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ORIGINAL ARTICLE

Cooperative cell transformation by Myc/Mil(Raf) involves induction of AP-1 and activation of genes implicated in cell motility and metastasis

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Avian fibroblasts transformed simultaneously by the v-*myc* and v-*mil*(*raf*) oncogenes of acute leukemia and carcinoma virus MH2 contain elevated levels of c-Fos and c-Jun, major components of the transcription factor complex AP-1. To define specific transcriptional targets in these cells, subtractive hybridization techniques were employed leading to the identification of strongly upregulated genes including *OPN* (osteopontin), *126MRP*, and *rac*2. OPN is a cytokine and cell attachment protein which has been implicated in human tumor progression and metastasis, the calcium binding 126MRP protein is related to the human S100 protein family involved in invasive cell growth, and the Rac2 protein belongs to the Rho family of small GTPases regulating actin reorganization and cell migration. Promoter analysis indicated that *OPN* activation is mediated by a non-consensus AP-1 binding site located close to the transcription start site. Electrophoretic mobility shift assays, chromatin immunoprecipitation and transcriptional reporter gene analyses showed that c-Fos and c-Jun bind specifically to this site and that c-Fos efficiently transactivates the *OPN* promoter. High-level expression of OPN, 126MRP, or Rac2 proteins from a retroviral vector led to partial cell transformation, documented by morphological changes and anchorage-independent growth. The specific activation in v-*myc*/v-*mil*(*raf*)-transformed cells of target genes with intrinsic oncogenic potential may provide an explanation for the longstanding observation that concomitant expression of these oncogenes leads to strongly enhanced oncogenicity *in vivo* and *in vitro* compared to cell transformation by v-*myc* or v-*mil*(*raf*) alone.
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Introduction

Oncogenes were originally discovered as single oncogenic determinants in the genomes of highly oncogenic retroviruses. The subsequent discovery of multiple oncogenes in single retroviral genomes, acquired by multiple transductions of cellular proto-oncogenes, provided strong genetic and molecular evidence in support of the notion that oncogenes cooperate in cell transformation and that multiple mutational events enhance tumorigenesis. One of the viruses containing more than one oncogene, avian leukaemia- and carcinoma-inducing retrovirus MH2, carries transduced v-*myc* and v-*mil* alleles derived from the cellular proto-oncogenes c-*myc* and c-*mil*, respectively (Jansen *et al*., 1983a, b, 1984; Bister and Jansen, 1986). The *myc* oncogene, originally identified as a single oncogenic determinant (v-*myc*) in avian acute leukemia virus MC29, encodes a bHLHZip protein (Myc), encompassing protein dimerization (helix-loop-helix, leucine zipper) and DNA contact (basic region) surfaces, that forms heterodimers with the Max protein and binds to specific DNA sequence elements (E-boxes) (Bister *et al*., 1977; Duesberg *et al*., 1977; Blackwell *et al*., 1990; Blackwood and Eisenman, 1991; Kerkhoff *et al*., 1991; Dang, 1999; Grandori *et al*., 2000; Eisenman, 2001). The Myc–Max transcription factor complex is a key regulator of genes critically involved in cellular growth, proliferation, metabolism, and differentiation (Blackwood and Eisenman, 1991; Dang, 1999; Grandori *et al*., 2000; Eisenman, 2001).

The v-*mil* oncogene identified in MH2 (Jansen *et al*., 1983a, b) is the avian ortholog of the mammalian v-*raf* oncogene (Jansen *et al*., 1984; Sutrave *et al*., 1984) that was independently defined as a single oncogenic determinant in murine retrovirus 3611-MSV (Rapp *et al*., 1983). The c-*mil*(*raf*) proto-oncogene encodes the serine/threonine-specific protein kinase c-Mil(Raf) that is an important part of a conserved signalling pathway transducing signals from the cell surface to the nucleus (Bister and Jansen, 1986; Wellbrock *et al*., 2004). Growth factor binding to cell surface receptors leads to activation of Ras and subsequently of Mil(Raf). Mil(Raf) then activates the dual-specificity protein kinases MEK1 and MEK2 which in turn activate mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) (Eferl and Wagner, 2003; Wellbrock *et al*., 2004). The concomitant

expression of the v-*myc* and v-*mil*(*raf*) oncogenes by the natural retroviral isolate MH2 and by the highly oncogenic recombinant murine retrovirus J2 (Rapp *et al*., 1985) correlates with the strongly enhanced oncogenic potential of these viruses including very rapid induction of hematopoietic neoplasms and liver tumors (Rapp *et al*., 1985; Graf *et al*., 1986).

An important nuclear effector in the ERK signaling pathway is the activator protein-1 (AP-1) transcription factor complex (Rapp *et al*., 1994; Suzuki *et al*., 1994; Shaulian and Karin, 2002; Eferl and Wagner, 2003) converting cytosolic signals into changes of gene expression programs. AP-1 represents a heterogeneous collection of dimers composed of members of the Jun, Fos, or activating transcription factor (ATF) protein families, and is a key regulator of genes involved in cell proliferation or differentiation (Curran and Franza, 1988; Ransone and Verma, 1990; Vogt and Bos, 1990; Shaulian and Karin, 2002; Vogt, 2002; Eferl and Wagner, 2003). Jun proteins can bind to DNA as functional homodimers, whereas Fos proteins need a member of the Jun protein family for dimerization (Curran and Franza, 1988; Ransone and Verma, 1990; Vogt and Bos, 1990; Vogt, 2002). AP-1 transcription factors bind with high affinity to the 12-*O*-tetradecanoyl-phorbol-13-acetate responsive element (TRE) with the consensus sequence 5′-TGA$^{C}/_{G}$TCA-3′, or to variants thereof, in the promoters of various target genes (Curran and Franza, 1988; Vogt and Bos, 1990; Vogt, 2002). The oncogenic v-*jun* or v-*fos* alleles were generated by retroviral transduction of the cellular c-*jun* or c-*fos* proto-oncogenes (Curran and Franza, 1988; Vogt and Bos, 1990).

To elucidate the molecular mechanisms of synergistic Myc/Mil(Raf) oncogenic signaling, we searched for specific genes differentially expressed in v-*myc*/v-*mil* (*raf*)-transformed avian cells. Here we report on the identification and functional characterization of three strongly activated genes: osteopontin (*OPN*) (Denhardt *et al*., 2001), *126MRP*, related to the *S100* gene family (Marenholz *et al*., 2004), and *rac*2, a member of the *Rho* gene family encoding small GTPases (Didsbury *et al*., 1989; Jaffe and Hall, 2005). Intriguingly, *OPN* and members of the *S100* and *Rho* gene families have been implicated in molecular processes linked to cell migration, invasion, and metastasis (Marenholz *et al*., 2004; Rittling and Chambers, 2004; Kurisu *et al*., 2005). We show that transcriptional activation of *OPN*, a typical marker gene for metastatic tumor cells (Denhardt *et al*., 2001; Weber, 2001; Moye *et al*., 2004; Rittling and Chambers, 2004), is dependent on c-Fos which is highly expressed in v-*myc*/v-*mil*(*raf*)-transformed cells. Furthermore, ectopic expression of OPN, 126MRP, or Rac2 proteins from a retroviral vector causes partial transformation of primary avian fibroblasts, suggesting that these gene products are critically involved in oncogenesis induced by Myc/Mil(Raf), possibly in a joint signaling pathway. Our data also suggest that AP-1 plays an important role in v-*myc*/v-*mil*(*raf*)-induced oncogenesis, and that concerted action of the oncogenic transcription factor complexes Myc–Max and Jun–Fos contributes to full neoplastic transformation.

Results

AP-1 activation and differential gene expression in v-myc/v-mil-transformed fibroblasts

Avian fibroblasts transformed by concomitant expression of the v-*myc* and v-*mil* oncogenes of avian acute leukemia virus MH2 display a fully transformed phenotype, manifested by rapid growth and extensive morphological changes of cells in culture and by a very high potential of rapid anchorage-independent growth. In these parameters, they surpass cells transformed by a single oncogene, like the v-*myc* oncogene of avian acute leukemia virus MC29, or the v-*mil* or v-*jun* oncogene, carried by retroviral constructs (Figure 1a and b). To elucidate the molecular mechanisms underlying the high oncogenic potential of MH2 both *in vitro* and *in vivo* (Rapp *et al*., 1985; Bister and Jansen, 1986; Graf *et al*., 1986), we used comparative gene expression analyses to identify genes specifically activated in v-*myc*/v-*mil*-transformed quail embryo fibroblasts.

To confirm oncoprotein expression in the cells used for comparison (Figure 1a and b), immunoprecipitation analysis of [^{35}S]methionine-labeled proteins from cellular extracts was carried out using antibodies raised against Myc and Mil, or against the AP-1 components Jun and Fos (Figure 1c). In the MC29-transformed quail embryo fibroblast (QEF) line Q8, v-*myc* is expressed as a 110-kDa Gag–Myc hybrid protein (Bister *et al*., 1977), and in the MH2-transformed QEF line MH2A10, v-*mil* is expressed as a 100-kDa Gag–Mil hybrid protein (Jansen *et al*., 1983a). The v-Myc protein in MH2A10 cells, the v-Mil protein in QEF transformed by retroviral construct RCAS-v-Mil, and the v-Jun protein in RCAS-v-Jun-transformed QEF are devoid of Gag sequences. In accordance with previous observations (Patschinsky and Bister, 1988; Hartl and Bister, 1998), the cellular proto-oncoproteins c-Myc or c-Jun were not detectable in Q8, MH2A10, or QEF/RCAS-v-Jun cells, respectively, which contain high levels of the corresponding viral oncoproteins v-Myc or v-Jun, whereas expression of c-Mil was observed at low levels in all cell types tested. Interestingly, expression levels of c-Jun and c-Fos were elevated in v-*myc*-transformed and particularly in v-*myc*/v-*mil*-transformed cells (Figure 1c), suggesting that cell transformation induced by MH2 may involve activation of AP-1. In contrast, normal QEF, or cells transformed by v-*mil* (QEF/RCAS-v-Mil) or v-*jun* (QEF/RCAS-v-Jun), did not contain detectable amounts of the 62-kDa c-Fos protein (Figure 1c). The Fos-specific antiserum raised against a recombinant GST-Fos hybrid protein also detected a 67-kDa non-specific protein band in all cell types as described previously (Hartl *et al*., 2001).

