Oncogene (2003) 22, 4047–4061
© 2003 Nature Publishing Group All rights reserved 0950-9232/03 $25.00
www.nature.com/onc

v-Jun downregulates the SPARC target gene by binding to the proximal promoter indirectly through Sp1/3

Sandrine Chamboredon[1], Joseph Briggs[2], Emmanuel Vial[1], Julien Hurault[1], Federico Galvagni[3], Salvatore Oliviero[3], Timothy Bos[2] and Marc Castellazzi*[,1]

[1]*Unité de Virologie Humaine, INSERM-U412, Ecole Normale Supérieure, 46 allée d'Italie, 69364 Lyon cedex 07, France;* [2]*Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, PO Box 1980, Norfolk, VA 23501, USA;* [3]*Dipartimento di Biologia Molecolare, Universita degli Studi di Siena, via Fiorentina 1, 53100 Siena, Italy*

Transformation of chick embryo fibroblasts by the v-Jun oncoprotein correlates with a downregulation of the extracellular matrix protein SPARC and repression of the corresponding mRNA. Repression of SPARC contributes to the oncogenic process by facilitating tumor development *in vivo*. A proximal promoter fragment, designated −124/+16, is responsible for high constitutive activity of the SPARC gene and is the target of repression by v-Jun. In this paper, using electrophoretic mobility shift and pull-down assays *in vitro*, and transient transfections and chromatin immunoprecipitation assays in Sp1/3-deficient *Drosophila* SL2 cells and in chick embryo fibroblasts, we show that (i) Sp1 and/or Sp3 is required for constitutive activation of SPARC transcription, by binding directly to the GGA-rich −92/−57 fragment; and (ii) v-Jun does not bind −124/+16 directly, but binds to the GGA-rich fragment indirectly, most likely through a physical interaction with Sp1/3. Moreover, a transactivation-proficient v-Jun derivative, designated v-Jun/cebp/glz, which cannot bind Jun DNA motifs anymore and cannot heterodimerize, is still capable of downregulating SPARC efficiently. Taken together, these data strongly suggest that v-Jun downregulates SPARC through the formation of a DNA–Sp1/3–v-Jun, chromatin-associated complex.
Oncogene (2003) **22,** 4047–4061. doi:10.1038/sj.onc.1206713

Keywords: cell transformation; v-Jun; Sp1; Sp3; SPARC; primary chick embryo fibroblasts

Introduction

Cell transformation by transcription factor Jun is generally assumed to take place through the activation or repression of a limited number of direct target genes. The characterization of these targets constitutes an important issue in order to decipher the oncogenic pathways downstream of this transcription factor. Direct targets are expected to contain a functional Jun-binding DNA element in their promoter and constitute a critical intermediate in cell transformation and tumorigenesis (Vogt *et al*., 1999; van Dam and Castellazzi, 2001; Vogt, 2001).

Studies from several laboratories led to the characterization of candidate targets in avian cells (Basso *et al*., 2000; Vial and Castellazzi, 2000; Bader *et al*., 2001) as well as in murine (Mettouchi *et al*., 1994; Fu *et al*., 2000) and human (Rinehart-Kim *et al*., 2000) cells. Although different techniques were used for screening, the same statistics was observed, that is, 2/3 of the genes are activated by Jun and 1/3 are repressed. Several genes already satisfy the conditions of direct targets and are activated during transformation, such as *toj3*, a gene encoding heparin-binding EGF-like growth factor, and *jac* (Fu *et al*., 2000; Bader *et al*., 2001; Hartl *et al*., 2001), or are repressed, such as c-*jun* and *p53* (Hussain *et al*., 1998; Piu *et al*., 2002).

We have recently characterized several putative targets repressed by v-Jun in chick embryo fibroblasts (CEFs) (van Dam and Castellazzi, 2001). Among these, repression of the SPARC gene has been shown to facilitate tumorigenesis *in vivo* (Vial and Castellazzi, 2000). The encoded product SPARC (secreted protein, acidic, and rich in cysteine) is a minor component of the extracellular matrix. Its sequence is highly conserved during evolution, from *Caenorhabditis elegans* to chicken, mouse, cow, and man. Although its precise function is poorly understood, SPARC is generally considered as a regulator of matrix–cell interactions, such as thrombospondin and tenascin (Bornstein, 1995; Brekken and Sage, 2000). SPARC has independently been isolated as a target of mammalian c-Jun in primary rat embryo fibroblasts (Mettouchi *et al*., 1994) and in the human breast cancer cell line MCF7 (Rinehart-Kim *et al*., 2000). Its expression is altered in most human cancers, including breast (Bellahcene and Castronovo, 1995), prostate (Jacob *et al*., 1999), ovarian (Mok *et al*., 1996), and colorectal (Porte *et al*., 1995) cancer, and melanoma (Ledda *et al*., 1997).

Previous studies from our laboratory (Vial *et al*., 2000) also showed that in v-Jun-transformed CEFs, repression of SPARC takes place within a short, proximal promoter fragment, designated −124/+16. This minimal promoter reproduces the behavior of the endogenous SPARC, and constitutes a reliable model for analysis of transcriptional regulation by v-Jun.

*Correspondence: M Castellazzi; E-mail: marc.castellazzi@ens-lyon.fr
Received 24 January 2003; revised 28 April 2003; accepted 28 April 2003

Intriguingly, the minimal −124/+16 promoter displays no consensus Jun-binding site, raising the interesting question of how this oncoprotein represses SPARC transcription.

Results

Sp1/3(-like) proteins bind the minimal −124/+16 SPARC promoter in vitro

We have previously shown (Vial *et al*., 2000) that in CEF cells, a short, proximal fragment of the avian SPARC promoter, designated −124/+16, displays over 80% of the high basal activity and responds to repression by v-Jun and, more efficiently, by v-Jun-ml (a mutant that exhibits enhanced tumorigenicity *in vivo*; Huguier *et al*., 1998). The organization of this promoter fragment displays the following main features: (i) no TATA and CAAT box; (ii) a single major transcriptional start point; (iii) a purine-rich region with a core sequence of eight GGA direct repeats in a row (referred to as the 'GGA box' hereafter); and (iv) no potential TPA-responsive (TRE) or cAMP-responsive (CRE) Jun-binding AP1 site (Figure 1a).

A first series of experiments was designed to characterize protein complexes that bind −124/+16 *in vitro*. Uninfected CEF cultures as well as CEF cultures chronically infected by the nontransforming virus R, or the transforming viruses R-v-Jun and R-v-Jun-ml were generated and nuclear extracts prepared for electrophoresis mobility shift analysis. As shown in Figure 1b, using −124/+16 as a radioactive probe, a pattern of four discrete bands was found. The corresponding protein complexes were designated Cl, C2, C3, and C4, according to their increasing mobilities. The closely linked C2 and C3 corresponded to the most intense bands, whereas C4 was of much lower intensity, and C1 a faint, sometimes barely visible band of the highest molecular weight (Figure 1b–d).

To determine which fragment(s) of −124/+16 bind(s) the various complexes, a competition experiment was carried out with an excess of cold probe 1–7 (Figure 1a) and nuclear extracts from R-v-Jun-infected CEFs. As shown in Figure 1c, probe 3, which extends from −92 to −57 and encompasses the GGA box, eliminated all four complexes. Probe 4 (−74/−38), which partially overlaps probe 3, specifically retained C4. The other probes affected only poorly, if not at all, the different complexes. Thus, this experiment clearly indicated that the −92/−57 sequence is required for the binding of all four complexes. The same result was also obtained with the other nuclear extracts (data not shown).

