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Targeted disruption of the c-fos gene demonstrates c-fos-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes

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The c-fos proto-oncogene is believed to play a pivotal role in transducing growth factor-mediated signals from the extracellular milieu into the nucleus. c-fos protein dimerizes with c-jun and related proteins and mediates transcription via AP-1 sites. Using c-fos-deficient mice generated through gene knockout techniques, we derived 3T3-type cell lines from primary embryonic fibroblasts. The c-fos-deficient cells grow normally under optimal culture conditions and show only a slight reduction in growth rate in low serum culture compared with control cells. They also express mRNA for most of the Fos and Jun family members at normal levels. The overall levels of AP-1 DNA binding activity are normal and several genes (c-jun, MCP1, metallothionein) known to contain functional AP-1 sites are expressed normally in the c-fos-deficient and control cells. In contrast, mRNA for the metalloproteases stromelysin (MMP-3) and type I collagenase (MMP-1), which are often induced by oncogenes and growth factors and have been implicated in tumor invasiveness, cannot be induced by epidermal growth factor or platelet-derived growth factor in c-fos-deficient cells. Transformation of mutant cells with polyoma middle T oncogene essentially restores wild-type levels of stromelysin expression, while transformation with v-src leads to only a weak induction of the metalloprotease. These results clearly demonstrate that some AP-1-dependent genes require c-fos for full expression while others do not; oncogenes may activate expression of metalloproteases via either fos-dependent or fos-independent mechanisms. These results also imply that c-fos may play an important regulatory role in the invasive behavior of malignant tumors, independent of any role this proto-oncogene might play in cell growth *per se*.

Key words: AP-1 factors/c-fos/c-fos-deficient cells/metalloprotease/oncogenes

Introduction

c-fos is a nuclear proto-oncogene that has been implicated in many important cellular events, including cell proliferation (Holt *et al.*, 1986; Nishikura and Murray, 1987; Riabowol *et al.*, 1988), differentiation (Distel *et al.*, 1987; Lord *et al.*, 1993) and tumorigenesis (Curran *et al.*, 1983; Miller *et al.*, 1984; Jenuwein *et al.*, 1985; Ruther *et al.*, 1989). c-fos encodes a 62 kDa protein that has been shown to form heterodimers with another proto-oncogene, c-jun, and the resulting complex binds DNA at AP-1 sites and stimulates transcription through these elements (Chiu *et al.*, 1988; Ransone and Verma, 1990). AP-1 sites have a consensus core sequence of TGACTCA. This sequence motif is also referred to as the TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element (TRE) since it has been found in many TPA responsive genes. A very large and diverse group of genes have been found to contain AP-1 sites in their regulatory regions, including c-jun (Angel *et al.*, 1988), MCP-1 (Rollins *et al.*, 1988), the metalloprotease genes stromelysin and type I collagenase (Kerr *et al.*, 1988; Schonthal *et al.*, 1988), metallothioneins (Lee *et al.*, 1987), adipocyte P2 (Distel *et al.*, 1987), proopiomelanocortin (Boutillier *et al.*, 1991) and interleukin 2 (Farrar *et al.*, 1989).

The Fos gene family now includes c-fos, fos B, fra-1 and fra-2. A variety of stimuli, including serum (Greenberg and Ziff, 1984), growth factors (Cochran *et al.*, 1984), tumor promoters (Angel *et al.*, 1987), cytokines (D.A. Brenner *et al.*, 1989) and UV radiation (Buscher *et al.*, 1988), induce their expression. The kinetics of the mRNA induction vary from 15 min to 2 h, with c-fos mRNA and protein generally being among the first to be expressed. The Fos family as a group can heterodimerize with members of the Jun family and result in Fos–Jun dimers with an enhanced affinity for DNA target sites relative to Jun–Jun homodimers. It has been suggested that different Fos–Jun complexes have different affinities for AP-1 sites with slightly different sequences around the core sequence (Ryseck *et al.*, 1991). Such complex protein–protein interactions may be a potential regulatory step in controlling complex patterns of gene expression (Falvey and Schibler, 1991).

c-fos and c-jun can also repress gene expression. Sassone *et al.* (1988) demonstrated that c-fos can inhibit its own promoter, as well as other early response genes such as Egr-1 (Gius *et al.*, 1990) and c-myc (Hay *et al.*, 1989). Recently, MHC class I and PEPCK were also shown to be negatively regulated by AP-1 factors (Gurney *et al.*, 1992; Howcroft *et al.*, 1993). The domain of c-fos responsible for gene repression is likely to be different from that responsible for gene activation (Gius *et al.*, 1990).

In many of the previous investigations, the importance of c-fos in a biological context was inferred from experiments that attempted to eliminate endogenous c-fos by

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using (i) anti-sense mRNA expression (Holt *et al.*, 1986; Nishikura and Murray, 1987), (ii) anti-c-*fos* antibodies (Adamson *et al.*, 1985; Riabowol *et al.*, 1988), (iii) a ribozyme that cleaves c-fos mRNA (Scanlon *et al.*, 1991), or (iv) a dominant negative mutant of c-fos (Okuno *et al.*, 1991). Our laboratory has utilized embryonic stem (ES) cell-mediated gene-knockout techniques to generate mice deficient in c-fos (Johnson *et al.*, 1992). The c-fos mutant mice are viable but display a range of tissue-specific developmental defects, including osteopetrosis, delayed gametogenesis, lymphopenia and behavioral abnormalities. In contrast, the loss of the c-jun allele leads to embryonic lethality and severe retardation of embryonic fibroblast cell growth (Johnson *et al.*, 1993; Hilberg *et al.*, 1993). To investigate further the functions of c-fos at the cellular and molecular levels, we have now generated several lines of fibroblasts from the fos-less and control mice and have examined a number of cellular and molecular properties. Our data indicate that c-fos is not required for cell proliferation and c-fos-deficient cells express most members of the fos and jun protein family at normal levels. However, the expression of two AP-1 site-containing genes, stromelysin and type I collagenase, is found to be abnormally low in growth factor-stimulated mutant cells. Transformation by certain oncogenes can restore stromelysin and collagenase expression, while others cannot. Taken together, these results demonstrated the existence of c-fos-dependent and -independent pathways for growth factor- and oncogene-regulated gene expression.

Results

c-fos-deficient cells retain the ability to proliferate in vitro

Many studies (Angel and Karin, 1991) have suggested that c-fos is required in cell cycle progression and cell proliferation. We decided to re-examine this issue using a c-fos-deficient cell line generated from mice carrying a null mutation at the c-fos locus. Immortal cell lines were established according to the standard 3T3 protocol (Todaro *et al.*, 1963) using embryonic fibroblasts derived from wild-type (+/+) or mutant (–/–) mice (see Materials and methods). We examined the ability of these cells to proliferate under normal or suboptimal serum conditions over a 4 day period (Figure 1). Under low serum concentration (0.1–1.0%), c-fos-deficient cells showed a slight retardation in growth rate relative to cells from control mice. In 5 or 10% fetal calf serum, wild-type or c-fos-deficient cells proliferate equally well. In addition to this work in immortalized cell lines, no significant differences were observed in growth rates of primary fibroblast cultures for the c-fos-deficient versus control cells (R.Johnson, E.Hu and B.M.Spiegelman, unpublished observations). These results, coupled with the fact that c-fos-deficient mice showed only a few specific developmental defects (Johnson *et al.*, 1992; Wang *et al.*, 1992), argue strongly that the c-fos gene is not required for the growth of most cells. These data do not rule out the possibility that growth may be dependent on the presence of c-fos under conditions that have not yet been explored.