Based on the observation of significantly elevated c-Fos and c-Jun protein levels in v-*myc*/v-*mil*-transformed cells, we were interested in the identification of

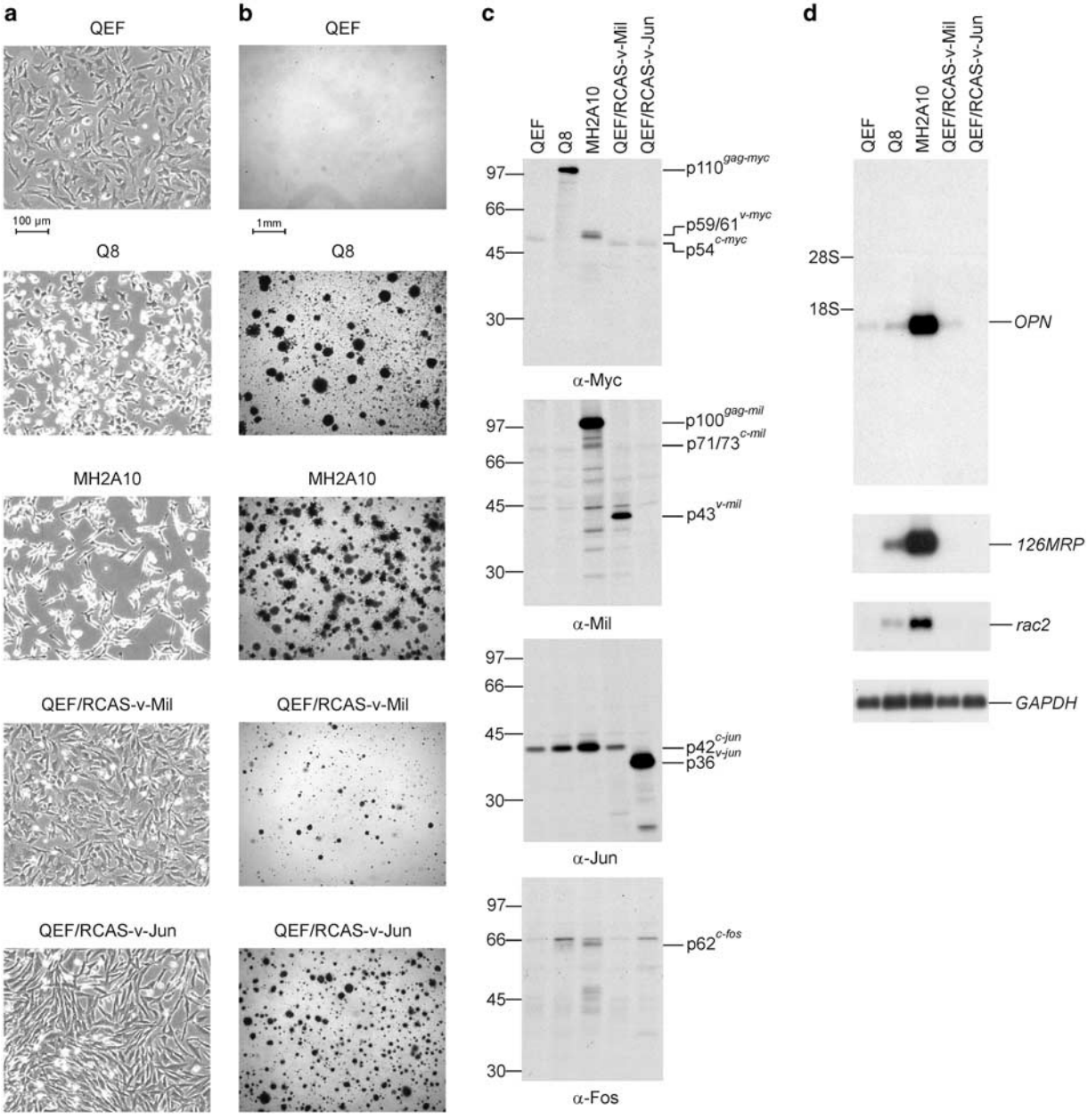

Figure 1 AP-1 activation and differential gene expression in v-*myc*/v-*mil*-transformed avian fibroblasts. (**a**) Phase-contrast micrographs of quail embryo fibroblasts (QEF), of the quail cell lines Q8 (Bister *et al*., 1977) and MH2A10 (Jansen *et al*., 1983a, b) transformed by MC29 (v-*myc*) or MH2 (v-*myc* and v-*mil*), respectively, and of QEF transfected with retroviral constructs carrying the coding regions of v-*mil* (pRCAS-v-Mil) or v-*jun* (pRCAS-v-Jun). (**b**) Agar colony formation of the cells shown in panel a. Equal numbers of cells (5×10^4) were seeded in soft agar on 35-mm dishes, and bright-field micrographs were taken after 14 days. (**c**) Expression analysis of the oncoproteins v-Myc (p110$^{gag-myc}$, p59/61^{v-myc}), v-Mil (p100$^{gag-mil}$, p43^{v-mil}), v-Jun (p36^{v-jun}), and of the proto-oncoproteins c-Myc (p54^{c-myc}), c-Mil (p71/73^{c-mil}), c-Jun (p42^{c-jun}), c-Fos (p62^{c-fos}), using extracts of [^{35}S]methionine-labeled cells shown in panel a. Proteins were analysed by immunoprecipitation using polyclonal rabbit antisera (α) directed against the indicated recombinant proteins, and precipitated proteins were resolved by SDS–PAGE (10%, wt/vol). Fluorographs were exposed for 4 d (α-Myc, α-Mil, α-Jun) or 6 d (α-Fos). The positions of molecular weight markers (in kDa) are indicated in the margin. (**d**) Differential expression of genes specifying osteopontin (*OPN*), the S100-related calcium binding protein (*126MRP*), or the Rho-family protein Rac2 (*rac*2) in v-*myc*/v-*mil*-transformed fibroblasts, monitored by Northern analyses using 2.5-μg aliquots of poly(A)$^+$-selected RNAs from the cells shown in panel a. Filters were hybridized with ^{32}P-labeled cDNA probes specific for *OPN* (0.9×10^6 cpm/ml, 6-h exposure), *126MRP* (0.8×10^6 cpm/ml, 6-h exposure), *rac*2 (1.3×10^6 cpm/ml, 240-h exposure), or *GAPDH* (0.8×10^6 cpm/ml, 20-h exposure). The positions of ribosomal RNAs on the filter shown in full size (*OPN*) are indicated in the margin. Autoradiography was done using an intensifying screen. The sizes of the mRNAs are 1.4 kb (*OPN*), 0.8 kb (*126MRP*), 1.2 kb (*rac*2), and 1.4 kb (*GAPDH*).

genes that are differentially expressed in these cells and possibly regulated by AP-1. Using mRNA preparations from MH2A10 cells and from normal QEF, subtractive hybridization technologies led to the identification of three genes that were expressed at high levels in the v-*myc*/v-*mil*-transformed MH2A10 cells, compared to

low or undetectable levels of expression in normal QEF or in cells transformed by v-*mil* or v-*jun* (Figure 1d). Expression of these genes was also slightly enhanced in the v-*myc*-transformed cell line Q8 (Figure 1d). Nucleotide sequence analyses of the isolated clones showed that the corresponding mRNAs encode quail homologs of proteins specified by the chicken genes osteopontin (*OPN*) (Castagnola *et al*., 1991), *126MRP* (Nakano and Graf, 1992), or by the human *rac*2 gene (Didsbury *et al*., 1989).

The deduced amino-acid sequences of the quail proteins were compared with those of their orthologs from chicken and man (Figure 2). Human homologs of OPN and Rac2 could be clearly defined. In the case of 126MRP, originally identified as the protein product of chicken clone MRP-126 (Nakano and Graf, 1992), sequence similarities with multiple human proteins were found, all belonging to the small calcium-binding protein family S100 (Donato, 1999; Marenholz *et al*., 2004). Of these, the S100A9 protein, also known as calgranulin B or migration inhibitory factor-related protein 14 (MRP-14), displays the closest relationship to quail or chicken 126MRP. In the compared sequences, functional protein domains are well conserved, including signal and cell adhesion sequences in OPN, EF hands in 126MRP/S100A9, and the GTPase domain in Rac2 (Figure 2). The quail and chicken orthologs share a high degree of identity at the amino-acid and nucleotide-sequence levels. Compared to their human orthologs, sequences outside the functional domains of avian osteopontin or 126MRP are less conserved. In contrast, human Rac2 displays a high degree of sequence similarity with its avian homologs throughout the entire protein sequences (Figure 2).