Recent reports (Ihn *et al*., 1997; Tone *et al*., 2000) suggested that the purine-rich repeats of the GGA box might bind members of the Sp1 family of transcription factors. Therefore, attempts to supershift the different bands were performed using antibodies specifically directed against avian proteins Sp1 and Sp3, and nuclear extracts from R-v-Jun-infected CEFs (Figure 1d). Remarkably, antibodies against Sp1 eliminated C1 and C3, whereas antibodies against Sp3 eliminated C1 and C2. C4 was not affected by any of the anti-Sp antibodies tested. An identical result was obtained with the other nuclear extracts (data not shown). These data indicated that the ubiquitous transcription factors Sp1 and Sp3 (or closely related family members) contribute to the C1, C2, and C3 complexes, but not C4. This conclusion was confirmed by a competition experiment with a cold Sp1 consensus, GC-rich probe to which these transcription factors bind with high affinity (Figure 1c).

In a second series of experiments, *in vitro* prepared avian Sp1 and Sp3 (isolated in the course of this work) were tested for their ability to bind to probe −124/+16. As shown in Figure 2a, a single retarded band was obtained with Sp1 and Sp3, in a pattern that was reminiscent of the C2 and C3 bands obtained with the nuclear extracts (Figure 1b–d). Interestingly, bands equivalent to C1 and C4 were not observed, supporting the hypothesis that C1 consists of a complex with Sp1/3 and (an) additional protein(s) and that C4 does not contain any Sp1/3(-like) protein. Finally, as expected, direct binding of *in vitro* prepared Sp1 and Sp3 was demonstrated on the GGA-rich probe 3 and not, for instance, on probe 5. This binding was however reduced in comparison with that on the consensus GC-rich probe for Sp1 (Figure 2c and Discussion).

In conclusion, the gel shift experiments presented above allowed us to draw the following conclusions: (i) four retarded complexes, designated C1–C4, bind −124/+16 *in vitro*; (ii) the binding of all four complexes requires the −92/−57 region which includes the GGA box; (iii) three high-molecular weight complexes Cl, C2, and C3 contain Sp1/3(-like) transcription factors that are likely to bind DNA directly.

v-Jun does not bind the minimal −124/+16 SPARC promoter directly in vitro

As already stated, computer analysis revealed no consensus or related Jun-binding motif in −124/+16. In agreement with this, we confirmed that *in vitro* prepared v-Jun and v-Jun-ml did not bind to this probe directly (Figure 2a), even in the additional presence of Sp1 or/and Sp3 (or/and in the presence of dimerization partners like c-Fos and ATF2; Vial and Castellazzi, unpublished result). However, both Jun proteins were shown to bind to a consensus AP1 probe efficiently (Figure 2b).

The presence of v-Jun in the retarded complexes described above (C1–C4) was also investigated. Competition assays with a cold consensus AP1 probe (Figure 1c) as well as antibody supershift assays with anti-c/v-Jun antibodies (Figure 1d) were performed. They revealed no gross change in the pattern of the retarded complexes.

Sp1 and Sp3 activate transcription of the minimal −124/+16 SPARC promoter in Drosophila *SL2 cells*

We next wanted to know whether avian Sp1 and Sp3 are required for transcriptional activation *in vivo*. To do this, we chose to analyse their effect in the *Drosophila*

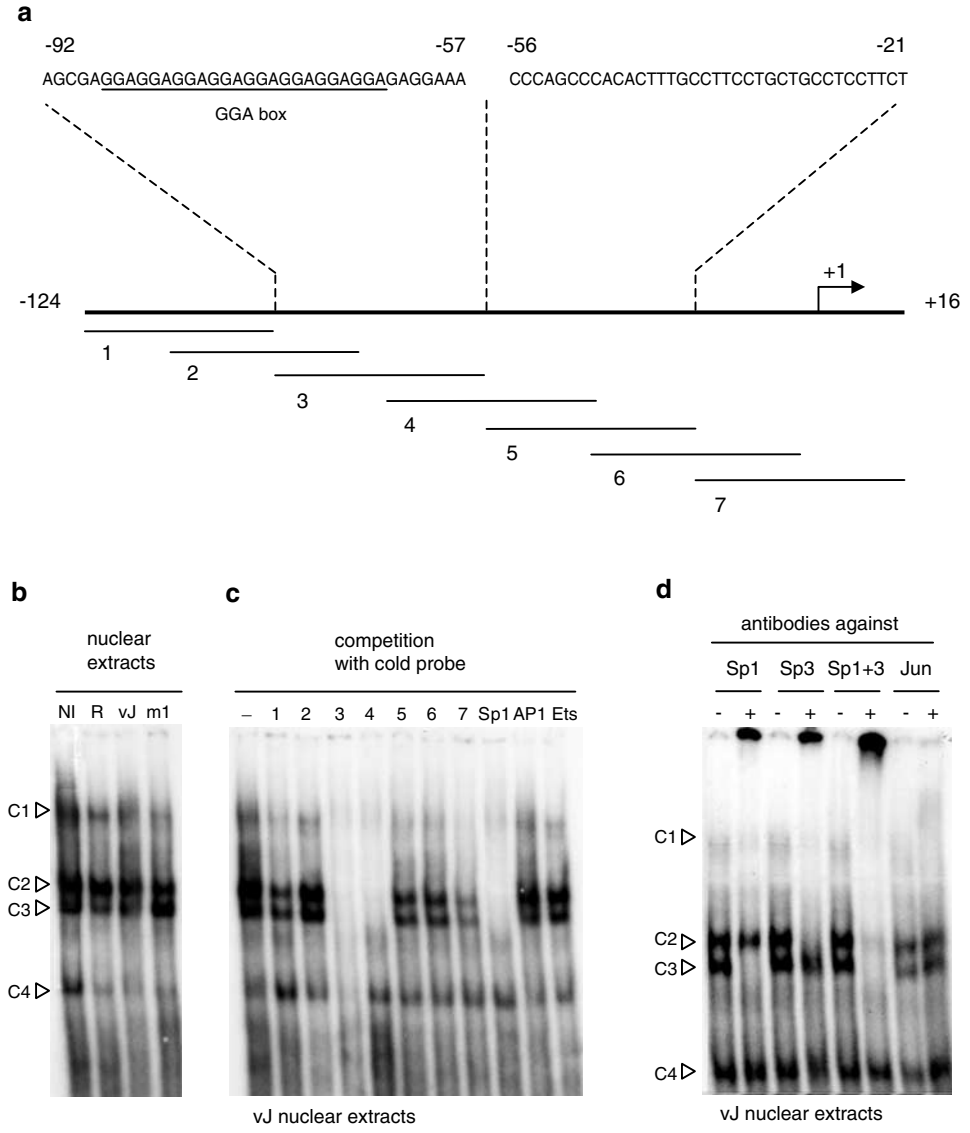
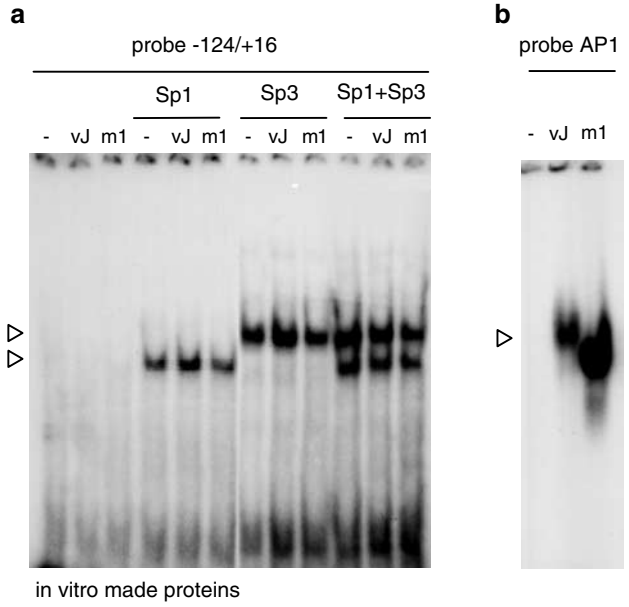