Expression of the AP-1 gene family in c-fos-deficient cells

To investigate whether the loss of c-fos resulted in any compensatory alterations in the expression of mRNA for

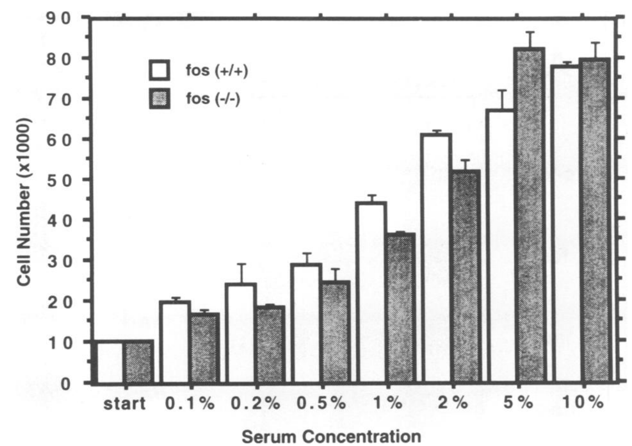

Fig. 1. Growth rate of c-fos-deficient (–/–) and wild-type (+/+) cells. c-fos-minus (–/–) and control (+/+) cell lines were derived, as described in Materials and methods, using a standard 3T3 protocol. Cell growth was measured by seeding 10 000 cells per 35 mm culture dish and feeding with media containing different serum concentrations every other day. Cells were then trypsinized and counted with a Coulter counter after 4 days. The experiments were repeated three times in both c-fos-deficient cell lines. Average cell number and standard errors were calculated.

other AP-1 factors, we treated c-fos-deficient 3T3 cells with epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or TPA, respectively. Cells were serum starved for 24 h, and after stimulation with the various agents, RNA was isolated at the indicated time points for Northern blot analysis. c-fos mRNA was completely absent in the cells containing the disrupted c-fos gene (Figure 2), as described previously for primary cell cultures (Johnson *et al.*, 1992). We observed a largely normal expression pattern for most of the mRNAs encoding known AP-1 factors, including fra-1, fra-2, c-jun, jun-B and jun-D (Figure 2). In both wild-type and c-fos-deficient cells, induction of c-jun was acute and reached a maximum level at ~1 h. The induction kinetics of fra-1, fra-2 and jun-B were slower, consistent with the published results for fibroblasts (Angel and Karin, 1991). jun-D expression underwent only a slight induction in both cell lines. fra-1 mRNA expression was sometimes observed to be slightly reduced in the c-fos-deficient cells, such as in Figure 2. However, this observation was not always consistent and most likely represents experimental variation. One consistent difference observed between wild-type and c-fos-deficient cells was that the quantitative level of fos-B in c-fos-deficient cells seemed to be reduced in all three treatments (PDGF, EGF or TPA) by ~80% (Figure 2).

AP-1 DNA binding activity is normal in c-fos-deficient cells

To assess whether the loss of c-fos affects total AP-1 binding activity in c-fos-deficient cells, gel electrophoresis mobility shift assays (EMSA) were performed using nuclear extracts from wild-type and fos-minus cells with or without growth factor stimulation (see Materials and methods). AP-1 oligonucleotides were derived from the AP-1 site of the stromelysin promoter (–70 to –84 bp). In unstimulated cells, AP-1 DNA binding activity in c-fos-deficient cells was indistinguishable from that of wild-type cells, qualitatively and quantitatively (Figure 3).

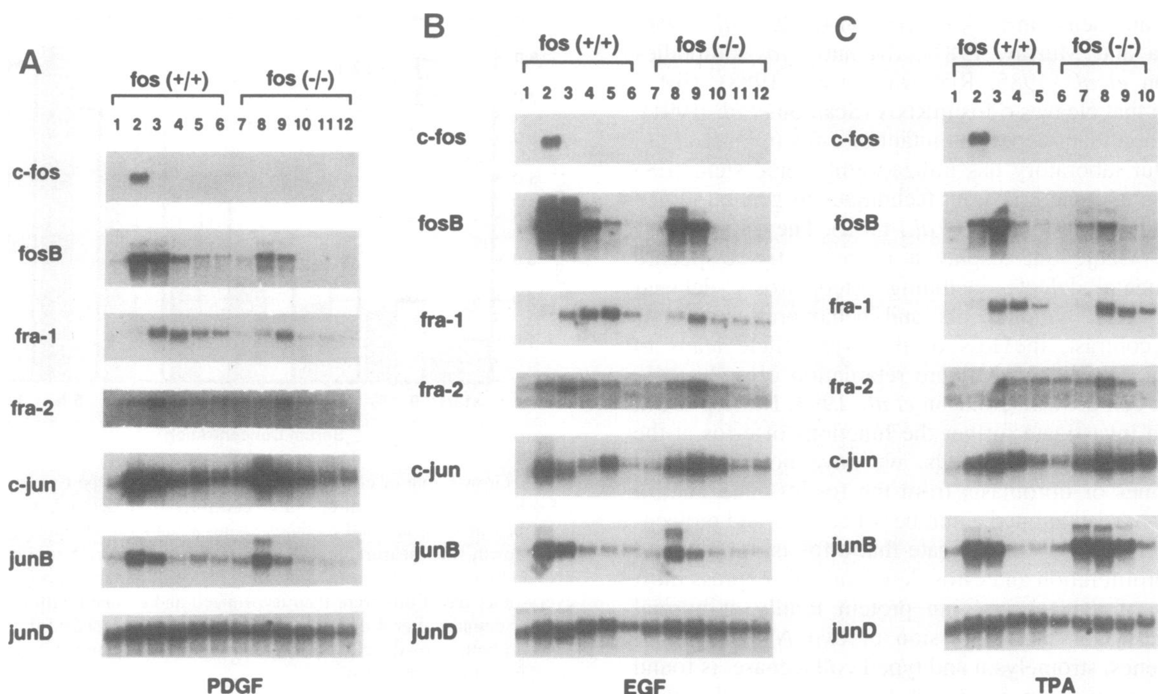

Fig. 2. Expression of AP-1 mRNAs in wild-type and c-fos-deficient cells. Confluent c-fos-deficient and control cells were serum starved for 24 h. PDGF, EGF or TPA was then added to the cell culture medium to a final concentration of 50, 20 or 100 ng/ml, respectively. At various time points, total cellular RNA was isolated and Northern blot analysis was performed. cDNA probes were sequentially hybridized to the Nylon membrane (ICN Corp.). (**A**) Northern blots for PDGF stimulation. Lanes 1–6 correspond to wild-type cells stimulated for 0, 0.5, 1, 2, 4 and 8 h, respectively. Lanes 7–12 represent c-fos-deficient cells stimulated for the same times. (**B**) Northern blots after EGF stimulation. Lanes 1–12 are designated in the same fashion as in (A). (**C**) Northern blots after TPA stimulation. Lanes 1–5 indicate RNAs from wild-type cells stimulated for 0, 0.5, 1, 4 and 8 h and lanes 6–10 are RNAs from c-fos-deficient cells stimulated for the same time points. 10 μg of total RNA were loaded in each lane in all cases. Ribosomal associated protein (RAP; Laborda, 1991) serves as a control for RNA loading (data not shown). The entire experiment was repeated twice for each of the two c-fos-deficient cell lines and controls, and a typical set of Northern blots is shown.