Mechanism of OPN activation in v-myc/v-mil-transformed fibroblasts

The cytokine OPN identified here as a putative target of v-*myc*/v-*mil* signaling has been implicated in cell transformation and metastasis, and is regarded as a candidate target for cancer therapy (Weber, 2001; Moye *et al*., 2004; Rittling and Chambers, 2004). Hence, we decided to study the regulation of the avian *OPN* gene

Figure 2 Multiple amino-acid sequence alignments (ClustalW) of quail (q) proteins encoded by genes specifically activated in v-*myc*/v-*mil*-transformed fibroblasts, and of their chicken (c) and human (h) orthologs. Amino acids are numbered in the margin. For the chicken and human orthologs, amino-acid residues are shown only at positions where they differ from the respective quail sequence. Gaps in the aligned sequences are indicated by dashes. (**a**) OPN, osteopontin; accession numbers AAF63330 (q), NP_989866 (c), AAH17387 (h); sequence identities (q)/(c) 91%, (q)/(h) 30%; signal peptide (yellow), cell adhesion sequence (orange). (**b**) 126MRP, migration inhibitory factor-related protein/S100A9 calcium-binding protein; accession numbers AAT01286 (q), XP_424012 (c), NP_002956.1 (h); sequence identities (q)/(c) 92%, (q)/(h) 44%; EF hands (pink). (**c**) Rac2, Ras-related C3 botulinum toxin substrate; accession numbers AAT01288 (q), XP_416280 (c), AAP36269 (h); sequence identities (q)/(c) 100%, (q)/(h) 98%; GTPase domain (blue).

and the putative function of the OPN protein in v-*myc*/v-*mil*-induced cell transformation in more detail. To confirm the specific activation of the *OPN* gene in the course of v-*myc*/v-*mil*-induced cell transformation and to corroborate the original observation obtained with the permanent cell line MH2A10 (cf. Figure 1d), primary chicken embryo fibroblasts (CEF) were freshly transformed by the MH2 virus (CEF/MH2) and passaged. mRNA was isolated from these cells and analysed by Northern hybridization in comparison with mRNAs from normal CEF and from CEF transformed by the ASV17 virus carrying the v-*jun* oncogene (Vogt and Bos, 1990). Furthermore, secreted OPN protein ($p52^{OPN}$) levels were analysed by immunoprecipitation using an antiserum directed against OPN recombinant protein (see below). The analysis showed that the *OPN* gene is specifically and efficiently activated at the mRNA and protein level in CEF transformed by the MH2 virus (Figure 3a).

The chicken *OPN* promoter was cloned and inserted into a chloramphenicol acetyltransferase (CAT) reporter plasmid yielding the pCAT-OPN construct. Transient transfection of the pCAT-OPN plasmid into the established cell line MH2A10 and subsequent CAT

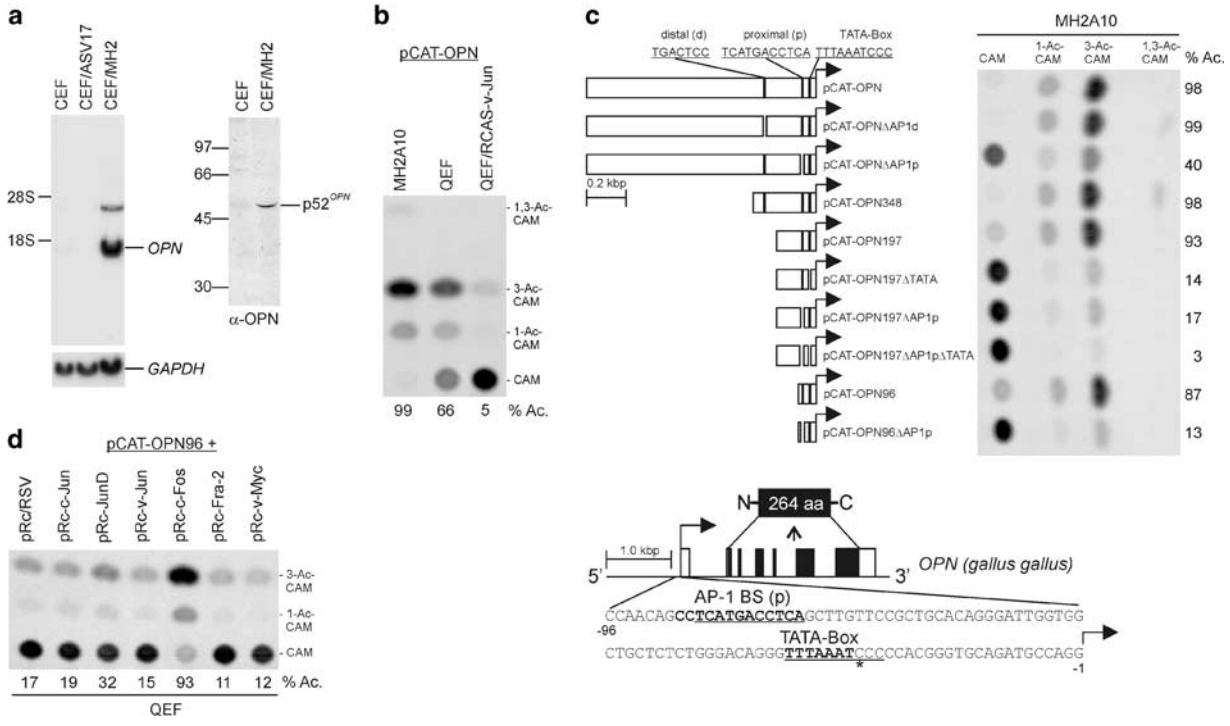

Figure 3 Transactivation of the osteopontin promoter. (**a**) *Left panel*: Northern analysis using 30-μg aliquots of total RNAs from chicken embryo fibroblasts (CEF), and from CEF transformed by the retroviruses ASV17 or MH2 carrying the v-*jun* or v-*myc*/v-*mil* oncogenes, respectively. Filters were hybridized with ^{32}P-labeled cDNA probes specific for *OPN* (1.0×10^6 cpm/ml, 22-h exposure) and *GAPDH* (1.2×10^6 cpm/ml, 20-h exposure). Autoradiography was done using an intensifying screen. The sizes of the mRNAs are 1.4 kb (*OPN*) and 1.4 kb (*GAPDH*). In addition to the 1.4-kb *OPN* mRNA also observed in MH2-transformed QEF (cf. Figure 1d), MH2-transformed CEF contain a minor *OPN*-specific transcript of 3.4 kb. The positions of ribosomal RNAs are indicated in the margin. *Right panel*: Analysis of the secreted OPN ($p52^{OPN}$) protein in the culture medium of normal CEF and of MH2-transformed CEF. Proteins were analysed by immunoprecipitation using an OPN-specific antiserum and SDS–PAGE (10%, wt/vol). The fluorograph was exposed for 10 days. The positions of molecular weight markers (in kDa) are indicated in the margin. (**b**) Five-microgram aliquots of DNA from a chloramphenicol acetyltransferase (CAT) gene construct containing the wild-type chicken *OPN* promoter (pCAT-OPN) were transiently nucleofected into MH2A10 or QEF/RCAS-v-Jun cells transformed by retroviruses specifying v-Myc/v-Mil or v-Jun oncoproteins, respectively, or into normal QEF. Extracts containing equal amounts of proteins (2.5 μg) were used for acetylation of [^{14}C]chloramphenicol. Reaction products were analysed by ascending thinlayer chromatography and autoradiography (4-h exposure). Positions of chloramphenicol (CAM), of acetylated products (Ac-CAM), and percentages of total acetylation are indicated in the margins and below the lanes, respectively. (**c**) Equal amounts of CAT gene constructs (5.0 μg) containing the wild-type *OPN* promoter (pCAT-OPN) or deletion derivatives thereof (pCAT-OPN348, pCAT-OPN197, pCAT-OPN96), or of constructs lacking distal (pCAT-OPNΔAP1d) or proximal (pCAT-OPNΔAP1p, pCAT-OPN197ΔAP1p, pCAT-OPN96ΔAP1p) AP-1 binding sites, or the TATA box (pCAT-OPN197ΔTATA), or the TATA box and the proximal AP-1 binding site (pCAT-OPN197ΔAP1pΔTATA) were nucleofected into MH2A10 cells and subsequently analysed as described for panel b. The structure of the chicken *OPN* locus is shown below. The seven exons are depicted as boxes with the coding region shown in black. The nucleotide sequence of the minimal promoter (OPN96) required for transcriptional activation is shown below the diagram. The proximal AP-1 binding site and the TATA box are indicated. Nucleotides deleted in the mutant promoter constructs (ΔAP1p, ΔTATA) are underlined. The asterisk denotes a T→C substitution in the chicken DNA used here compared to the chicken genome sequence (http://www.ncbi.nlm.nih.gov). The transcriptional start site is marked by the arrow. (**d**) Two-microgram aliquots of pCAT-OPN96 DNA were transfected by the calcium phosphate method into QEF together with 2.0-μg aliquots of DNA from pRc/RSV-derived protein expression constructs carrying the c-*jun*, *junD*, v-*jun*, c-*fos*, *fra*-2, or v-*myc* coding regions as indicated. Cell extracts containing equal amounts of proteins (50 μg) were analysed as described for panel b.