Figure 1 Electrophoretic mobility shift assays using nuclear extracts from nontransformed CEFs and CEFs stably transformed by v-Jun, and the ^{32}P-labeled $-124/+16$ minimal promoter fragment as a probe. (**a**) Schematic representation of the minimal $-124/+16$ promoter fragment and overlapping probes 1–7 covering the entire promoter. The sequences of probes 3 and 5 are shown. (**b**) Nuclear extracts from noninfected (NI), R-infected (R), R-v-Jun-infected (vJ), and R-v-Jun-ml-infected (ml) CEFs were used. Arrowheads show the retarded bands that correspond to protein complexes C1–C4. (**c**) Competition experiments were carried out with cold probes 1–7, cold consensus probe for Sp1/3 (sense: 5′-ATTCGATCGGGGCGGGGCGAGC) (Sp1), cold consensus probe for AP1 (sense: 5′-AGCTAGCATGAGTCAGACAC) (AP1), and cold nonrelevant, Ets probe (sense: 5′-TCGGGCTCGAGATAACCAG-GAAGTGGGC) (Ets); – indicates no probe. (**d**) Supershift experiments were performed using rabbit polyclonal antibodies directed against avian Sp1, Sp3, and v/c-Jun. – and + correspond to pre-immune serum and anti-serum of the same rabbit, respectively. Nuclear extracts in (**c**) and (**d**) were from R-v-Jun-infected CEFs

embryonic SL2 cell line (Schneider, 1972), which lacks homologs of mammalian transcription factors Sp1 and Sp3 (Courey and Tjian, 1988; Santoro *et al*., 1988). Transient transfection experiments were performed. The $-124/+16$ promoter was placed in front of the luciferase gene and the Sp1 and Sp3 genes were expressed under the control of the ubiquitous *Drosophila* actin AC5 promoter.

First, increasing amounts of Sp1 and Sp3 expression vectors were cotransfected with $-124/+16$ (and $-56/+16$ as a control). As shown in Figure 3a, there was no detectable activity in the absence of Sp1 and Sp3, whereas, independently, these factors activated the promoter in a dose-dependent manner. The reduced activation at the highest concentration of Sp1 might be due to the sequestration of a specific coactivator.

Second, the activation of $-124/+16$ was compared to that of $-124\ \Delta GGA/+16$ deleted from the $-92/-57$ sequence containing the GGA box. As shown in Figure 3b, the activity of the deleted form was strongly reduced. Taken together, these results indicated that (i) the avian Sp proteins efficiently activate the minimal SPARC promoter and that (ii) this activation requires the GGA box region of the promoter.



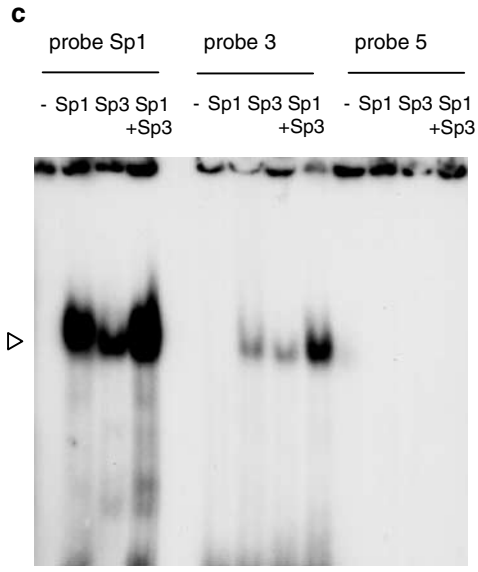


Figure 2 Electrophoretic mobility shift assays using *in vitro* synthesized chicken proteins. (**a**) Binding of Sp1, Sp3, and a 1 : 1 mixture of Sp1 and Sp3, in the presence or not of v-Jun or v-Jun-ml. The arrowheads indicate the retarded bands. (**b**) Binding of v-Jun (vJ) and v-Jun-ml (ml) on the consensus AP1 probe. (**c**) Binding of Sp1, Sp3, and a 1 + 1 mixture of Sp1 and Sp3 on the consensus Sp1 probe, and on probes 3 and 5 (see Figure 1 and legend)

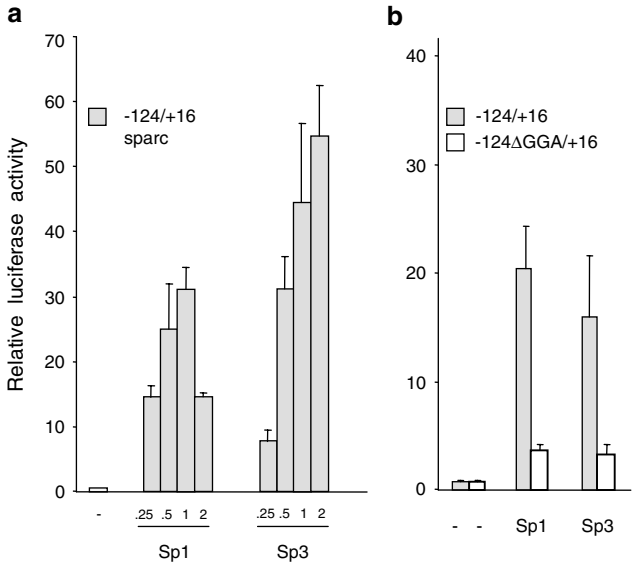


Figure 3 Sp-mediated transactivation of the minimal SPARC promoter (5 μg/plate) in *Drosophila* SL2 cells. (**a**) Effect of various concentrations (μg/plate) of Sp1 and Sp3, as indicated. (**b**) Comparison of the wild type $-124/+16$ and the deleted -124 ΔGGA$/+16$ promoter lacking the GGA box (5 μg/plate, each) with Sp1 and Sp3 (0.25 μg/plate, each)

v-Jun represses Sp1- and Sp3-induced transcription in SL2 cells

We next asked whether v-Jun can repress Sp1- and Sp3-mediated transcription in this heterologous system. To do this, the activity of $-124/+16$ was assessed in SL2 cells that were cotransfected by Sp1 or Sp3, and by v-Jun or v-Jun-ml at two different concentrations. As shown in Figure 4a, in the absence of Sp proteins, neither v-Jun nor v-Jun-ml affected the promoter activity significantly. In contrast, when Sp proteins were present, both Jun proteins repressed the promoter in a dose-dependent manner.

Two control experiments were also performed. First, a transfection was carried out with Jun and the $1 \times$ coll-tata promoter in front of a luciferase gene. This artificial promoter contains a single consensus Jun-binding site from the human collagenase gene and a tata box (van Dam *et al*., 1998). As seen in Figure 4b, both Jun were strong activators, showing unambiguously that these oncoproteins were transcriptionally fully active and, consequently, that the absence of activation without Sp1/3 and the repression with Sp1/3 were promoter-specific phenomena. Second, the accumulation of Sp1 and Sp3 was tested by Western blotting followed by immunodetection with specific antibodies. As shown in Figure 4f, no significant differences were observed between these proteins under the various cotransfection conditions. This ruled out the possibility that the observed repression might be due to a reduced accumulation of the Sp transcription factors in the presence of Jun. Taken together, the above data clearly showed that repression by v-Jun and v-Jun-ml, previously reported in CEFs, can also be established in heterologous SL2 cells.

v-Jun-mediated repression does not require direct and specific DNA binding in SL2 cells

Experiments were designed to rule out the possibility that v-Jun(-ml) requires direct and specific DNA binding to establish repression in the presence of Sp1/3. To this aim, we took advantage of a v-Jun-derivative,