Competitions using an unlabeled identical probe (TRE) totally abolished binding (Figure 3A, lanes 3 and 8), while a mutant stromelysin AP-1 oligonucleotide (mTRE) did not affect binding (Figure 3A, lanes 5 and 10), indicating that DNA binding activity is specific for the AP-1 site. Competition with another AP-1 oligonucleotide from the adipocyte P2 gene (FSE2; Distel *et al.*, 1987) promoter also completely abolished binding (Figure 3A, lanes 4 and 9). Even in the absence of c-fos in mutant cells, the binding activity is likely to contain fos-related antigens (Fras), since a polyclonal antibody against the DNA binding domain of c-fos (which recognizes c-fos and fos-related proteins) can disrupt binding (Figure 3B, lanes 2, 6, 10 and 14). Preimmune serum caused no disruption of binding (Figure 3B, lanes 4, 8, 12 and 16). Nuclear extracts were also prepared from EGF- or TPA-stimulated cells and no apparent differences were observed between wild-type and c-fos-deficient fibroblasts in identical gel retardation assays (Figure 3B). In data not shown, PDGF-stimulated cells also showed no difference in total AP-1 binding activity.

Expression of AP-1-regulated genes: MCP-1, metallothioneins and metalloproteases

A large number of genes have been shown to have functional AP-1 sites in their regulatory regions. Typical examples of AP-1 site-containing genes include adipocyte P2 (Distel *et al.*, 1987), the cytokine MCP-1 (Rollins *et al.*, 1988), metallothionein (Lee *et al.*, 1987), type I collagenase (Schonthal *et al.*, 1988) and stromelysin (Kerr *et al.*, 1988). AP-1 sites have been suggested to play important roles in regulating basal, as well as hormonally induced, gene expression (Angel and Karin, 1991). Figure 2 suggested that at least one gene with a functional AP-1 site (Angel *et al.*, 1988), c-jun, is expressed at relatively normal levels. However, it can be argued that an immediate early response gene such as c-jun may not require c-fos for normal expression, since it has been documented that transcription is not necessary for this gene induction. Therefore, it is of particular interest to examine both the expression of genes that are not members of the AP-1 family and those that respond later to growth factors and other agents.

We first examined the MCP-1 gene. MCP-1 was originally identified as an immediate early PDGF responsive gene, called JE in fibroblasts (Cochran *et al.*, 1983; Rollins *et al.*, 1988). PDGF induces MCP-1 mRNA several-fold and the maximal mRNA accumulation is achieved ~2–3 h after growth factor administration. AP-1 sites have been identified in the promoter region of MCP-1 gene and these sites have been suggested to play an important role in controlling basal level gene expression (Timmers *et al.*, 1990). As shown in Figure 4, MCP-1 mRNA expression was identical for both cell types in the basal state and after PDGF stimulation.

We then studied the expression of metallothioneins in c-fos-deficient and control cells. Metallothioneins are known to be inducible by agents such as glucocorticoid and heavy metals. The AP-1 site from metallothioneins has been studied extensively and shown to be important

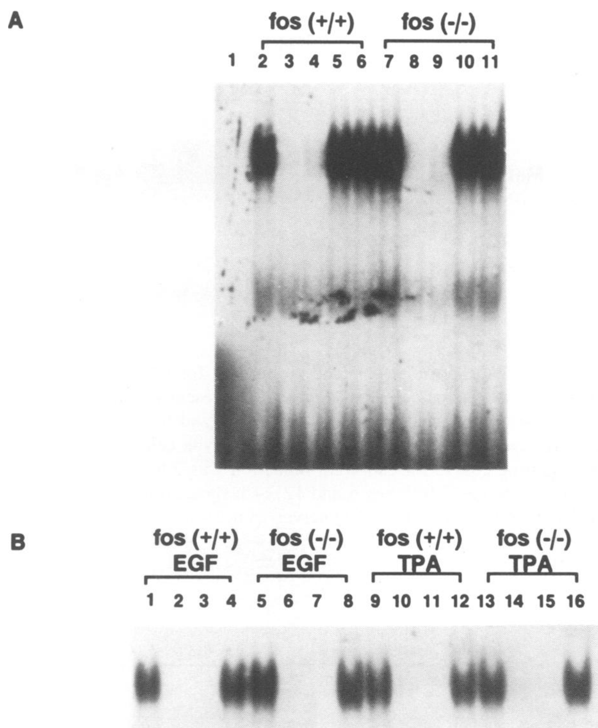

Fig. 3. AP-1 DNA binding activity is not altered in c-fos-deficient cells. An electrophoretic mobility shift assay (EMSA) was performed as described in the text (Materials and methods) using extracts from either wild-type or c-fos-deficient cells. An equal amount of protein (5 µg) was used in each binding reaction. The labeled probe (TRE) contained the AP-1 site from the stromelysin gene (see Materials and methods). In (**A**), protein extracts from unstimulated wild-type (lanes 2–6) or c-fos-deficient cells (lanes 7–11) were used for EMSA. Competitions were carried out using unlabeled probe itself (stromelysin TRE) (lanes 3 and 8), the AP-1 site from the adipocyte P2 gene (FSE 2) (lanes 4 and 9), a mutant stromelysin TRE oligonucleotide (lanes 5 and 10) or an unrelated oligonucleotide (c-ets binding site) (lanes 6 and 11). Lane 1 in (A) is a control in which no protein extract was added. In (**B**), specific antibodies were used to disrupt the formation of DNA–protein complexes. Protein extracts from both types of cell stimulated with EGF or TPA were used. Cells were first serum starved for 24 h before stimulation with EGF or TPA, as described previously (legend to Figure 2). Two hours later, protein extracts were prepared from both types of cell for EMSA. The sources of extracts are listed above the lanes in (B). Anti-c-fos serum or a control preimmune serum were used to disrupt the formation of DNA–protein complexes. Lanes 2, 6, 10 and 14 were antibody disruption experiments using 0.2 µl of anti-fos antiserum. Lanes 3, 7, 11 and 15 were the same experiments using 1 µl of anti-fos antibody. Lanes 4, 8, 12 and 16 were EMSA using 1 µl of preimmune serum. Film exposure time was ~4 h.

in promoter function (Hamer, 1986). Both wild-type and c-fos-deficient cells were stimulated with 1 µM of dexamethasone or 50 µM of cadmium ($CdCl_2$), respectively. mRNAs for both metallothioneins I and II were expressed in response to dexamethasone or cadmium treatment with maximum levels reached at 2–4 h after induction (Figure 5). No differences in the kinetics or magnitude of induction were observed between wild-type and c-fos-deficient cells. c-fos was induced in wild-type cells by cadmium but not dexamethasone (data not shown).