analysis revealed that the *OPN* promoter becomes strongly activated in these cells (Figure 3b). No or moderate promoter activities were seen in v-*jun*-transformed cells or in normal QEF in correlation with no or low mRNA expression in these cells, respectively (cf. Figure 1d). The basal activation of the *OPN* promoter in normal QEF may be due to endogenous AP-1 components like c-Jun or JunD (see below) which are absent in v-*jun*-transformed cells (cf. Figure 1c and data not shown). Bioinformatic analysis of the chicken *OPN* promoter region revealed the presence of three potential non-consensus AP-1 binding sites (5′-TGA CTCC-3′, 5′-CCTCATGAC-3′ and 5′-TGACCTCA-3′) which have recently been described as functional binding sites located in the promoters of the monocyte chemotactic protein 1 or mouse osteopontin genes (Shyy *et al*., 1995; Bidder *et al*., 2002), or in the bovine leukemia virus genome (Merezak *et al*., 2001), respectively. The latter two sequence motifs overlap in the chicken *OPN* promoter giving rise to a 5′-CCTCATG ACCTCA-3′ sequence element termed here the proximal AP-1 binding motif (Figure 3c). Site-directed mutagenesis leading to deletion of the distal or disruption of the proximal AP-1 binding motifs in pCAT-OPN showed that only mutation of the proximal AP-1 binding motif led to significant reduction in CAT-activity (Figure 3c). Progressive 5′-deletion analysis of pCAT-OPN demonstrated that a 96-bp region (pCAT-OPN96) containing the critical proximal AP-1 motif and a TATA box was sufficient to confer almost full promoter activity (Figure 3c). The enhancer and basal promoter motifs are located at nucleotide positions −89 to −77 and −30 to −24, respectively. No potential binding sites for the Myc/Max transcription factor complex were found in this minimal promoter segment. Deletion of the proximal AP-1 binding motif, of the TATA box, or of both sites, led to a drastic reduction of CAT activity (Figure 3c) indicating that these binding sites are required as essential promoter elements for activation of the *OPN* gene in v-*myc*/v-*mil*-transformed cells. To test directly if AP-1 components are necessary for transcriptional activation, the promoter construct pCAT-OPN96 was co-transfected with eukaryotic expression vectors specifying v-Myc, v-Jun, c-Jun, c-Fos, or the Jun- and Fos-related proteins JunD and Fra-2, respectively, into normal QEF (Figure 3d). The analysis revealed that high-level expression of c-Fos led to strong activation of the minimal *OPN* promoter, whereas no or only moderate activation was observed with ectopically expressed c-Jun, v-Jun, Fra-2, v-Myc, or JunD proteins, respectively.

To directly demonstrate specific interaction of an AP-1 complex containing c-Fos and c-Jun with the proximal AP-1 binding site in the *OPN* promoter, a double-stranded oligodeoxynucleotide encompassing this motif was used for electrophoretic mobility shift analysis (EMSA). A heterodimer composed of *in vitro* co-translated c-Jun/c-Fos proteins was able to bind to this DNA probe with equal specificity compared to a control probe containing the consensus AP-1 site present in the promoter of *JAC*, a direct Jun target gene (Hartl *et al*., 2001) (Figure 4a). The specificity of protein-DNA interactions was demonstrated by competitive inhibition of binding using the unlabeled DNA probe, and by specific interference with binding using antibodies directed against Jun or Fos proteins (Figure 4a). As reported previously, Jun or Fos antibodies inhibit DNA binding in the EMSA analysis, rather than inducing supershifts (Suzuki *et al*., 1994; Hartl *et al*., 2001). Antibodies directed against the v-Myc protein or normal rabbit serum did not interfere with DNA binding or induce supershifts (Figure 4a). Using the OPN-specific DNA probe, significant binding activity was also detected in nuclear extracts prepared from MH2-transformed CEF compared to nuclear extracts derived form normal CEF (Figure 4b). As shown for the *in vitro* translated c-Jun/c-Fos proteins, specificity of binding was demonstrated by unlabeled probe competition and antibody inhibition (Figure 4b).

To obtain additional evidence that c-Fos/c-Jun complexes bind directly to the *OPN* promoter, chromatin immunoprecipitation (ChIP) analysis was employed using cross-linked chromatin from CEF/MH2 cells, Fos- and Jun-specific antibodies, and a PCR primer pair for amplification of a 112-bp DNA segment encompassing the proximal AP-1 binding site of the *OPN* promoter. The PCR analysis specifically produced a 112-bp DNA fragment when Fos- or Jun-specific antibodies were used for the chromatin immunoprecipitation (Figure 4c). This corroborates the finding that c-Fos and c-Jun bind specifically to the proximal AP-1 motif present in the *OPN* promoter. As a control, ChIP was performed using chromatin from ASV17-transformed CEF and a primer pair for amplification of a 201-bp segment from the *JAC* promoter containing the consensus high-affinity AP-1 binding site (Hartl *et al*., 2001). The analysis confirmed that v-Jun but not c-Fos binds efficiently to the *JAC* promoter. Accordingly, the chicken c-Fos protein ($p62^{c\text{-}fos}$) activated in MH2-transformed CEF was not detectable in ASV17-transformed cells or in normal CEF (Figure 4d) demonstrating the specificity of the ChIP data (Figure 4c) using the Fos-specific antiserum and OPN-specific primers in the case of MH2-transformed cells.

OPN, 126MRP, and Rac2 have intrinsic oncogenic potential and cooperate in cell transformation

To investigate whether the proteins encoded by the three activated target genes are involved in cell transformation, the coding regions of *OPN*, *126MRP*, and *rac2* were inserted into replication-competent retroviral vectors (RCAS) and the constructs transfected into avian fibroblasts (Figure 5). To facilitate detection of the protein products by using FLAG antibodies (Hartl *et al*., 2001), the FLAG epitope was inserted between the start methionine and the second amino-acid residue of each protein (Figure 5a). Since the human homologs of both *OPN* and *126MRP* have been implicated in tumorigenesis (Donato, 1999; Denhardt *et al*., 2001; Marenholz *et al*., 2004; Moye *et al*., 2004), bicistronic retroviral constructs for the simultaneous overexpression

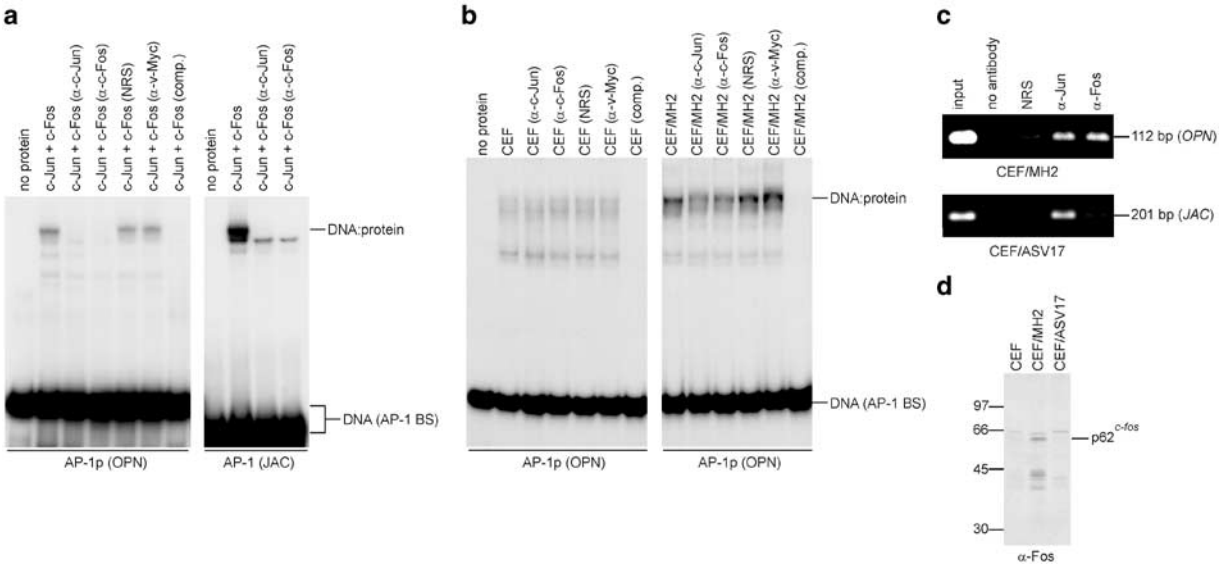

Figure 4 Specific binding of AP-1 to the osteopontin promoter. (**a**, **b**) ^{32}P-labeled double-stranded oligodeoxynucleotides (3 nM) derived from the chicken *OPN* promoter containing the proximal composite non-consensus AP-1 binding motif (5′-CCTCATGACCTCA-3′) (AP-1p), or from the chicken *JAC* promoter containing the consensus AP-1 binding site (5′-TGAGTCA-3′) (Hartl *et al*., 2001) were used as probes (2.5×10^4 cpm) for electrophoretic mobility shift assays (EMSA). The probes in panel a were incubated with equal aliquots from an *in vitro* transcription/translation reaction using DNA constructs carrying the coding regions for the chicken c-Jun and c-Fos proteins as templates, and the probes in panel b were incubated with 10 μg each of nuclear proteins extracted from normal CEF or from MH2-transformed CEF (CEF/MH2). Incubations were also carried out in the presence of 1-μl aliquots of specific antisera (α) directed against the Jun, Fos, or Myc proteins, of 1 μl of normal rabbit serum (NRS), or of unlabeled AP-1p (OPN) competitor (comp.) DNA (300 nM) as indicated. Protein-DNA complexes were resolved by polyacrylamide (7%, wt/vol) gel electrophoresis. Autoradiography was performed using an intensifying screen (66-h exposure for panel a, 6-h exposure for panel b). The positions of free DNA and of DNA:protein complexes are indicated in the margins. (**c**) Chromatin-immunoprecipitation (ChIP) analysis of sheared extracts derived from formaldehyde-treated CEF/MH2 or CEF/ASV17 cells. Immunoprecipitations were carried out using Jun- or Fos-specific antibodies followed by PCR amplification of DNA segments encompassing the AP-1 binding sites of the chicken *OPN* or *JAC* promoters as described for panel a. Control immunoprecipitations included samples treated with no antibody or with NRS. PCR products were analysed by agarose(1.4%, wt/vol) gel electrophoresis, and visualized by ethidium-bromide staining. The sizes of the PCR products (in bp) are indicated in the margin. (**d**) Analysis of the c-Fos (p62$^{c\text{-}fos}$) protein in normal CEF and in MH2- or ASV17-transformed CEF. Proteins were analysed by immunoprecipitation using a Fos-specific antiserum and SDS–PAGE (10%, wt/vol). The fluorograph was exposed for 5 days. The positions of molecular weight markers (in kDa) are indicated in the margin.