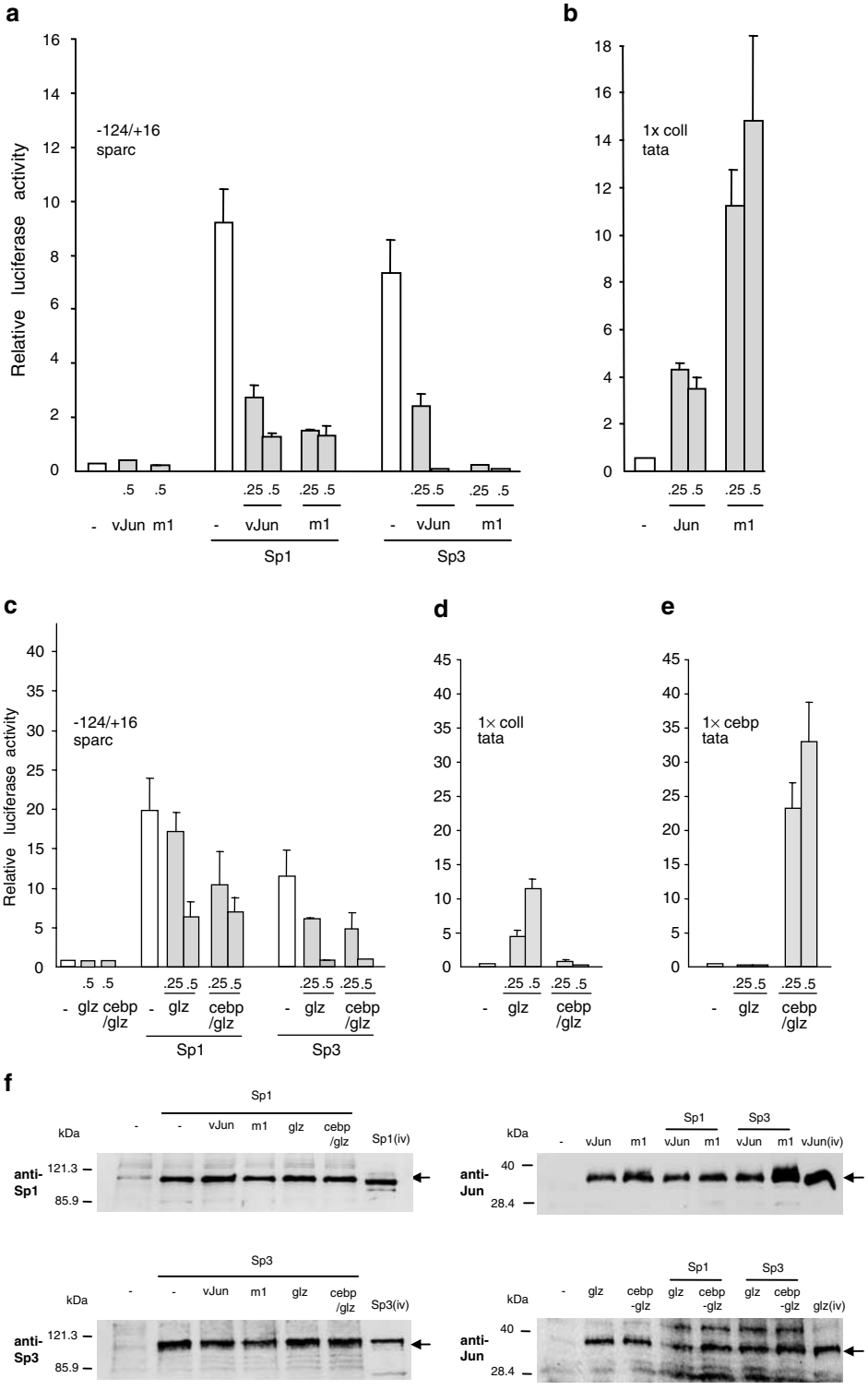


Figure 4 Sp-mediated transactivation of the minimal SPARC promoter (5 μg/plate) in *Drosophila* SL2 cells and repression by v-Jun and derivatives. Sp1 and Sp3 were always transfected at 0.25 μg/plate in each experiment and v-Jun and derivatives were transfected at the indicated concentration (μg/plate). (**a**) Effect of v-Jun and v-Jun-ml alone, and in the presence of Sp1 or Sp3. (**b**) Control experiment: effect of v-Jun and v-Jun-ml on a 1 × coll-tata minimal promoter containing a consensus AP1 site. (**c**) Same experiment as in (a) with v-Jun/glz and v-Jun/cebp/glz. (**d**) and (**e**) Control experiments: effect of v-Jun/glz and v-Jun/cebp/glz on 1 × coll-tata and on 1 × cebp-tata containing a c/EBP consensus site. (**f**) Control experiment: Western blots showing the accumulation of Sp1, Sp3, and v-Jun and derivatives in the transfected extracts. Antibodies specifically directed against the different proteins were used, as indicated. Arrows indicate the corresponding proteins, (iv) *In vitro* prepared proteins. In this particular experiment and in the presence of Sp3, a significantly higher accumulation of v-Jun-ml was observed in comparison with v-Jun and this might have contributed to the abnormally strong repression by v-Jun-ml at 0.25 μg/plate in (**a**)

named v-Jun/cebp (Basso *et al*., 2000), in which the basic DNA-binding domain of Jun was replaced by the DNA-binding domain of c/EBP. Like v-Jun, c/EBP belongs to the b-zip family of transcription factors but binds a distinct, unrelated DNA motif (5′-ATTGCGCAAT instead of the consensus AP1 motif 5′-TGACTCA) (Johnson, 1993; Suckow *et al*., 1993). Interestingly, v-Jun/cebp has been shown to heterodimerize with avian Jun and Fos to generate heterodimers that bind both to an AP1 and a c/EBP motif *in vitro* (Basso *et al*., 2000) and this might also happen with *Drosophila* Jun and Fos (Kockel *et al*., 2001). In fact, to eliminate this possibility, a v-Jun/cebp-derivative, designated v-Jun/cebp/glz (Basso *et al*., 2000), was used in which the natural dimerization domain of v-Jun was replaced by the homodimerization domain of GCN4 (glz for GCN4 leucine zipper). This transcription factor is another member of b-zip family isolated in yeast that binds DNA strictly as a homodimer and cannot form stable dimers with any known Jun and Fos proteins (Hughes *et al*., 1992; Oliviero *et al*., 1992; Castellazzi *et al*., 1993).

Transactivation experiments similar to those presented in Figure 4a, b were conducted with v-Jun/cebp/glz (and v-Jun/glz as a control). The following results were obtained: (i) in the absence of Sp proteins, neither v-Jun/glz nor v-Jun/cebp/glz activated the SPARC promoter; (ii) in the presence of Sp proteins, the two v-Jun derivatives repressed the promoter in a dose-dependent manner, reaching values close to those obtained with v-Jun (Figure 4c); (iii) the two v-Jun derivatives were still capable of activating transcription, however on distinct DNA-binding elements; indeed, whereas v-Jun/glz specifically activated $1\times$coll-tata (Figure 4d), v-Jun/cebp/glz activated $1\times$cebp-tata, a minimal promoter with a single c/EBP motif (Figure 4e); and finally, (iv) no obvious differences in the accumulation of Sp1/3 were observed in the various cotransfections (Figure 4f). Taken together, these data strongly suggested that v-Jun-mediated repression does not require any specific DNA binding of the oncoprotein. They further showed that, at least in SL2 cells, heterodimerization with another b-zip partner is not required for this repression to take place.

v-Jun and v-Jun-ml physically interact with Sp1 and Sp3 in vitro

To test a possible direct interaction between v-Jun and Sp proteins, a typical "GST pull-down" assay was performed. Bacterially purified GST, as well as GST-v-Jun, and GST-v-Jun-ml fusion proteins were coupled to glutathione–agarose beads and incubated with an equal amount of *in vitro* prepared ^{35}S-labeled Sp1 or Sp3. After extensive washing, the amount of Sp protein retained on the beads was estimated by SDS–PAGE followed by Western blotting and scanning. As shown in Figure 5, both v-Jun and v-Jun-ml were capable of interacting with Sp1 and Sp3 protein with the same efficiency.