Stromelysin and type I collagenase are AP-1-dependent genes induced by growth factors in fibroblastic cells. Figure 6A and B show that basal expression of mRNA for these proteases is observed only in the wild-type cells but not in c-fos-deficient cells. Furthermore, both stromelysin and collagenase mRNAs were stimulated by PDGF, EGF and TPA in the wild-type cells. mRNAs for the metalloprotease genes were markedly elevated 8 h after stimulation (Figure 6). In c-fos-deficient cells, however, EGF- and PDGF-stimulated expression of stromelysin and collagenase was completely abrogated (Figure 6, lanes 7–12). TPA, on the other hand, still elicited some response. While the time course of TPA induction was similar to that in wild-type cells (Figure 6, lanes 5 and 10), the magnitude was reduced by ~50%. The total loss of basal as well as PDGF- and EGF-stimulated metalloprotease expression suggests a pivotal and highly specific role for c-fos in the regulation of these genes.

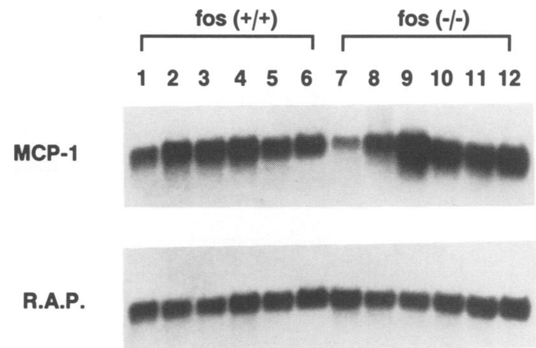

Fig. 4. PDGF stimulates the expression of MCP-1 mRNA in c-fos-deficient cells. c-fos-deficient and wild-type control cells were serum starved for 24 h and stimulated with PDGF as described in the legend to Figure 2. Subsequently, RNA was isolated for Northern blot analysis. Lanes 1–6 correspond to RNA isolated from wild-type control cells at 0, 0.5, 1, 2, 4 and 8 h after PDGF stimulation. Lanes 7–12 correspond to RNA isolated from c-fos-deficient cells at the same time points of PDGF stimulation. Ribosomal associated protein (RAP; Laborda, 1991) serves as a control for RNA loading. 10 µg of total RNA was loaded in each lane. Northern analysis was repeated for both c-fos-deficient cell lines and controls, and a typical Northern blot is shown.

Expression of exogenous c-fos restores basal stromelysin expression and response to PDGF

If the loss of c-fos is responsible for the observed reductions in stromelysin and collagenase expression, then forced expression of c-fos from a transfected vector should correct most if not all of these alterations. We utilized two vectors to achieve exogenous c-fos expression: PLJ-fos and PMV-fos-ER (Superti *et al.*, 1991). In PLJ-fos, constitutive c-fos expression is driven by a viral LTR promoter (Druker *et al.*, 1990). In PMV-fos-ER, a fos–estrogen receptor fusion protein is placed under the control of a viral LTR. The hormone binding region of the estrogen receptor contains a ligand-dependent transactivation (Webster *et al.*, 1988) and dimerization (Kumar and Chambon, 1988) domain. In the absence of hormones, these domains also inhibit DNA binding and dimerization activities via interaction with an abundant heat shock protein, Hsp90 (Superti *et al.*, 1991). The DNA binding and transactivation activity of the fusion protein is inducible by the addition of 17-β-estradiol (E2). Similar constructs have been shown to be active in gene regulation and transformation assays (Superti *et al.*, 1991). These DNA constructs were transfected in the cell and neomycin-resistant clones were selected by G418 resistance. Several

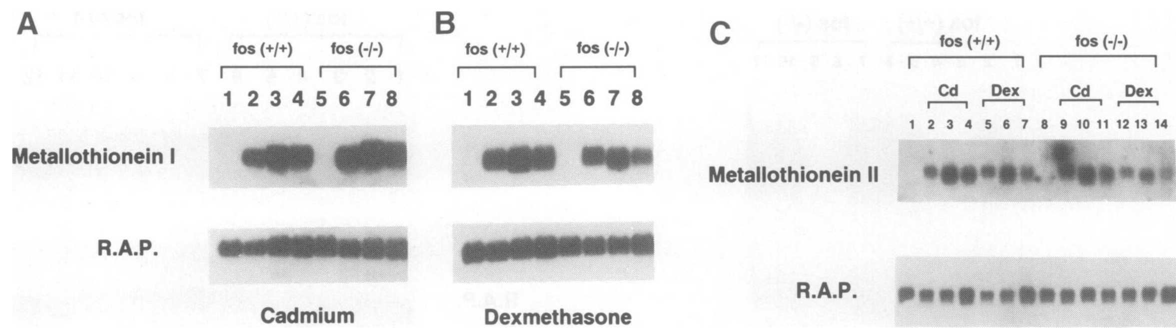

Fig. 5. Metallothionein mRNA expression in wild-type and c-fos-deficient cells. (**A** and **B**) Northern blot of metallothionein I for RNA isolated from cadmium- ($CdCl_2$) or dexamethasone-treated cells. Lanes 1–4 indicate RNAs isolated from wild-type cells and lanes 5–8 represent RNAs prepared from a c-fos-deficient cell line. Time courses for cadmium or dexamethasone inductions are 0 h (lanes 1 and 5), 2 h (lanes 2 and 6), 4 h (lanes 3 and 7) or 8 h (lanes 4 and 8) for both (A) and (B). (**C**) Northern blot for metallothionein II. Lanes 1–7 are for RNAs from wild-type cells. Lanes 8–14 are for RNA from a c-fos-deficient cell line. Uninduced controls are lanes 1 and 8. The time course for cadmium induction was 2 h (lanes 2 and 9), 4 h (lanes 3 and 10) and 8 h (lanes 4 and 11). The time course for dexamethasone induction was 2 h (lanes 5 and 12), 4 h (lanes 6 and 13) and 8 h (lanes 7 and 14). 10 μg of RNA was loaded for each lane. Ribosomal associated protein (RAP; Laborda, 1991) serves as a control for RNA loading.