of *OPN* and *126MRP*, and also of *OPN* and *rac*2, were engineered to test for possible synergistic effects in cell transformation. Protein expression in transfected and metabolically labeled cells was monitored by immunoprecipitation using antibodies directed against the FLAG peptide or against a 220-amino-acid recombinant OPN protein (Figure 5b). FLAG-126MRP (M_r 15 082) and FLAG-Rac2 (M_r 22 528) displayed apparent molecular weights of 18 000 and 24 000, respectively. FLAG-OPN (M_r of 33 445) migrated with an apparent size of 53 000. A similar anomalous electrophoretic mobility was also observed for the *in vitro* translated and immunoprecipitated non-tagged full-length OPN (M_r 29 335) and the amino terminally truncated recombinant protein OPN220 (M_r 24 311) displaying apparent molecular weights of 52 000 and 41 000, respectively (Figure 5b). The aberrant electrophoretic mobility may be due to the acidic character of OPN proteins (Denhardt *et al*., 2001) (the calculated pI of quail OPN is 4.25). In contrast to the FLAG-OPN protein, the non-tagged OPN protein encoded by the bicistronic FLAG-126MRP-IRES-OPN and FLAG-Rac2-IRES-OPN constructs was expressed in multiple forms (apparent molecular weights of 52 000, 56 000, 62 000, and 70 000), presumably due to post-translational modification (Sørensen *et al*., 1995). This is in agreement with previous observations of multiple forms of fowl OPN with sizes ranging from 43 000 to 70 000 under denaturing SDS–PAGE conditions (Castagnola *et al*., 1991; Kuykindoll *et al*., 2000).

Fibroblast cultures fully infected with retroviruses harvested from the transfected cells displayed morphological alterations compared to cells infected with the empty RCAS virus. QEF expressing FLAG-OPN or FLAG-126MRP became slightly more elongated, whereas cells expressing FLAG-Rac2 displayed a more round-shaped morphology. Simultaneous expression of FLAG-126MRP and OPN, or FLAG-Rac2 and OPN led to more pronounced morphological changes of the cells. Control cells transformed by the MH2 virus specifying a v-Myc protein and a Gag–Mil hybrid protein displayed the typical spindle-shaped morphology of v-*myc*/v-*mil*-transformed fibroblasts which is clearly distinguishable from the round-shaped morphology of v-*myc*-transformed cells (Figure 5c, cf. Figure 1a). Most significantly, CEF expressing

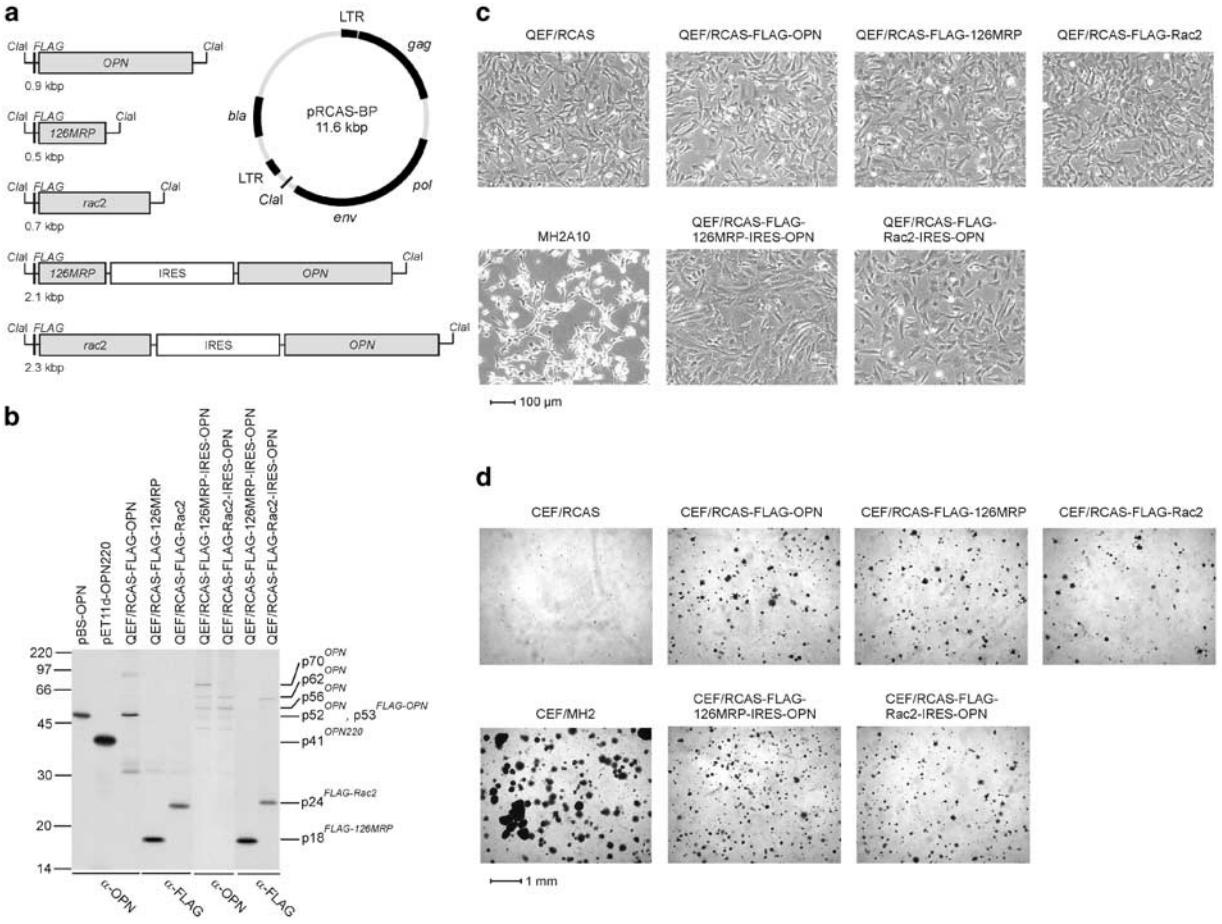

Figure 5 Cell transformation induced by ectopic expression of genes specifically activated in v-*myc*/v-*mil*-transformed fibroblasts. (**a**) Coding sequences of the *OPN*, *126MRP*, and *rac2* genes were inserted into the replication-competent retroviral pRCAS-BP vector via the unique *Cla*I restriction enzyme site. *bla*, *β*-lactamase coding sequence; LTR, long terminal repeat; *gag*, group-specific antigen; *pol*, polymerase; *env*, envelope glycoprotein; kbp, kilobasepairs; FLAG, 8-amino acid epitope; IRES, internal ribosomal entry site. (**b**) Immunoprecipitation and SDS–PAGE (12%, wt/vol) analysis of OPN, FLAG-OPN, FLAG-126MRP, and FLAG-Rac2 proteins using polyclonal antibodies directed against a 220-amino-acid recombinant OPN polypeptide, or a monoclonal antibody directed against the amino terminal FLAG epitope. Equal aliquots (2×10^7 cpm) of extracts from [^{35}S]methionine-labeled QEF transfected with pRCAS-FLAG-OPN, pRCAS-FLAG-126MRP, pRCAS-FLAG-Rac2, pRCAS-FLAG-126MRP-IRES-OPN, or pRCAS-FLAG-Rac2-IRES-OPN were analysed. Proteins from *in vitro* transcription and translation reactions using the plasmid constructs pBS-OPN or pET11d-OPN220 were analysed as a control (lanes 1 and 2). Fluorographs were exposed for 15 h (lanes 1 and 2), 6 days (lane 3), 2 days (lanes 4, 5, 8 and 9), or 10 days (lanes 6 and 7). Positions of protein size markers (in kDa) are indicated in the margin. (**c**) Phase-contrast micrographs of MH2A10 cells, and of QEF transfected with vector DNA (QEF/RCAS) or with the retroviral constructs shown in panel a. (**d**) Agar colony formation of CEF infected by the MH2 virus or by viruses derived from the RCAS vector or the retroviral constructs shown in panel a. Equal numbers of cells (2.5×10^4) were seeded in soft agar on 35-mm dishes, and bright-field micrographs were taken after 16 days.

FLAG-OPN, FLAG-126MRP, or FLAG-Rac2 formed colonies in soft agar, with OPN and 126MRP displaying the higher capacity of inducing anchorage-independent growth (Figure 5d). Colony formation by MH2-transformed CEF was used as a control. Simultaneous expression of OPN and FLAG-126MRP led to a significant increase in colony numbers suggesting that these proteins act in a synergistic manner. A similar, but less pronounced effect was observed for the coexpression of OPN and FLAG-Rac2. The results suggest that the activation of *OPN*, *126MRP* and possibly *rac*2 is involved in v-*myc*/v-*mil*-induced cell transformation.