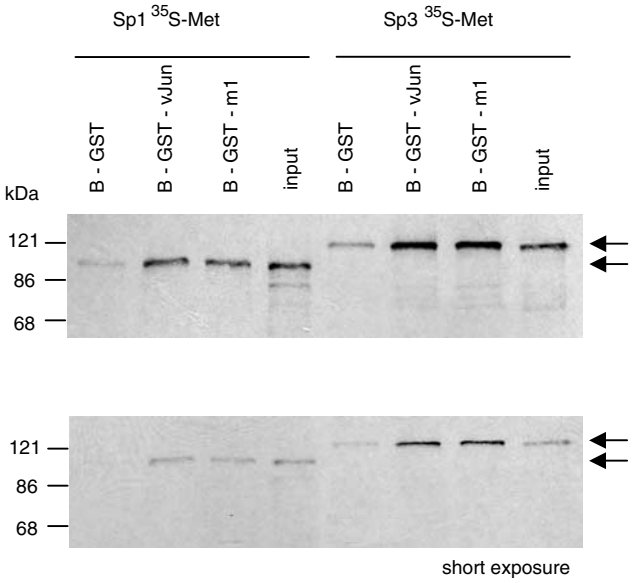


Figure 5 Physical interaction between v-Jun and Sp1/3. The interaction was tested between ^{35}S-labeled Sp1 or Sp3, and the bacterially expressed GST, GST-v-Jun, and GST-v-Jun-ml. Radiolabeled bound proteins (as indicated by the arrows) were separated and analysed by SDS–PAGE. The lower panel represents a short exposure. The inputs represent 5% of the *in vitro* prepared ^{35}S-labeled proteins used in the interaction experiments. B-GST: GST-coupled glutathione–agarose beads. B-GST-v-Jun(m1): GST-v-Jun(m1) fusion protein coupled to the glutathione–agarose beads

v-Jun and v-Jun-ml interact with the −124/+16 promoter indirectly in SL2 cells

The results of the pull-down assays opened the possibility that v-Jun could repress SPARC by binding to the $-124/+16$ promoter indirectly, as a component of a multimeric Sp1/3-containing complex associated with chromatin. To test this hypothesis, chromatin immunoprecipitation (ChIP) assays were performed in transiently transfected SL2 cells. At 48 h after transfection, SL2 cells were fixed with formaldehyde, sonicated, the DNA–protein complexes recovered and immunoprecipitated with anti-v/c-Jun polyclonal antibodies. The presence of promoter DNA was assessed by PCR amplification and migration on a conventional agarose gel followed by quantification of the stained bands.

As shown in Figure 6a, nonspecific background bands were always obtained in the absence of immune serum, most likely because of the high amount of plasmid copies in the transfected cells. However, the amount of precipitated promoter was significantly enhanced by an 8–10-fold factor when anti-Jun antibodies were used instead of nonimmune serum, in two independent cotransfections with v-Jun-ml and either Sp1 or Sp3. This clearly supported the view that v-Jun is a component of the chromatin bound to the $-124/+16$ promoter fragment.

Additional control experiments were also carried out with the following results. First, as expected from the gel shift assays and the transactivation data reported above, anti-Sp1 and -Sp3 antibodies were also found to

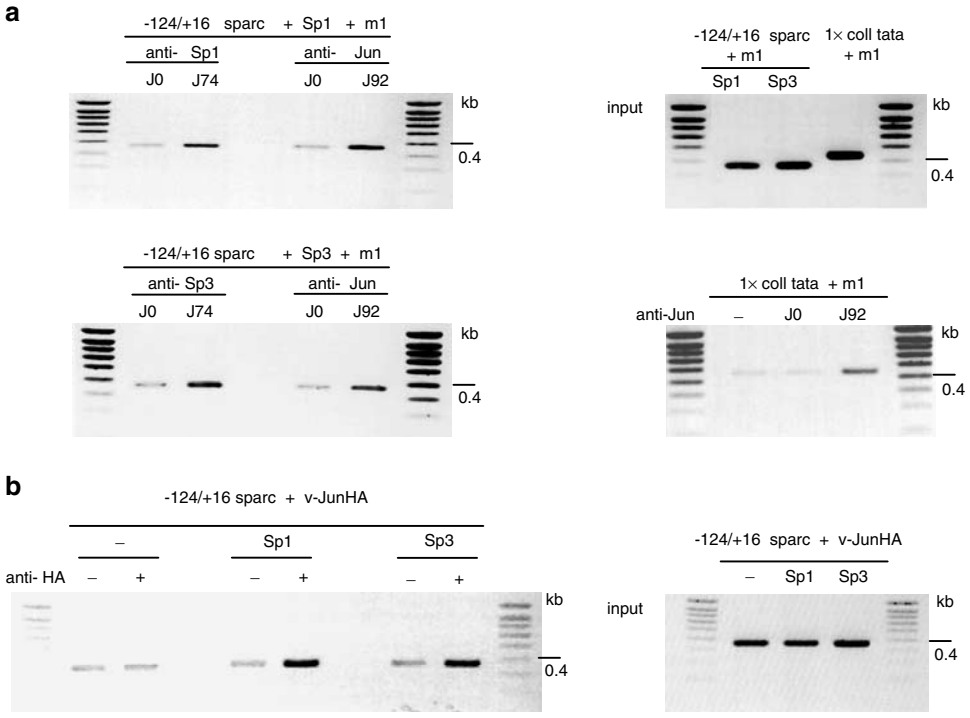


Figure 6 ChIP assays in SL2-transfected cells. A 380-base-pair plasmid fragment containing the entire $-124/+16$ SPARC promoter was amplified by PCR from input chromatin, immunoprecipitated chromatin with specific antibodies or control antibodies, and visualised by ethidium bromide staining on a 1.5% agarose gel. (**a**) The minimal promoter was cotransfected with an expression plasmid for Sp1 or Sp3, and v-Jun-ml, as indicated. A control experiment with 1 x coll-tata and expression plasmid for v-Jun-ml is also presented; the amplified PCR product is 435-base-pair long and contains the entire minimal promoter. Anti-Sp/J0 and anti-Sp/J74 are polyclonal sera at day 0 of immunization (control serum) and at day 74 (immune serum) from the same rabbit, respectively. Anti-v-Jun/J0 and anti-vJun/J92 are control serum and immune serum from the same rabbit, respectively. The same samples were used in the supershift experiment in Figure 1c. (**b**) The SPARC promoter was cotransfected with expression plasmid for v-JunHA alone (−), v-JunHA and Sp1 (Sp1), or v-JunHA and Sp3 (Sp3), as indicated. Immunoprecipitation was performed with a mouse monoclonal anti-HA antibody diluted in PBS (+) and control sample was with PBS alone (−). In both panels, inputs represent PCR amplification from 4% of the fragmented, nonimmunoprecipitated chromatin

precipitate the promoter. Second, an independent transfection with $1\times$ coll-tata and v-Jun-ml also confirmed the direct binding of Jun to this artificial promoter. Third, the total sonicated input DNA was estimated by the amplification of a fraction (1/25) of the DNA before immunoprecipitation. As expected, the total input was found to be the same in the different experiments. By comparing the intensities of the amplified bands recovered from the input and the immunoprecipitated DNA, the amount of immunoprecipitated promoter was estimated to be in the range of 1–4% of the total input DNA, a value in agreement with independent data (E Manet and A Sergeant, unpublished result).

Finally, a ChIP assay was conducted with a C-terminal, HA-tagged v-Jun, designated v-JunHA, in the presence or not of transfected Sp1 or Sp3. As shown in Figure 6b, a significant increase of promoter recovery was obtained with anti-HA antibodies, but only when Sp1 or Sp3 was cotransfected with v-JunHA.

The results obtained so far, in particular those in the SL2 system, invited us to propose a working hypothesis in which v-Jun and the various derivatives v-Jun-ml, v-Jun/glz, and v-Jun/cebp/glz repress SPARC by binding to the $-124/+16$ promoter DNA indirectly through Sp1/3 (Figure 7 and Discussion).