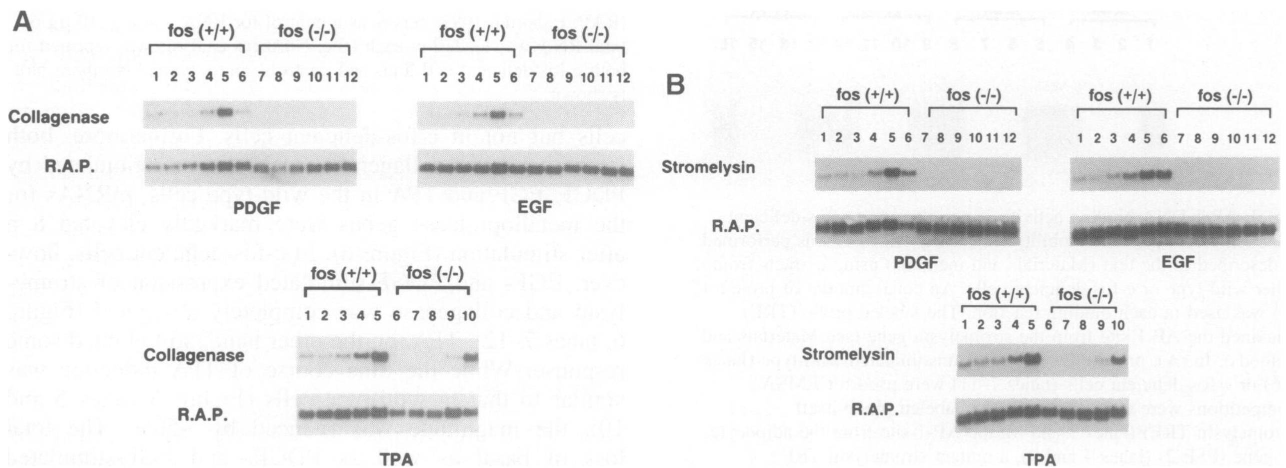

Fig. 6. Expression of the metalloproteases stromelysin and type I collagenase in wild-type and c-fos-deficient cells. (**A** and **B**) Northern blots probed for type I collagenase and stromelysin, respectively. Wild-type or c-fos-deficient cells were serum starved for 24 h before being stimulated with PDGF, EGF or TPA. Lanes 1–6 (PDGF and EGF treatment) and lanes 1–5 (TPA treatment) are RNAs from wild-type cells; lanes 7–12 (PDGF and EGF treatment) and lanes 6–10 (TPA treatment) are RNAs from c-fos-deficient cells. For PDGF and EGF treatments, RNAs were isolated at 0 h (lanes 1 and 7), 0.5 h (lanes 2 and 8), 1 h (lanes 3 and 9), 2 h (lanes 4 and 10), 4 h (lanes 5 and 11) and 8 h (lanes 6 and 12) after stimulation. In TPA treatment, time courses of induction are 0 h (lanes 1 and 6), 0.5 h (lanes 2 and 7), 2 h (lanes 3 and 8), 4 h (lanes 4 and 9) and 8 h (lanes 4 and 10). RAP was used as loading controls and ~10 μg of total RNA were loaded for each lane.

cell lines that expressed exogenous c-fos were then examined for stromelysin and type I collagenase expression. As shown in Figure 7, clones that constitutively expressed exogenous c-fos also expressed high levels of stromelysin. Moreover, β-estradiol can induce stromelysin expression several-fold from clones transfected with PMV-fos-ER constructs (Figure 7, lanes 5 and 6). These data argue strongly that the deficiency in c-fos is responsible for the reduced stromelysin and collagenase expression in the c-fos-deficient fibroblast cell lines.

Since c-fos is induced by growth factors and these same factors also induce metalloprotease expression, it is possible that the growth factor responsiveness of stromelysin and collagenase is primarily due to the induction of c-fos. We asked whether PDGF could induce the metalloprotease expression when c-fos was constitutively expressed. Surprisingly, PDGF can still induce the stromelysin expression in cells stably transfected with the c-fos expression vector (Figure 7, lanes 3 and 4). These data indicate that c-fos is required for PDGF induction of stromelysin; however, the ability of PDGF to induce stromelysin in the presence of constitutively expressed c-fos could be due to either the simultaneous induction of additional transcriptional regulators or the modification of c-fos and other regulatory molecules.

Oncogenic transformation in c-fos-deficient cells

Deregulated c-fos expression results in tumorigenesis in animals (Ruther *et al.*, 1987) and transformation in cell culture (Miller *et al.*, 1984; Jenuwein *et al.*, 1985). In addition, several cytoplasmic oncogenes, such as Ha-ras, v-src, v-raf and polyoma middle T, have been suggested to act on AP-1 pathways (Angel and Karin, 1991) and may exert at least part of their transforming activities through AP-1 proteins. It is therefore important to ask whether cells lacking c-fos can be transformed efficiently by these oncogenes.

We used a standard focus-formation assay to assess the ability of various oncogenes to transform c-fos-deficient cells. Activated Ha-ras, c-jun, v-jun, v-raf, v-src, polyoma

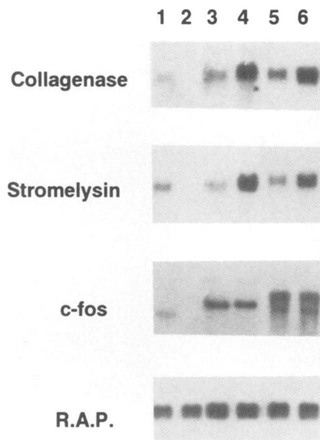

Fig. 7. Expression of exogenous c-fos restores stromelysin and type I collagenase expression in c-fos-deficient cells. c-fos expression vectors PLJ-fos and PMV-fos-ER were transfected into c-fos-deficient cells and G418-resistant clones were selected. One clone from PLJ-fos (lanes 3 and 4) and one clone from PMV-fos-ER (lanes 5 and 6) were analyzed for exogenous c-fos, stromelysin and collagenase mRNA expression. The PLJ clone was further analyzed for the ability of PDGF to induce metalloprotease expression while PMV-fos-ER clone was analyzed for β-estradiol-induced metalloprotease expression. Cells were serum starved for 24 h before PDGF or β-estradiol induction. RNA was isolated before or 8 h after stimulation and subjected to Northern blot analysis. Lanes 1 and 2 are RNAs from the parent untransfected wild-type and c-fos-deficient cells, respectively. Lanes 3 and 4 are the PLJ clone that expresses exogenous c-fos without (lane 3) or with (lane 4) PDGF stimulation. Lanes 5 and 6 are PMV-fos-ER clone that expresses exogenous c-fos without (lane 5) or with β-estradiol stimulation (lane 6).

middle T antigen and SV40 large T antigen were used in a transfection experiment. Virus derived from polyoma middle T (PLJ-MT) and v-src retroviral vector (PLJ-v-src) (see Materials and methods) were used in infection experiments. In both methods, transformed foci were visible 2–3 weeks after transfection or infection and ~30–60 foci were counted for each 100 mm culture dish. DNA vector alone (in the transfection experiment) or Y2 supernatant without retroviral vector (in infection experiments) did not yield any visible foci after methylene blue stain. Of the oncogenes tested (Table I), no significant differences in the number and size of foci were observed between wild-type and c-fos-deficient cells. The morphologies of transformed foci were also very similar (Figure 8). We conclude, therefore, that the formation of transformed foci by v-jun, Ha-ras, SV40 large T, polyoma middle T, v-src and v-raf are not dependent on c-fos expression.

Metalloprotease expression is elevated in a number of cancers and their synthesis and secretion are thought to be an important component of the invasive and metastatic ability of tumors (Liotta and Stetler, 1990; Matrisian and Bowden, 1990; McDonnell and Matrisian, 1990). In addition, these molecules have been shown to be elevated by certain oncogenes and their expression is dependent upon functional AP-1 sites in their promoters (Matrisian *et al.*, 1986; Kerr *et al.*, 1988). We thus examined stromelysin expression in cell lines that were transformed with v-src or polyoma middle T antigen, since it has been shown that these two oncogenes elevate stromelysin gene expression (Matrisian *et al.*, 1986). Stromelysin was in fact originally isolated based on its enhanced expression

Table I. Oncogene transformation of c-fos-deficient and wild-type cells

Oncogenes	Number of foci/100 mm dish	
	Fos (+/+)	Fos (–/–)
Vector(plen)	0	0
v-src	95 ± 10	90 ± 12
Polyoma middle T	84 ± 13	91 ± 9
Ha-ras	52 ± 6	45 ± 11
v-jun	40 ± 9	48 ± 14
v-raf	68 ± 13	60 ± 17
SV40 large T	72 ± 15	80 ± 12

A standard focus formation assay was used to assess the ability of various oncogenes to transform the c-fos-deficient and wild-type cells. Foci were observed in both types of cell transformed with the oncogenes listed. Vector DNA did not produce visible foci in identical assays. The number of foci per 100 mm dish was counted after methylene blue staining. The experiments were repeated four times and average foci number and the standard errors (±) are listed.

in a polyoma middle T antigen transformed cell line (Matrisian *et al.*, 1985). As shown in Figure 9, stromelysin expression was enhanced by both v-src and polyoma middle T, but to markedly different degrees. Compared with wild-type cells (Figure 9, lane 2), polyoma middle T elevated stromelysin to similar levels, but v-src only slightly stimulated stromelysin expression in c-fos-deficient cells (Figure 9). These data indicate that there must be fos-dependent and fos-independent pathways by which oncogenes activate stromelysin expression.