Cooperative cell transformation by the myc and mil oncogenes

Analysis of the genome structure of the natural retroviral isolate MH2 had provided strong support to the notion that oncogenes cooperate in cell transformation (Jansen *et al*., 1983a, b). This was further supported by the oncogenic spectrum of the retroviral construct J2 combining v-*myc* and v-*mil*(*raf*) oncogenes in a murine vector (Rapp *et al*., 1985). To directly demonstrate that the two oncogenes *myc* and *mil* cooperate in cell transformation, the coding sequence of v-*mil* and an internal ribosomal binding site (IRES) were inserted into the replication-defective RCAS-MC29 vector

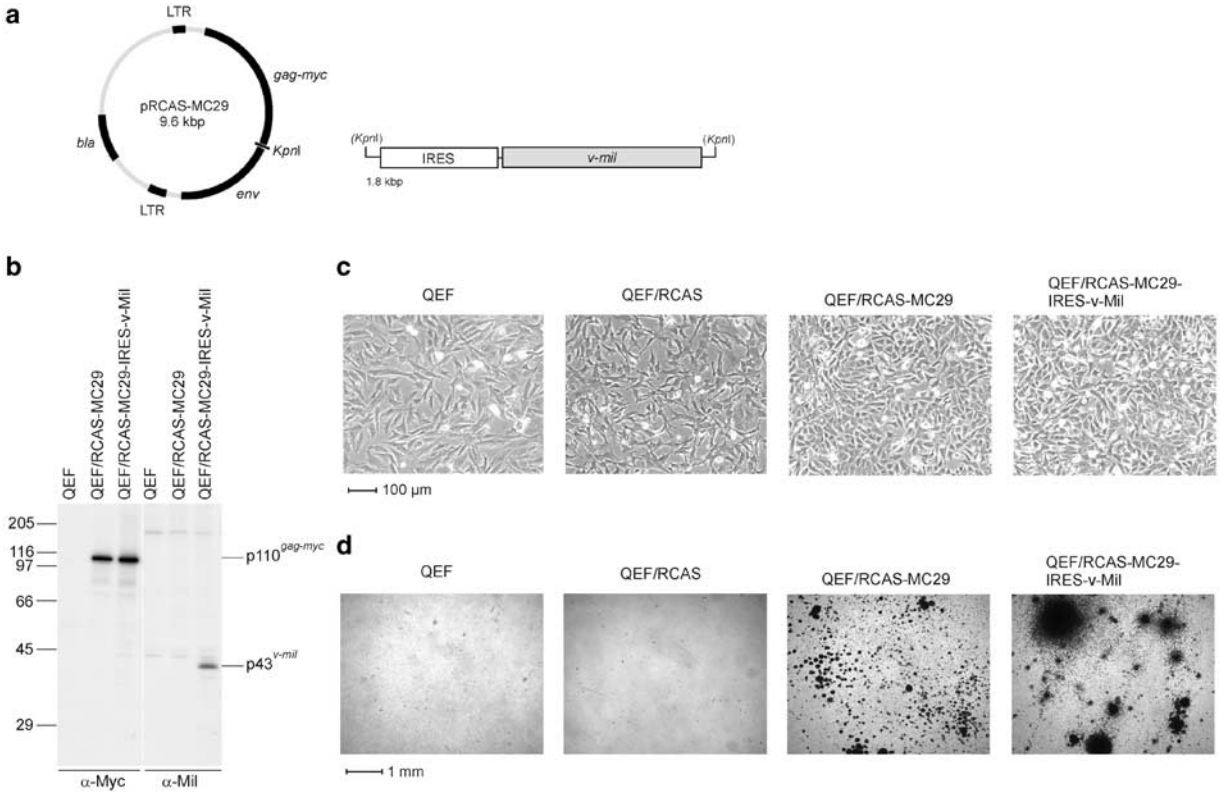

Figure 6 Cooperative cell transformation by *myc* and *mil* oncogenes expressed from a bicistronic construct. (**a**) Insertion of the IRES element and the coding sequence of v-*mil* into the retroviral construct pRCAS-MC29 (Tikhonenko and Linial, 1992) yielding pRCAS-MC29-IRES-v-Mil. (**b**) Immunoprecipitation and SDS–PAGE (10%, wt/vol) analysis of Gag–Myc and v-Mil proteins using polyclonal antibodies directed against Myc and Mil proteins. Equal aliquots (2×10^7 cpm) of extracts from [^{35}S]methionine-labeled QEF transfected with pRCAS-MC29 or pRCAS-MC29-IRES-v-Mil were analysed. The fluorograph was exposed for 23 h. Positions of protein size markers (in kDa) are indicated in the margin. (**c**) Phase-contrast micrographs of QEF and of QEF transfected with vector DNA (QEF/RCAS) or with the retroviral constructs shown in panel a. (**d**) Agar colony formation of the cells shown in panel c. Cells (5×10^4) were seeded in soft agar on 35-mm dishes, and bright-field micrographs were taken after 14 days.

(Tikhonenko and Linial, 1992) specifying the p110 Gag–Myc hybrid protein (Figure 6a). The construct (RCAS-MC29-IRES-v-Mil) was co-transfected into QEF together with the replication-competent RCAS vector allowing the generation of infective virus particles. As controls, RCAS-MC29 and RCAS plasmids were co-transfected analogously. Protein analysis of the transfected cells showed that the Gag–Myc and v-Mil proteins were efficiently translated from the bicistronic MC29-IRES-v-Mil mRNA, whereas in RCAS-MC29-transfected cells only the p110 Gag–Myc polypeptide was detectable (Figure 6b). Both constructs, RCAS-MC29-IRES-v-Mil and RCAS-MC29, significantly enhanced cell proliferation accompanied by characteristic morphological changes in the transfected cells (Figure 6c), and induced the capacity of anchorage-independent growth (Figure 6d). No colonies were obtained with normal QEF or QEF transfected with the empty RCAS vector. Colonies induced by RCAS-MC29-IRES-v-Mil-transformed cells were significantly larger in size compared to colonies from RCAS-MC29-transformed cells. This corroborates the results obtained with established cell lines and with cells transformed with RCAS-v-Mil (cf. Figure 1a and b), demonstrating that the oncogenes *myc* and *mil* synergistically cooperate in cell transformation.

Discussion

Abnormal cell signaling leads to modified cytoskeletal structures and transcriptome alterations manifested by aberrantly regulated target genes. Several transcription factors positioned at the end of mitogenic signaling casacades have oncogenic potential, and mutant or deregulated forms of such transcriptional regulators are able to induce cancer (Dang, 1999; Grandori *et al*., 2000; Eisenman, 2001). A deregulated Ras/Raf(Mil)/MEK/ERK signaling pathway is involved in many cancer types and leads to the activation of nuclear effectors at the transcriptional or post-transcriptional level, for instance by phosphorylation of critical gene regulators. A prototypic nuclear effector of this signaling cascade is AP-1 (Rapp *et al*., 1994; Suzuki *et al*., 1994; Eferl and Wagner, 2003), but there is also evidence that other transcription factors like Myc could participate in this mitogenic signaling pathway (Kerkhoff and Rapp, 1998; Lefevre *et al*., 2003; Polsky and Cordon-Cardo, 2003),

although the molecular mechanisms of Myc activation remain still obscure. There is clear evidence that Myc and Mil(Raf) act synergistically in cell transformation. The strikingly high oncogenic potential of avian leukemia and carcinoma virus MH2 (Bister and Jansen, 1986; Graf *et al*., 1986) and of its constructed murine counterpart J2 (Rapp *et al*., 1985) is apparently due to the combination of the v-*myc* and v-*mil*(*raf*) oncogenes in one retroviral genome leading to concomitant high-level expression of the v-Myc and v-Mil(Raf) oncoproteins. However, v-Myc expression does not lead to increased c-Mil protein levels, and, *vice versa*, v-Mil does not induce elevated c-Myc levels (cf. Figure 1c). A possibly complex mechanism of synergism could be based on the acceleration of cell division and proliferation by v-Myc, strongly enhancing the v-Mil(Raf)-induced aberrant mitogenic signaling pathway (Kerkhoff and Rapp, 1998; Grandori *et al*., 2000).