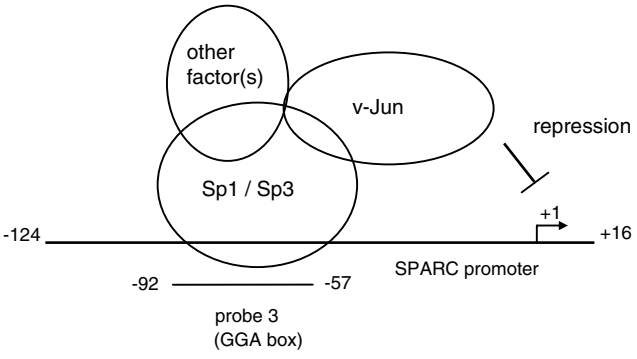


Figure 7 Working hypothesis. In this hypothesis, v-Jun acts as a corepressor on SPARC transcription by binding to the GGA box indirectly via Sp1/3, and (an)other factor(s)

Sp1/3-mediated transcriptional control, repression by v-Jun/cebp/glz, and indirect binding of v-Jun also take place in CEF cells

Experiments were conducted in CEFs to support the model in Figure 7. First, cotransfections of $-124/+16$ with Sp1, Sp3, or ΔN Sp1 were carried out. (ΔN Sp1 is a truncated form of Sp1, which lacks the serine/threonine-

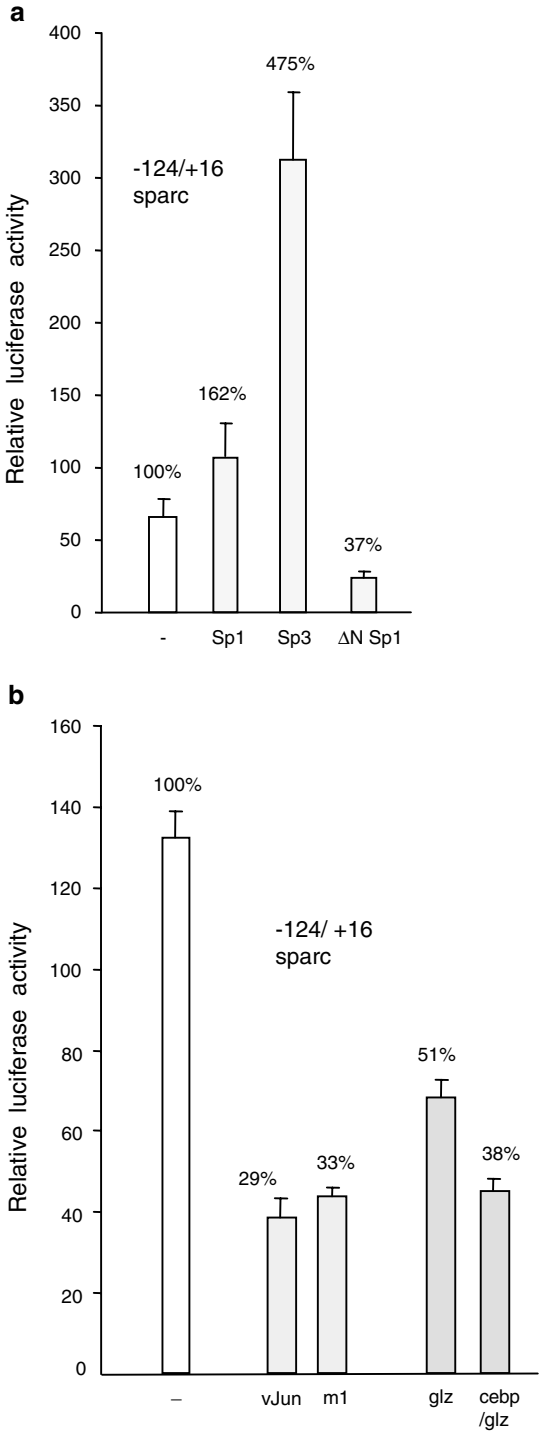


Figure 8 Transient transfections in CEF cells. (**a**) Sp-mediated transactivation of the SPARC promoter (3 μg/plate), activation by Sp1 (3 μg/plate) and Sp3 (3 μg/plate), and repression by the dominant negative ΔN Sp1 (3 μg/plate). (**b**) Repression of the SPARC promoter (1 μg/plate) by v-Jun, v-Jun-ml, v-Jun/glz, and v-Jun/cebp/glz (0.5 μg/plate, in each case)

and glutamine-rich transactivation domains, but retains the DNA-binding domain. It was found to bind Sp1 sites efficiently *in vitro* and to counteract the transactivation induced by Sp1 and Sp3 in SL2 cells; data not shown). As shown in Figure 8a, the constitutively high promoter activity could be activated slightly by Sp1, more efficiently by Sp3, and repressed by the dominant negative form of Sp1. This result reinforced the view that Sp1 and Sp3 are direct transcriptional regulators of $-124/+16$.

Next, cotransfections of $-124/+16$ with v-Jun, v-Jun-ml, and the mutated v-Jun/glz and v-Jun/cebp/glz were also performed (Figure 8b). As expected, all four constructs repressed $-124/+16$, thus confirming that this repression phenomenon does not require specific DNA binding of Jun to an AP1 (-like) site (or heterodimerization with (an)other b-zip partner(s) present in CEFs). As already reported (Vial *et al*., 2000; Basso *et al*., 2000; Huguier *et al*., 1998), the different mutants accumulated like v-Jun in CEFs. Moreover, they did not affect the accumulation of endogenous Sp1 and Sp3 (data not shown; Figure 11).

Indirect binding of v-Jun to $-124/+16$ was also confirmed using the ChIP assay. Two physiological situations were considered. In Figure 9a, a transient cotransfection of $-124/+16$ and v-Jun-ml was performed with normal CEFs. As expected, anti-Jun antibodies (as well as anti-Sp1 and anti-Sp3) were capable of precipitating $-124/+16$. In Figure 9b, transient transfection of $-124/+16$ was carried out in Jun-transformed CEFs, chronically infected by either R-v-Jun or R-v-JunHA. Both viruses were shown to express the corresponding oncoprotein at an identical high level, and were potent transforming retroviruses, as judged from their capacity to establish a typically altered, fusiform morphology and to stimulate cellular proliferation (data not shown) (Bos *et al*., 1990; Castellazzi *et al*., 1990). As expected, polyclonal anti-Jun antibodies could precipitate the promoter from either transformed cell line, whereas monoclonal anti-HA could specifically precipitate the promoter from R-v-JunHA-transformed CEFs. This latter result, together with the fact that in v-Jun-transformed CEFs c-Jun was downregulated (Bos *et al*., 1990; Hussain *et al*., 1998) (Figure 11), demonstrated the predominant (if not exclusive) presence of the oncoprotein in the precipitated chromatin complex.

As all ChIP assays presented up to now were based on transient transfections, we wanted to confirm some results with the endogenous SPARC gene. We therefore chose to reproduce the results in Figure 9a and b, using the same DNA samples, but a PCR amplifying the $-203/+50$ promoter sequence not present in the pGL3 $-124/+16$ plasmid. As expected, antibodies against Jun, Sp1, and Sp3 precipitated the endogenous sequence efficiently (Figure 10a, b). Curiously, anti-HA antibodies were poorly efficient, a result that might reflect some minor change in the promoter-associated chromatin complexes between plasmid and cellular DNA.