Discussion

c-fos was among the first nuclear proto-oncogenes shown to participate in the control of gene transcription (Distel *et al.*, 1987; Rauscher *et al.*, 1988). Shortly thereafter it became clear that the c-fos family (Curran and Franza, 1988) was functionally related to the Jun gene family. These multiple factors bind as dimers to DNA involving the core sequence TGACTCA, commonly called an AP-1 site. If homodimers of the Jun family and heterodimers between the Jun and Fos families all bind to similar target sequences, it is important to determine if there is any functional significance to the apparent redundancy of these factors.

Hints that various types of biological specificity reside in the AP-1 factors come from several sources. First, the binding specificities of different complexes within these families are not absolutely identical. For example, *in vitro*-translated c-Jun and related proteins exhibit significant differences in their binding affinities to oligonucleotides that contain an identical TGACTCA core but with different flanking sequences (Ryseck and Bravo, 1991). In this *in vitro* DNA binding system, c-fos, fos-B and fra-1 can greatly enhance the binding affinity of Jun proteins, but the different degrees of enhancement depend on the nature of the Fos–Jun interaction. On the other hand, some members of the Jun family, notably Jun-B, can inhibit the AP-1 binding activity of c-Jun.

Secondly, Bravo and colleagues (Kovary and Bravo, 1991a) have documented that *in vivo*, different Fos–Jun complexes are clearly present in different stages of the cell cycle in synchronized Swiss 3T3 fibroblasts. c-jun,

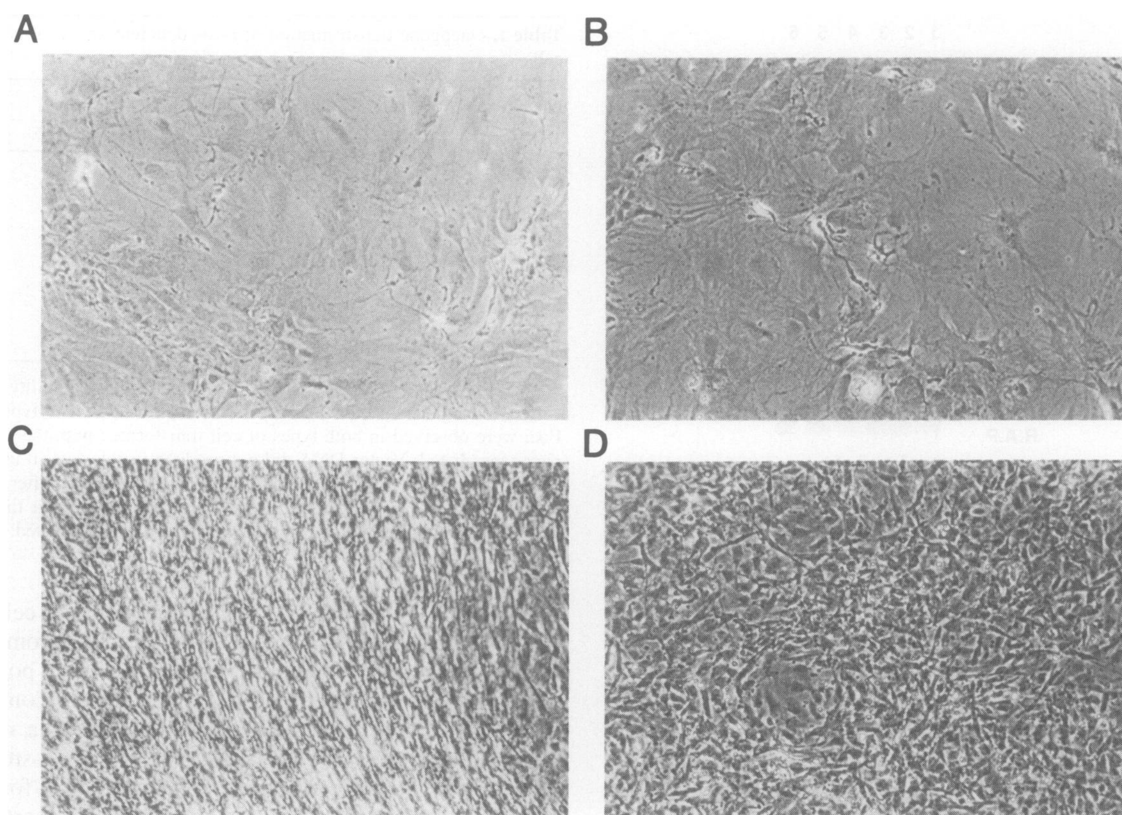

Fig. 8. Morphology of v-src-transformed c-fos-deficient cells. c-fos-deficient and wild-type control cells were transfected with expression vector v-src (PLJ-v-src). Cells were split 1:3 after transfection, and maintained in 5% FCS at 37°C for 2–4 weeks. A typical v-src-transformed focus from c-fos-deficient and control cells is shown. (**A** and **B**) Untransformed wild-type and c-fos-deficient cells. (**C** and **D**) v-src-transformed wild-type and c-fos-deficient cells, respectively.

for example, dimerizes with itself and jun-B with different kinetics and stability after serum induction. fra-1, on the other hand, can complex with all the members of the Jun family after stimulation. In addition, injection of fos- and jun-specific antibodies into synchronized fibroblasts results in different degrees of inhibition of DNA synthesis (Kovary and Bravo, 1991b). Recently, Boise *et al.* (1993) demonstrated that fra-1 and jun-B may be responsible for the DNA binding activity to an AP-1 sequence from an IL-2 gene in activated T lymphocytes. These results suggested the presence of functionally distinct Fos–Jun complexes *in vivo*.

Finally, although c-fos is induced in a wide variety of cell types by a large assortment of inducers, the experimental deficiency of c-fos in mice causes a striking but limited range of defects, including osteopetrosis, mild lymphopenia, delayed or deficient gametogenesis and behavioral abnormalities (Johnson *et al.*, 1992; Wang *et al.*, 1992). This implies that the functions of c-fos that can be described genetically are a limited subset of those that have been ascribed to AP-1 activity.