To investigate the molecular basis of the strong enhancement of malignancy in the course of v-*myc*/v-*mil*-induced cell transformation and tumorigenesis, we aimed at the isolation of genes that were specifically activated in MH2-transformed cells. Intriguingly, the screen led to the isolation of specifically overexpressed genes that represent the avian homologs of genes previously implicated in human cancer, providing a well-defined experimental cell transformation system to study the function and regulation of these important cancer-related genes. The OPN protein product of the osteopontin gene (*OPN*) is an acidic hydrophilic glycophosphoprotein that functions both as a cell attachment protein displaying a GRGDS cell adhesion sequence (cf. Figure 2a) and as a cytokine that signals through integrin and CD44 cell adhesion molecules (Denhardt *et al*., 2001; Rittling and Chambers, 2004). Originally discovered as a phosphoprotein secreted by transformed cells in culture (Senger *et al*., 1979), it is now recognized as a multifunctional protein implicated in development, neoplastic change, tumor progression and metastasis, but is also abundant in the mineral matrix of bones binding tightly to hydroxyl apatite (Mi *et al*., 2004; Rittling and Chambers, 2004). OPN not only represents a classical marker for metastatic cells, but has proven intrinsic potential to enhance the metastatic phenotype of tumor cells (Moye *et al*., 2004; Rittling and Chambers, 2004; Teramoto *et al*., 2005). Although there is strong evidence that overexpression of OPN at the mRNA and protein level represents a key molecular event in tumor progression and metastasis, the molecular mechanisms and signaling pathways leading to its transcriptional activation and its precise biochemical function in these disease-related processes are largely unknown (Mi *et al*., 2004; Rittling and Chambers, 2004). In this study we have identified *OPN* as a strongly and specifically activated gene in v-*myc*/v-*mil*-transformed cells that also contain markedly elevated levels of the AP-1 components c-Jun and c-Fos (cf. Figure 1), and we show that activation of the *OPN* promoter is indeed mediated by binding of AP-1 complexes to a specific site, and that c-Fos may be the principle activating component. Intriguingly, there is strong evidence that Fos proteins are required for malignant progression (Saez *et al*., 1995) and that they trigger a transcriptional program responsible for cell invasion, a process crucial for morphogenesis and cancer metastasis (Sherwood *et al*., 2005). Recently, analyses of murine *OPN* promoters have also identified AP-1 binding sites as critical elements for transcriptional activation (Bidder *et al*., 2002; El-Tanani *et al*., 2004; Mi *et al*., 2004; Sakata *et al*., 2004). Furthermore, it has been suggested that AP-1 is, at least in part, responsible for the overexpression of *OPN* in human breast cancer (El-Tanani *et al*., 2004).

Ectopic expression of *OPN* from a retroviral vector leads to agar colony formation in primary chicken fibroblasts (cf. Figure 5d), demonstrating intrinsic oncogenic potential of this gene in a direct cell transformation assay. This extends previous results showing that this gene promotes metastatic potential when introduced into established rat mammary epithelial cell lines (Moye *et al*., 2004; Teramoto *et al*., 2005). The transforming potential of *OPN* is enhanced by concomitant ectopic expression of the *126MRP* gene that is also activated in v-*myc*/v-*mil*-transformed cells, suggesting that *OPN* and *126MRP* are able to act in a synergistic manner. The 126MRP protein belongs to the S100 family of small proteins that contain EF-hand calcium-binding domains and are implicated in intracellular and extracellular regulatory activities (Donato, 1999; Marenholz *et al*., 2004). Interestingly, both OPN and S100 proteins bind Ca^{2+}, and it has been suggested that calcium-ion binding of phosphorylated OPN influences its conformation in combination with the high density of negative charges around the GRGDS cell-binding sequence (Denhardt *et al*., 2001). Free calcium levels may therefore have an impact on the interaction between OPN and its integrin or CD44 receptors (Denhardt *et al*., 2001; Weber, 2001). Intriguingly, the human homologs of both *OPN* and *126MRP* are implicated in cell invasion and metastasis. *OPN* expression, as discussed above, strongly correlates with the metastatic phenotype, and the *126MRP*-related *S100A4* gene encodes a metastasis-associated cytoskeletal calcium-binding protein, termed metastasin (Marenholz *et al*., 2004). Strikingly, in a *neu*/*S100A4* transgenic model of metastatic breast cancer, ectopic expression of *S100A4* leads to induction of the *126MRP* ortholog *S100A9* and of *OPN* (Simpson *et al*., 2003), corroborating our finding that these two genes are implicated in cell transformation.

The highly conserved Rac guanosine triphosphatases are members of the Rho family within the superfamily of Ras-related small GTPases, and play distinct roles in actin organization, cell proliferation, polarity, and migration. Rac signaling is also implicated in invasive and metastatic cell growth (Kurisu *et al*., 2005), and it was recently suggested that autocrine activation of an OPN-CD44-Rac pathway in Ras-transformed cells enhances transformation and motility (Teramoto *et al*., 2005). Based on the observation that ectopic expression of *rac*2 leads to agar colony formation in CEF (cf. Figure 5d), it is conceivable that the Rac2 protein

accelerates aberrant autocrine signaling pathways and thus contributes to v-*myc*/v-*mil*-induced cell transformation. The results reported here demonstrate that cooperative v-*myc*/v-*mil*(*raf*)-induced cell transformation involves activation of genes and pathways implicated in human cancer progression and metastasis, providing an experimental system for further molecular analyses.

Materials and methods

Cells and retroviruses

Sources of fertile Japanese quail (*Coturnix japonica*) or chicken (*Gallus gallus*) eggs, cell culture, DNA transfection, and preparation and transformation of chicken or quail embryo fibroblasts (CEF, QEF) have been described (Hartl and Bister, 1998; Bader *et al*., 2001; Hartl *et al*., 2001, 2003). Colony assays performed in 0.3%(wt/vol) agarose were done as described (Hartl *et al*., 2001). The established QEF cell lines Q8 and MH2A10 are transformed by the v-*myc* oncogene of avian acute leukemia virus MC29 (Bister *et al*., 1977), and by the v-*myc* and v-*mil* oncogenes of avian acute leukemia virus MH2 (Jansen *et al*., 1983a), respectively. Infection of CEF with avian retroviruses was performed as described previously (Hartl *et al*., 2001). QEF transformed by the retroviral constructs pRCAS-MC29 (Tikhonenko and Linial, 1992) or pRCAS-v-Jun (pRCAS-VJ1) carrying the v-*myc* or v-*jun* oncogenes, respectively have been described (Hartl and Bister, 1998). For construction of pRCAS-v-Mil, a 1134-bp PCR-amplified segment of *gag-mil* (Sutrave *et al*., 1984) specifying the last 377 amino acids of v-Mil including the stop codon was inserted into the pA-CLA12NCO adaptor plasmid (Hughes *et al*., 1987) yielding pA-v-Mil. The v-*mil* insert was then excised with *Cla*I and ligated into the replication-competent retroviral pRCAS vector (subgroup A) (Hughes *et al*., 1987). For construction of pRCAS-FLAG-OPN, pRCAS-FLAG-126MRP, and pRCAS-FLAG-Rac2, the coding sequences without the start codon but including the stop codon of quail *OPN*, *126MRP*, and *rac*2 were first ligated into the *Eco*RV and *Bam*HI sites of the adaptor plasmid pA-FLAG and subsequently transferred into the pRCAS-BP vector as described (Hartl *et al*., 2001). To generate pA-FLAG-Rac2-IRES-OPN, the 1127-bp *Sma*I/*Pvu*II fragment from pA-OPN, consisting of an 1194-bp *OPN* cDNA *Eco*RI-fragment ligated into pA-CLA12 (Hughes *et al*., 1987), was inserted into pA-FLAG-Rac2-IRES cut by *Sma*I. To construct pA-FLAG-Rac2-IRES, the 612-bp *Nco*I/*Bam*HI(filled in) fragment of pA-FLAG-Rac2 was ligated into pA-IRES cut by *Nco*I/*Eco*RI(filled in). To generate pA-IRES, the 630-bp *Eco*RI-*Bam*HI fragment of pIRES (BD Biosciences, Vienna, Austria) encompassing the internal ribosome entry site (IRES) of encephalomyocarditis virus (ECMV) (Jackson *et al*., 1990) was inserted into pA-CLA12NCO. To construct pA-FLAG-126MRP-IRES-OPN, a 1327-bp *Mlu*I(filled in)/*Pvu*I fragment from pA-FLAG-Rac2-IRES-OPN containing the FLAG-Rac2 coding sequence was replaced by the corresponding 1097-bp *Bam*HI(filled in)/*Pvu*I fragment from pA-FLAG-126MRP. A 141-bp *Sac*II(blunted)/*Sma*I fragment containing the original polyadenylation signal of the *OPN* cDNA was subsequently removed from pA-FLAG-Rac2-IRES-OPN and from pA-FLAG-126MRP-IRES-OPN. For construction of pRCAS-MC29-IRES-v-Mil, a 1840-bp *Eco*RI(filled in) fragment containing the IRES element and the coding sequence of v-*mil* was excised from pIRES-0-v-Mil and ligated into pRCAS-MC29 which has been linearized by *Kpn*I und blunt-ended using the 3′–5′ exonucleolytic activity of T4 DNA polymerase. To construct pIRES-0-v-Mil, the 1224-bp *Xba*I fragment encompassing the v-*mil* coding sequence was released from pA-v-Mil and ligated into pIRES cut by *Xba*I.

Target gene isolation and nucleic-acid analyses

cDNA library construction, DNA labelling, plaque hybridization, subcloning, Northern analysis, and nucleotide sequencing were performed as described (Hartl and Bister, 1998; Bader *et al*., 2001; Hartl *et al*., 2001, 2003). Subtractive probe generation and representational difference analysis (RDA) were done as described previously (Hartl *et al*., 2001; Bader *et al*., 2001) using RNAs isolated from QEF and from MH2A10 cells for generation of driver and tester cDNAs, respectively. For isolation of full-length cDNA clones representing the *OPN* and *126MRP* genes with insert sizes of 1170 and 509 bp, respectively, a MH2A10-specific subtracted probe was used to screen a cDNA library generated from MH2A10 cells. For isolation of a *rac*2-specific 895-bp full-length clone from the MH2A10 cDNA library, an appropriate RDA fragment was used as a probe. Hybridization probes for Northern analyses included cDNA fragments derived from quail *OPN*, *126MRP*, and *rac*2 isolated in this screen, and from quail glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as described previously (Hartl and Bister, 1998). Bioinformatic analyses of nucleotide and amino-acid sequences of the genes identified and sequence interpretation were performed as described (Hartl and Bister, 1998; Bader *et al*., 2001; Hartl *et al*., 2001). To scan the chicken *OPN* promoter for potential transcription factor binding sites, the computer programs AliBaba2 (Biobase) and TESS (www.cbie.upenn.edu/tess) were applied. For multiple sequence alignments, the program ClustalW (DS/Gene, Accelrys) was used. The nucleotide and deduced amino-acid sequences reported here have been deposited in the GenBank database (accession nos. AF239805, AY583752, AF583754).