Downregulation of SPARC takes place in R-v-Jun/cebp/glz-infected, nontransformed CEF cells

The model in Figure 7 predicted that repression of the endogenous SPARC promoter can be established by a

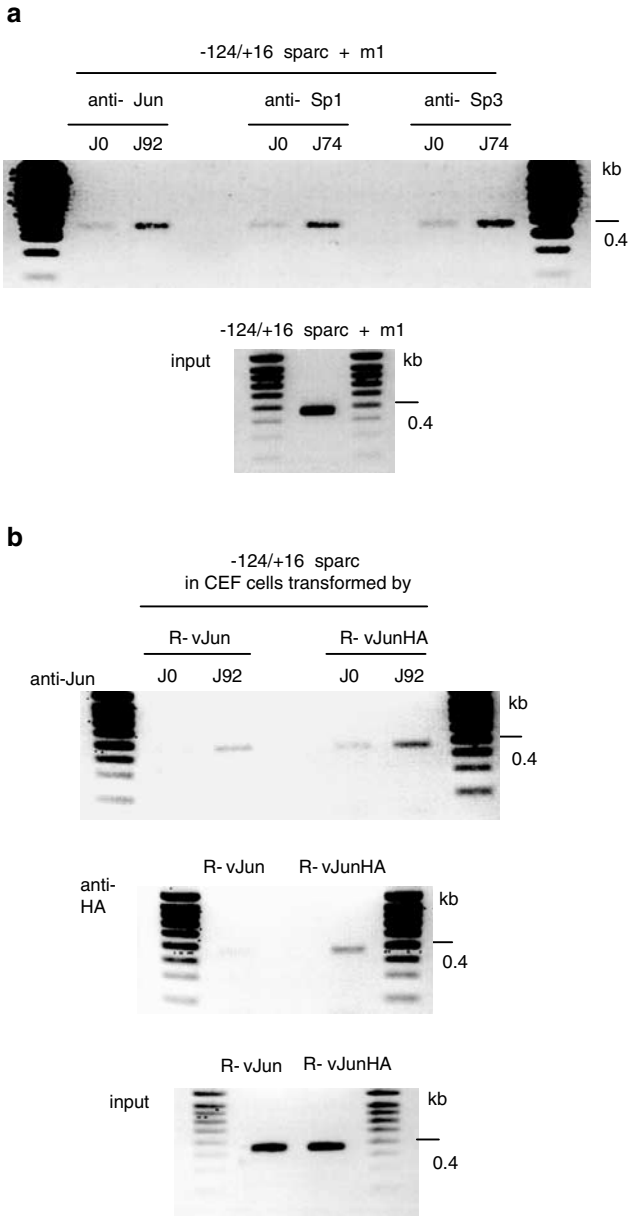


Figure 9 ChIP assays in CEFs. A 380-base-pair plasmid fragment containing the entire $-124/+16$ SPARC promoter was amplified by PCR from input chromatin, immunoprecipitated chromatin with specific antibodies or control antibodies, and visualized by ethidium bromide staining on a 1.5% agarose gel. For antibodies and input, see legend of Figure 6. (**a**) The SPARC promoter was cotransfected with an expression plasmid for v-Jun-ml in primary CEF cells. (**b**) The SPARC promoter was transfected into transformed CEF cells stably infected by either Rcas-v-Jun or Rcas-v-JunHA

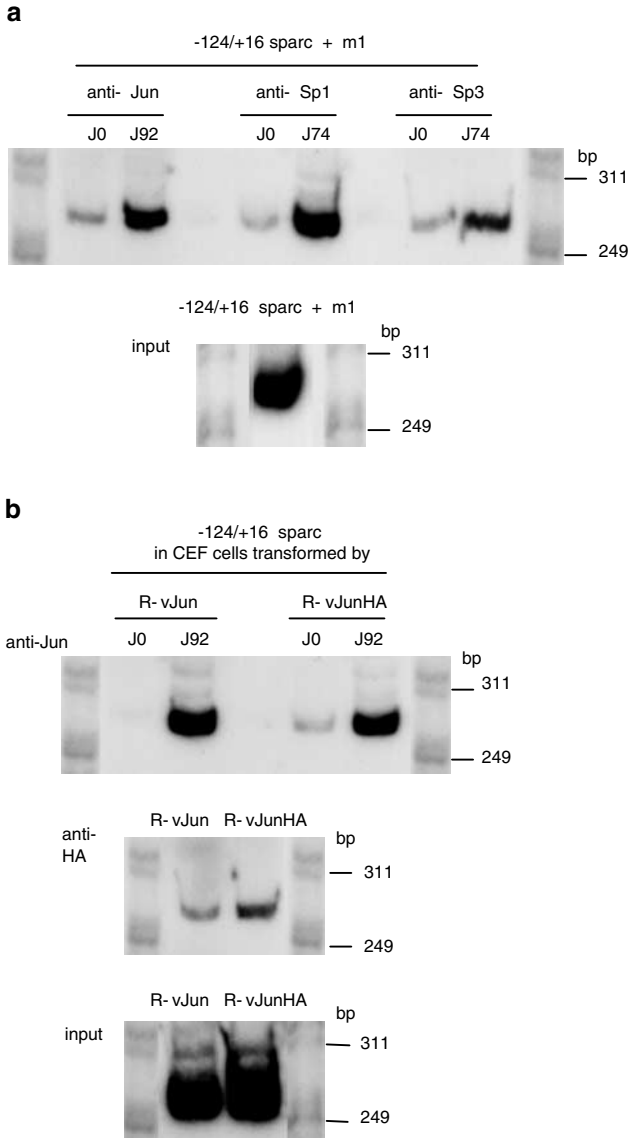


Figure 10 ChIP assays in CEFs. A 253-base-pair fragment containing the $-203/+50$ portion of the endogenous SPARC promoter was amplified by PCR in the presence of traces of [α^{32}P]dCTP. The radioactive PCR products were resolved on a nondenaturing, 5% polyacrylamide gel. Experiments in panels a and b were performed with the DNA samples from experiments presented in Figure 9a, b, respectively

direct interaction between v-Jun and Sp1/3. We therefore wondered whether v-Jun/cebp/glz, the Jun double mutant that cannot bind AP1 sites, cannot heterodimerize, and cannot transform (Basso *et al*., 2000) (data not shown), would be capable of repressing the endogenous SPARC in chronically infected CEFs. To answer this question, cell extracts from either noninfected CEFs or CEFs infected by R-, R-v-Jun, R-v-Jun-ml, and R-v-Jun/cebp/glz were analysed for the steady-state accumulation of SPARC. As shown in Figure 11, cells expressing v-Jun/cebp/glz indeed displayed a reduced accumulation of SPARC. This reduction however was not as efficient as with v-Jun and v-Jun-ml. It is interesting to note that in the same experiment, in contrast to v-Jun and v-Jun-ml, the double mutant could not downregulate endogenous c-Jun. This is in agreement with the fact that, in this latter case, repression is established by a direct binding of the Jun oncoprotein to the proximal Jun-binding site in the c-*jun* promoter (Hussain *et al*., 1998).

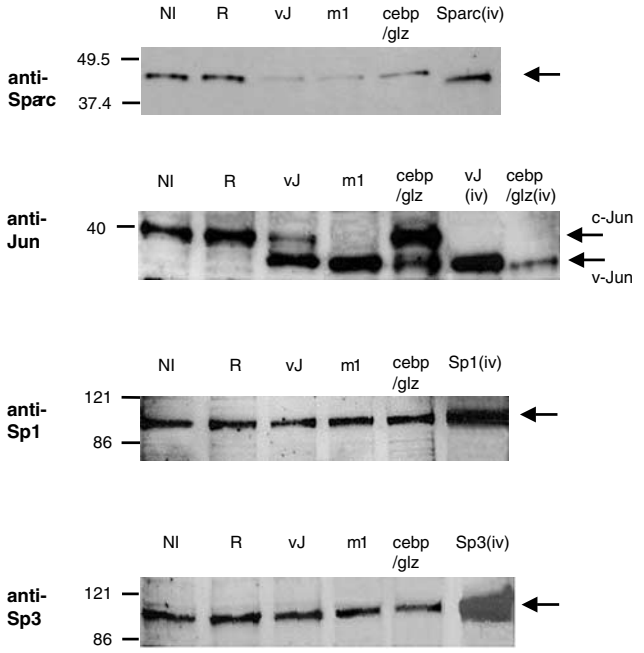


Figure 11 Western blots showing the steady-state level of various proteins from cell extracts in noninfected CEF cultures (NI) and in CEF cultures chronically infected by R, R-vJun, R-v-Jun-ml, and R-v-Jun/cebp/glz. Antibodies directed against avian SPARC, c/v-Jun, Sp1, and Sp3 were used. Arrows indicate the corresponding proteins. (iv) *In vitro* prepared proteins. Note that vJ(iv) and cebp/glz(iv) are loaded equally but recognized differently by anti-Jun