This latter point is shown fairly conclusively in the current data. The deficiency in c-fos does not significantly affect fibroblast growth at normal serum concentrations and has only a small effect at suboptimal growth conditions. This is in contrast to many studies using a variety of less definitive techniques that have implied that AP-1 factors generally, or c-fos specifically, are required for fibroblast growth. The c-fos-deficient fibroblasts have normal AP-1 DNA binding activity both quantitatively

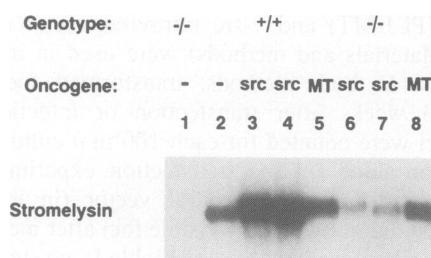

Fig. 9. Oncogenes can stimulate stromelysin expression in c-fos-deficient cells. Viral expression vectors for v-src and polyoma middle T antigen were used to infect both wild-type and c-fos-deficient cells. G418-resistant clones were selected and analyzed for metalloprotease expression. RNAs were isolated from wild-type (lanes 2–5) and c-fos-deficient cells (lanes 1 and 6–8). Two clones corresponding to v-src-transformed cells (lanes 3, 4, 6 and 7) and one clone corresponding to middle T transformed cells (lanes 5 and 8) of each genotype were analyzed. Lanes 1 and 2 are parent uninfected wild-type and c-fos-deficient cells, respectively. 10 μg of RNA were loaded in each lane.

and qualitatively. This is true when protein is isolated from quiescent cells or those that have been stimulated by growth factors. Similarly, genes whose full expression is dependent on functional AP-1 sites, such as metallothioneins or the growth factor-inducible MCP-1, are expressed quite normally. In striking contrast, we show that expression of the metalloprotease genes stromelysin and type I collagenase is severely affected by c-fos deficiency. The greatly reduced expression of these mRNAs is observed in quiescent cells or when PDGF or EGF are used as inducers. Some expression can be restored

when TPA is used, although there is still a quantitative reduction. That this aberration in the expression of these proteases really is a consequence of the absence of c-fos rather than some other abnormality related to the establishment of these cell lines is demonstrated by the restoration of metalloprotease mRNAs upon introduction of exogenously expressed c-fos into these cells.

From these data it could be argued that the metalloproteases are growth factor-inducible because they depend upon c-fos, a growth factor-responsive transcription factor. The situation is shown to be more complicated, however, because the protease mRNAs remain growth factor-inducible even when c-fos is expressed from a constitutive promoter. Therefore, the growth factor responsiveness of these genes is likely to derive from the ability of PDGF and EGF to induce other required factors, in addition to the requirement for c-fos. The ability of TPA to partially restore normal metalloprotease expression clearly shows that c-fos-independent pathways leading to the activation of these genes exist. Since TPA is known to activate c-jun at the transcriptional level and at the level of increasing the affinity of this protein for DNA (Lamph *et al.*, 1988; Binetruy *et al.*, 1991), c-jun may be important in this partial compensation via the formation of homodimers or through complexes with other Fos-related proteins.

Taken together, these data argue strongly for a specific transcriptional role for c-fos in activating certain genes, independent of the overall levels of AP-1 factors. This apparent functional distinction is reminiscent of the distinctions observed in transformation assays between c-fos and c-jun versus GCN4, the yeast AP-1 factor, despite the fact that all of these factors were very effective in stimulating transcription through multimerized AP-1 sites in mammalian cells (Oliviero *et al.*, 1992). A c-fos-specific effect in stimulating stromelysin or collagenase expression could, in theory, be the result of a subtle distinction between the AP-1 sites in these genes and many other AP-1 responsive genes. However, several pieces of data argue against this. First, the gel retardation assay (Figure 3) showed that DNA binding activity in nuclear extracts from fos-less cells interacts very well with the stromelysin TRE. Secondly, Ryseck *et al.* (Ryseck and Bravo, 1991) showed that AP-1 oligonucleotide, derived from stromelysin, collagenase and aP2 genes, exhibits similar affinities for several *in vitro*-translated Fos–Jun complexes including c-fos–c-jun, c-fos–jun-B and c-fos–jun-D. It is therefore more likely that the requirement for c-fos reflects an interaction with another protein factor, especially with the basal transcriptional machinery. Current evidence suggests that upstream transcriptional activators work, at least in part, through contacts with proteins interacting with the TATA binding protein (TBP; Hoey *et al.*, 1993). There is also evidence that this basal transcriptional machinery is not identical for all genes (Roeder, 1991). c-fos may be much more efficient in interacting with TBP or TAFs (TBP-associated factors; Dynlacht *et al.*, 1991) in the metalloprotease promoters than are the other AP-1 factors.

It is worth noting that Kerr *et al.* (1988) and later others (Buttice *et al.*, 1991) demonstrated a role for c-fos in the induction of stromelysin (transin) by growth factors or tumor promoters. These authors used antisense expression vectors to show that c-fos induction was required for metalloprotease expression. In these studies, the distinction between an effect on c-fos *per se* versus a reduction in overall AP-1 activity by suppression of c-fos was not considered, probably because the complexity of the Fos and Jun families was not fully appreciated at this time. In addition, Kerr *et al.* (1988) observed a dramatic reduction in PDGF and TPA responsiveness of the stromelysin promoter when endogenous c-fos was inhibited, while a relatively normal EGF response was observed under the same conditions. This is in contrast to our results in which we demonstrated that in c-fos-deficient cells, both PDGF and EGF responses are completely ablated while TPA response is only partially reduced. It is not entirely clear why the discrepancies exist in these two different experimental approaches, although it is possible that expression of the anti-sense mRNA had some effects not entirely specific for the c-fos molecule.

The specific dependence of stromelysin and collagenase type I expression on c-fos, even in the presence of relatively normal total AP-1 activity, is of particular interest because these proteases have been implicated in embryonic tissue development (C.A.Brenner *et al.*, 1989), injured tissue remodeling (Hasty *et al.*, 1990; Woessner and Gunja, 1991), tumor progression and metastasis (Liotta and Stetler, 1990). Much evidence indicates that malignant transformation results in elevated stromelysin expression and especially correlates with tumor expansion and metastasis (Matrisian and Bowden, 1990; Goldberg and Eisen, 1991). Tumor metastasis is a complex, multi-step process that involves degradation of cellular matrix, tumor cell locomotion, cell adhesion, cell recolonization and angiogenesis (Bishop, 1991; Liotta *et al.*, 1991). Proteases, including gelatinase, stromelysin, collagenase and cathepsin, have been implicated in many of these processes, especially tumor expansion and progression. In addition, oncogenes as well as a number of growth factors have been shown to be activators of metalloprotease gene expression (Matrisian *et al.*, 1986). Our data indicate that at least one oncogene, polyoma middle T, can activate stromelysin expression in the absence of c-fos, while v-src requires c-fos for maximal induction. These results demonstrate that oncogenes use both fos-dependent and fos-independent pathways for stromelysin gene induction. The involvement of c-fos in regulating metalloprotease expression in response to oncogenic transformation implies a potential link between c-fos expression and the malignant behavior of tumor cells. That this is more specific than an overall effect on total AP-1 activity indicates a step at which it may be possible to interfere with certain aspects of malignant behavior.

Materials and methods

Materials

PDGF, EGF and TPA were obtained from Sigma Chemical Co., St Louis, MO. PDGF was also purchased from Gibco-BRL Corp. A c-fos-specific antibody was a generous gift from Dr R.Bravo of Bristol-Meyer Squibb Pharmaceutical Corp. Another anti-c-fos antibody was provided by Dr Robert Distel. This antibody is against the M-peptide (YGKVEQLSPEEEEKRIRRERNKMAAA), which is from a conserved region of the c-fos DNA binding domain (Curran *et al.*, 1985). This antibody has been shown to recognize c-fos as well as several other members of the Fos family, including fos-B, fra-1 and fra-2.