Transactivation analysis

For construction of the chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT-OPN, a 1242-bp DNA fragment encompassing the chicken *OPN* promoter including 86 bp of the first exon was amplified from chicken genomic DNA by PCR and then inserted into the *Sph*I/*Sal*I sites of the pCAT-Basic vector (Promega, Mannheim, Germany). This *OPN* promoter segment corresponds to nucleotides 16015255–16014014 of contig 6 (accession number NW_060347.1) from chicken chromosome 4. To construct the deletion mutants pCAT-OPN348, pCAT-OPN197, pCAT-OPN96, the *OPN* promoter was progressively deleted using *Pst*I, *Dpn*I, or *Hae*III restriction enzyme sites leaving 348, 197, or 96 bp promoter segments upstream of the transcription start site which has been mapped previously (Rafidi *et al*., 1994). Site-directed deletion mutagenesis was performed as described (Hartl *et al*., 2001) using pCAT-OPN, pCAT-OPN197, or pCAT-OPN96 as templates, and mutator oligodeoxynucleotides leading to deletion of the sequences 5′-TGACTCC-3′ (pCAT-OPNΔAP1d), 5′-TCATGACCTCA-3′ (pCAT-OPN ΔAP1p, pCAT-OPN197ΔAP1p, pCAT-OPN96ΔAP1p), or 5′-TTTAAATCCC-3′ (pCAT-OPN197ΔTATA) that contain distal (d) or proximal (p) AP-1 binding motifs, or the TATA -box, respectively. The double mutant pCAT-OPN197 ΔAP1pΔTATA was constructed using pCAT-OPN197ΔTA TA as a template. The eukaryotic expression vectors pRc-c-Jun, pRc-JunD, pRc-v-Jun, pRc-c-Fos, pRc-Fra-2, pRc-v-Myc have been described (Hartl *et al*., 2001, 2003). DNA transfer into cells by the nucleofection technology (Axama

Biosystems, Cologne, Germany) was performed as described (Hartl *et al*., 2003). For all nucleofections, liposome solution V was applied in combination with the electroporation program T-20. CAT-analysis and quantification of radioactive signals using a phosphor imager were performed as described (Hartl *et al*., 2001, 2003).

Protein-DNA interaction analyses

In vitro translation of proteins, preparation of nuclear extracts from subconfluently grown cells, and electrophoretic mobility shift assays (EMSA) were carried out as described (Hartl and Bister, 1998; Hartl *et al*., 2001). A ^{32}P-labeled 51-bp double-stranded oligodeoxynucleotide corresponding to nucleotide positions −110 to −60 upstream of the *OPN* transcription start site and containing the two overlapping non-consensus AP-1 binding motifs was prepared as described (Hartl and Bister, 1998). The DNA probe encompassing the consensus AP-1 binding site from the chicken *JAC* promoter has been described previously (Hartl *et al*., 2001). Binding reactions were performed at 25°C in a buffer containing 10 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM NaCl, 10%(vol/vol) glycerol, 2 mM DTT, 2 mM spermidine (for *in vitro* translated proteins), or a buffer containing 12 mM HEPES pH 7.9, 4 mM Tris-HCl pH 7.9, 60 mM KCl, 12%(vol/vol) glycerol, 1 mM EDTA, 1 mM DTT (for nuclear extract proteins), each supplemented with 133 ng/μl of poly(dI-dC)·poly(dI-dC). To test for binding specificity, 1-μl aliquots of polyclonal antisera directed against Jun, Fos, or Myc proteins were added 30 min after the binding reaction was initiated, and incubation was continued for another 15 min.

Chromatin immunoprecipitation analysis (ChIP) was carried out as described previously (Chamboredon *et al*., 2003) with the following modifications. Samples of 2.5×10^7 cells grown on 100-mm dishes were cross-linked with formaldehyde (final concentration 1%). The washed and pelleted material was then resuspended in 1 ml of SDS-lysis buffer containing 1%(wt/vol) sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl pH 8.1 supplemented with protease inhibitors. Chromosomal DNA was sheared to yield fragments with sizes between 0.2 and 1.0 kbp using an ultrasonifier (Branson, Danbury, CT, USA) equipped with a microtip at constant 40 W (25% power) for 6×10 s. Sonicated lysates were centrifuged, and the supernatant was diluted ten-fold with ChIP dilution buffer containing 0.01%(wt/vol) SDS, 1.1%(wt/vol) Triton®X-100 (Merck), 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, and 167 mM NaCl. For immunoprecipitation, 1 ml of this solution was precleared by adding 80 μl of a 50%(wt/vol) protein A sepharose CL-4B (Amersham Biosciences, Vienna, Austria) bead slurry supplemented with 0.2 μg/μl sonicated salmon sperm DNA and 0.5 μg/μl BSA, and gentle shaking for 60 min at 4°C. The beads were pelleted, and the supernatant was incubated with 2 μl of specific antiserum at 4°C for 16 h. Protein A sepharose slurry was added and samples processed as above. The pellets were washed twice with washing buffer 1 (0.5% sodium deoxycholate, 1% Nonidet P40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl), once with washing buffer 2 (0.05% sodium deoxycholate, 0.1% Nonidet P40, 50 mM Tris-HCl pH 7.5, 500 mM NaCl), once with washing buffer 3 (0.05% sodium deoxycholate, 0.1% Nonidet P40, 50 mM Tris-HCl pH 7.5), and finally twice with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Immunoprecipitated cross-linked protein-DNA complexes were incubated for 30 min at 25°C in 450 μl of elution buffer containing 1% SDS and 100 mM $NaHCO_3$. NaCl was then added to a final concentration of 192 mM and incubation was continued at 65°C for 4 h. As a control, a 450-μl input sample containing 40 μl of sonicated and diluted cell lysate and 410 μl of elution buffer was treated analogously. Then, EDTA, Tris-HCl pH 6.5, and proteinase K were added to final concentrations of 9.4, 36 mM, and 3.6 μg/ml, respectively, and incubation was continued for 1 h at 45°C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Thirty-three PCR cycles (94°C/1 min; 54°C (*OPN*), 56°C (*JAC*)/1 min; 72°C/1 min) were carried out using 20% each of the recovered DNAs with primers flanking functional AP-1 binding sites present in the *OPN* or *JAC* (Hartl *et al*., 2001) promoters. The *OPN*- and *JAC*-specific PCR primer pairs were 5′-CTTCAGAAGCCACAAAGC-3′/5′-GAGAGCAGCCACCAATCC-3′ and 5′-ATCCTATCT GGTAAAACACC-3′/5′-TGAATTTGTCCTCACTGC-3′, respectively.

Recombinant protein purification and antiserum generation

OPN-specific antiserum was generated using a purified recombinant polypeptide as immunogen containing the carboxyl terminal 219 amino acids of quail OPN and a vector-encoded start methionine (OPN220). For construction of pET11d-OPN220, a PCR fragment encompassing the coding region for amino-acid residues 46–264 and the stop codon was ligated into the bacterial expression vector pET-11 d (Novagen, Vienna, Austria). Transformation of *Escherichia coli* BL21(DE3)pLysS with pET11d-OPN220, and induction of recombinant protein synthesis using isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1.0 mM were performed as described (Kerkhoff *et al*., 1991; Hartl and Bister, 1998; Bader *et al*., 2001). Cells were pelleted and resuspended in 30 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA) per liter of the original bacterial culture, and lysed by a freeze–thaw cycle. DNA was sheared by passing the lysate through a 20 G needle. After centrifugation at 26 000 g for 30 min, the supernatant was fractionated by ammonium sulfate precipitation. The precipitate formed between 40 and 50% saturation was dissolved and dialyzed at 4°C in phosphate-buffered saline (PBS) containing 1 mM DTT, and then loaded onto a FPLC MonoQ anion exchange column (Amersham Biosciences, Vienna, Austria). Proteins were eluted with a sodium chloride gradient generated by mixing PBS (150 mM NaCl) and high salt PBS (1 M NaCl). Fractions containing OPN220 were concentrated using a Centriprep-10 ultrafiltration device (Amicon, Vienna, Austria), and the protein was further purified using a Superdex-75 gelfiltration column (Amersham Biosciences, Vienna, Austria). The final yield of purifed OPN220 protein was 3.6 mg per liter of bacterial culture. Immunization and antiserum preparation were done as described (Hartl and Bister, 1998; Bader *et al*., 2001). Polyclonal antisera directed against recombinant Jun, Fos, or Myc proteins, or against a carboxyl terminal Mil undecapeptide, and a monoclonal antibody directed against the FLAG epitope have been described (Hartl and Bister, 1998; Patschinsky and Bister, 1988; Hartl *et al*., 2001).

Protein analysis

To construct pBS-OPN, the *Cla*I insert of pA-OPN containing the coding region of OPN (see above) was inserted into pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). pBS-OPN and pET11d-OPN220 specifying the truncated OPN protein (see above) were used for *in vitro* transcription–translation. Immunoprecipitations under denaturing conditions of L-[^{35}S]methionine-labeled proteins from cell lysates or supernatants, or from *in vitro* transcription–translation reactions using polyclonal or monoclonal antibodies, and SDS–PAGE were done as described (Hartl and Bister, 1998; Hartl *et al*., 2001, 2003).

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