Representative results are presented in the figures. We have previously described the following recombinants that are illustrated in Figure 1: pG1 (lane 1), pG2, pG2Py (lane 2), pRCM, pRCBx2, pΔRCM, pΔRCBx2 (lane 3), pβCM, pβCBx2 (lane 4,11,13), CW12 (lane 9) pβCM (lane 11) (Wasylyk *et al.*, 1987; Imler *et al.*, 1988a). See, for pEIASV, Borrelli *et al.* (1984), for pSG5, Green *et al.* (1988), for pSVcmyc1, Land *et al.* (1983), for pSVLT and pSVMT Rassoulzadegan *et al.* (1982). pG1PB4 and pG1PAB4 (lane 1) contain four head-to-tail copies of the WT and MUT oligonucleotides in the *Xho*I site at −109 directly upstream from the β-globin promoter of pG1 (see Figure 1A,B). pvSRC5 (lane 5) contains the *Bam*HI−*Sal*I (site repaired with Klenow) RSV v-*src* fragment from pSP6VSRC (Piwnica-Worms *et al.*, 1987) in the *Bam*HI (one end repaired) site of pSG5. pTCM (lane 10) contains the Klenow repaired 1-kb *Xba*I mouse immunoglobulin heavy chain enhancer-containing fragment in the Klenow repaired *Bam*HI site upstream from the conalbumin promoter of pTCT (Wasylyk and Wasylyk, 1986). pvMCBx2 contains the *Hin*dIII (Klenow repaired)−*Eco*RI fragment containing the conalbumin−SV40 enhancer promoter fragment of pβCBx2 (lane 4) between the *Xba*I (Klenow repaired) and *Eco*RI sites of pVMTV-1 (van der Hoorn and Muller, 1985) in the place of the MMTV promoter. pcFOS5 contains the c-*fos* *Bam*HI−*Sal*I (Klenow repaired) fragment from p19/1 (Rüther *et al.*, 1985) in the *Bam*HI site of pSG5.

pEIANEO was constructed from pAG60 (Colbere-Garapin *et al.*, 1981) by replacing the thymidine kinase promoter with the EIA promoter from EIASV. The FR3T3-derived cell lines used here were: EJ c-Ha-*ras*-transformed FRRAS1 and FRRAS2 (Matrisian *et al.*, 1985); RSV-transformed FR-RSV and FR-RSV1 (Matrisian *et al.*, 1985); Py virus-

transformed Py-WT-A2 (Seif and Cuzin, 1977) and Py T21 (Rassoulzadegan *et al.*, 1982); Py-tsa virus-transformed Py tsa A1 (Seif and Cuzin, 1977); Py-MT-transformed MTT4 (Rassoulzadegan *et al.*, 1982); Py-LT-immortalized LT-1 (Glaichenhaus *et al.*, 1986); c-*myc*-immortalized FRcmyc20 (Mougneau *et al.*, 1984); v-*myc*-immortalized FRvmyc23 (Mougneau *et al.*, 1984); SV40-immortalized SV-WT-A1 (Rassoulzadegan *et al.*, 1978) and SV40 tsa 30-immortalized SV-TSA30-A1 (Perbal and Rassoulzadegan, 1980).

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