Oligonucleotides and DNA constructs

The sequences of the double-stranded oligonucleotides used were as follows (only one strand is shown): TRE from the AP-1 site of the

stromelysin promoter (–70 to –84; Kerr et al., 1988), 5′-AATTCATG-AGTCAGATCTG-3′; mutant TRE (mTRE), 5′-AATTCACGAGTGAG-ATCTG-3′; FSE2, AP-1 site of the aP2 gene (Distel et al., 1987), 5′-CATGACTCAGAGGAAAACATAC-3′; C-ets, 5′-GTCAGTTAAGC-AGGAAGTGACTAAC-3′; c-ets oligonucleotide was used as an unrelated control oligomer in the competition experiment. The c-ets sequence was derived from the stromelysin promoter as described previously (Wasylyk et al., 1991). PLJ-v-src (Piwnica-Worms et al., 1987) and PLJ-MT (polyoma middle T; Druker et al., 1990) were gifts from Dr Brian Druker of the Dana-Farber Cancer Institute. Both are retroviral vectors and contain an endogenous neomycin resistance gene in the constructs. Expression vectors for c-jun and v-jun (CJ-3 and VJ-0) were supplied by Dr Iain Morgan and Dr Peter Vogt of the University of Southern California (Havarstein et al., 1992; Morgan et al., 1992). Activated Ha-ras (pEJ6.6) was obtained from Dr Robert Weinberg (MIT, Cambridge, MA). SV40 transforming vector (pM-8) was supplied by Dr James DeCaprio of the Dana-Farber Cancer Institute (DeCaprio et al., 1988). v-raf transforming vector (pMSV-p90) (Jamal and Ziff, 1990) was provided by Dr Edward Ziff of New York University, NY. The c-fos expression vector PLJ-fos was constructed by digesting Plen-fos with *Bam*HI (Oliviero et al., 1992) and moving the digested c-fos-containing *Bam*HI–*Bam*HI insert into the *Bam*HI site of the PLJ vector (Piwnica-Worms et al., 1987; Druker et al., 1990). PMV-fos-ER was a generous gift from Dr Busslinger of the Research Institute of Molecular Pathology, Vienna, Austria (Superti et al., 1991). Murine fra-1 cDNA clone (Cohen and Curran, 1988) was provided by Dr Tom Curran. Human Fra-2 plasmid (Matsui et al., 1990) was a gift from Dr Nobuo Nomura, Laboratory of Molecular Biology, Institute of Gerontology, Nippon Medical School, Kanagawa, Japan. Plasmids containing mouse fos-B, jun-B, jun-D, metallothionein 1 and human metallothionein 2 were obtained from ATCC. MCP-1 plasmid (pcJE) was provided by Dr Charles Stiles of the Dana-Farber Cancer Institute (Rollins et al., 1988). Plasmids containing mouse stromelysin (Matrisian et al., 1986) and type I collagenase were gifts from Dr Lynn Matrisian of the Department of Cell Biology, Vanderbilt University.

Establishment of wild-type and c-fos-deficient 3T3 cell lines

Embryos (15–16 days old) were used to generate primary embryonic fibroblast according to Robertson et al. (Robertson, 1987) as described (Johnson et al., 1992). Genotyping of the cells was performed by PCR and Southern blot analysis (Saiki et al., 1988). Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS), glutamine and penicillin-streptomycin. Every 3 days, cells were trypsinized and reseeded at a density of 1×10^6 per 100 mm dish (1.2×10^4 cells/cm^2). Crisis was observed at about passage 15 and further passage resulted in the development of immortalized 3T3-type cell lines (Todaro and Green, 1963). Two c-fos-deficient and one wild-type cell lines were obtained through the 3T3 protocol.

Cell growth measurements

To measure the growth rate of the c-fos-deficient cells at optimal and suboptimal conditions, 10^4 cells per well were seeded into six-well culture plate (Nunc) in 10% fetal calf serum (FCS), and 2 h later, medium was changed to DME containing various serum concentrations. Four days later cells were trypsinized, diluted 1:50 with 1× Isoton buffer and counted on a Coulter counter.

RNA isolation and Northern blot analysis

Northern blot analysis was performed as described (Sambrook et al., 1989). The guanidine–isothiocyanate method was used to make total RNA as described (Chomczynski and Sacchi, 1987).

Electrophoretic mobility shift assay (EMSA)

Single-stranded oligonucleotides were annealed and end-labeled as described previously (Sambrook et al., 1989). Nuclear extract was prepared according to Dignam et al. (1987) from both wild-type and c-fos-deficient cells. The cells were initially serum starved for 24 h and then stimulated for 2 h with PDGF, EGF or TPA, as described in the legend to Figure 2. Extracts were isolated from unstimulated as well as growth factor-stimulated cells. In an EMSA reaction, nuclear extracts with 5 μg protein were incubated with 10 000 c.p.m. of end-labeled oligonucleotide TRE in a 30 μl volume containing 20 mM HEPES–KOH (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM DTT, 0.05% (v/v) NP-40 and 10% (v/v) glycerol. After incubating at room temperature for 20 min, bound and unbound oligonucleotides were resolved on a 4% polyacrylamide gel (30:1 acylamide:bisacrylamide) in 0.5× TBE buffer (45 mM of Tris-borate and 1 mM EDTA, pH 8.0). Competitions were performed with a 50-fold excess of unlabeled TRE oligonucleotides or with excess unlabeled c-ets, mTRE or FSE2 oligonucleotides, respectively. In an antibody disruption experiment, 1 μl of anti-fos antiserum was added to the binding reaction, and incubated 2 h at 4°C before adding the labeled oligonucleotide probe. Film exposure time was 2–4 h.

Generation of stably transfected cell lines that express exogenous c-fos

The c-fos-deficient cells were transfected by the calcium phosphate precipitation method as described (Oliviero et al., 1992) using 20 μg of each DNA construct per transfection. After transfection, G418 (400 μg/ml) selection was applied to the cell culture. Individual colonies were picked after 2–3 weeks of selection and propagated subsequently. Total cellular RNA for individual clones was isolated for Northern blot analysis.

Virus production and viral infection

Two retroviral constructs, PLJ-MT (polyoma middle T) and PLJ-v-src, were transfected into a viral packaging cell line Y2 as described (Mann et al., 1983; Cepko et al., 1984). Two days after transfection, virus containing supernatant was harvested and filtered through 0.45 μm filters and used immediately for infection. c-Fos-deficient cells and control cells were seeded at 25% confluence 24 h before infection and medium containing the viral particles was added to culture. Polybrene was added to a final concentration of 8 μg/ml to enhance the infection efficiency. Virus containing medium was removed 3 h later and cells were maintained in fresh DMEM for an additional 24 h. Cells were then split 1:3 into new dishes and maintained for focus formation assay. Alternatively, G418 selection was applied to select clones that harbored the v-src and polyoma middle T (MT) oncogenes.

Focus formation assay

A focus formation assay was performed essentially as described (Oliviero et al., 1992). Both transfections (for c-Ha-ras, c- and v-jun, c-fos, v-raf, v-src, polyoma middle T, SV40 large T) and infections (for v-src, polyoma middle T) were used for focus formation assay.

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