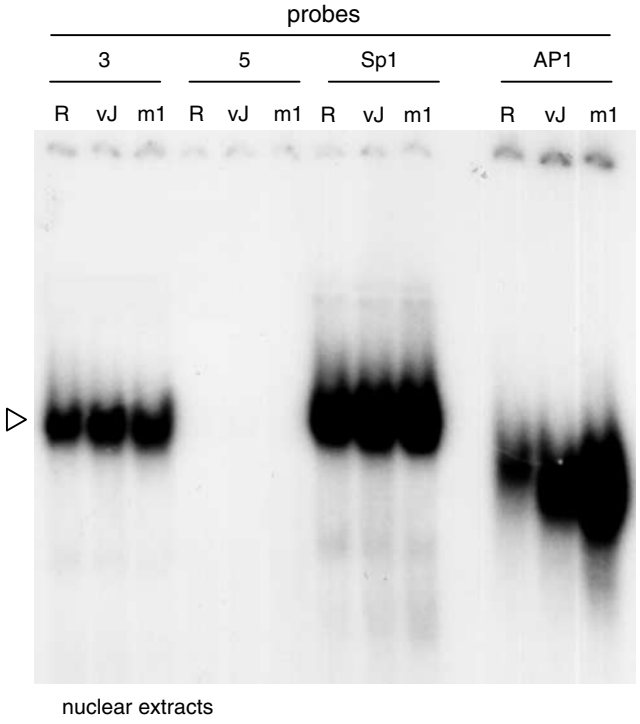


Figure 12 Electrophoretic mobility shift assays using nuclear extracts from R-infected nontransformed CEFs and CEFs stably transformed by v-Jun or v-Jun-ml, and the ^{32}P-labeled, probes 3 and 5, consensus probe AP1, and consensus probe Sp1

Discussion

The work presented in this paper was aimed at understanding how the v-Jun oncoprotein and, more efficiently, the highly tumorigenic mutant v-Jun-ml, repress the SPARC target gene in stably transformed CEF cells. Previous reports (Vial and Castellazzi, 2000; Vial *et al*., 2000) showed that both high constitutive activity in normal CEFs and reduced activity in v-Jun-transformed CEFs take place within a proximal promoter fragment, designated $-124/+16$, which otherwise does not seem to contain a classical Jun-binding element. We propose that the data obtained from electrophoretic mobility shift and pull-down assays *in vitro* and from transient transfections and ChIP assays in SL2 and CEF cells are best explained by a working hypothesis, illustrated in Figure 7, which stipulates the following points:

(i) Sp1 or Sp3 is required for constitutive activation of the $-124/+16$ promoter, by binding to the GGA-rich, $-92/-57$ fragment directly;
(ii) v-Jun and v-Jun-ml do not bind to the $-124/+16$ promoter directly, but repress transcription by binding to the $-92/-57$ sequence indirectly, and this is likely to involve a direct physical interaction with Sp1 or Sp3;
(iii) one or several additional, unknown factors contribute to the stabilization of the DNA–Sp1/3–v-Jun(-ml) chromatin-associate complex.

This working hypothesis calls for several comments.

First, the GGA repeat constitutes an unusual DNA-binding motif for Sp1 and Sp3. Indeed, these transcription factors are generally considered as binding GC- and GT-rich motifs (Lania *et al*., 1997; Philipsen and Suske, 1999). Moreover, gel shift assays with *in vitro* prepared avian Sp1 and Sp3 as well as nuclear extracts from normal and Jun-transformed CEFs indicated that the GGA-rich ($-92/-57$) probe 3 is less efficiently recognized in comparison to the Sp1 consensus probe (Figures 2c and 12). A reduced affinity for GGA motifs has also been reported with human Sp1 (Ihn *et al*., 1997; Tone *et al*., 2000) and is in agreement with the fact that chicken Sp1 and Sp3 are highly homologous to the human factors, particularly in the zinc-finger, DNA-binding domain (Figure 13). Besides, it is interesting to note that, as shown in Figure 12, nuclear extracts from nontransformed R-infected, or from R-v-Jun(-ml)-transformed CEFs, displayed the same binding affinity to probe 3 (and this also is true for the Sp1 consensus probe), showing that a reduced binding of Sp1/3 cannot explain the repression of SPARC mediated by v-Jun(-ml). In the same experiment, as expected, a slight, but clear increase of AP1 binding activity took place in the Jun-transformed CEFs.

Second, a purine-rich region with GGA repeats is present in the TATA-less, homologous SPARC promoter from man. We have previously shown that a short proximal fragment of the human promoter, designated $-120/+28$, homologous to the avian $-124/+16$ fragment and which does not contain any Jun consensus

```
Sp1(h)     GKKKQHICHIQGCGKVYGKTSHLRAHLRWHTGER  finger 1
Sp1(ch)    ----------P--------------------
Sp3(h)     ----------P-------------------S---
Sp3(ch)    ----------P-------------------S---

Sp1(h)          PFMCTWSYCGKRFTRSDELQRHKRTHTGEK  finger 2
Sp1(ch)         --I-G-ML-------------------
Sp3(h)          --V-N-M---------------R-------
Sp3(ch)         --V-N-MF--------------R-------

Sp1(h)          KFACPECPKRFMRSDHLSKHIKTHQNKK    finger 3
Sp1(ch)         ----------------------------
Sp3(h)          --V----S-----------V-------
Sp3(ch)         --V----S-----------V-------
```

Figure 13 Protein sequence alignment of the DNA binding, zinc-finger domains of Sp1 and Sp3 from human (h) and chicken (ch) origin. Dashes represent identical residues. The cystein and histidine residues that are involved in zinc coordination are in bold

binding element, is responsible for repression by mammalian c-Jun in primary cultures of rat embryo fibroblasts (Vial *et al*., 2000). Recent results in human breast cancer MCF7 cell line further showed that the activity of the human promoter requires the purine-rich sequence (within the −120/−83 fragment) and is dependent upon human Sp1 and Sp3 in *Drosophila* SL2 cells (Briggs *et al*., 2002). These latter results strongly suggest that constitutive transcription of SPARC might be regulated by the same mechanism in avian and human cells. Furthermore, it is tempting to speculate that the postulated 'indirect' activation of the human SPARC promoter by c-Jun (Briggs *et al*., 2002) might take place through a mechanism analogous to that depicted in Figure 7.

Third, indirect activation or repression of transcription by a functional interaction between c-Jun and Sp1 has been suggested to take place in mammalian cells, at the level of Sp-controlled promoters regulating the expression of the cell cycle inhibitor $p21^{WAF1/Cip1}$ (Kardassis *et al*., 1999; Wang *et al*., 2000), the 12(S)-lipoxygenase (Chen and Chang, 2000), the neuronal nicotinic acetylcholine receptor (Melnikova and Gardner, 2001) and the cytosolic phospholipase A_2 (Blaine *et al*., 2001). As also reported in the present study, no convincing evidence of a DNA–Sp1(-like)–c-Jun complex in gel shift assays could be demonstrated, further supporting the existence of (an) additional, stabilizing component(s) as suggested in the working hypothesis (Figure 7). In contrast to the present study, however, no ChIP assay had been performed to demonstrate a physical link between c-Jun, Sp1 (-like), and a specific promoter segment *in vivo*.

Fourth, positive versus negative control by Jun through Sp1/3 might, among other parameters, depend upon the composition and activity of the pool of b-zip factors in a given cell type. For example, in CEF cells, v-Jun-mediated repression of SPARC has been shown to be exacerbated by excess Fra2 and antagonized by excess ATF2; and most of the Fos, Jun, ATF, and Maf family members known in CEFs have been shown to either activate or repress SPARC transcription (Vial *et al*., 2000). Although we have shown in this paper that v-Jun does not require heterodimerization with another b-zip partner for repression and, thus, might function as a monomer or a homodimer in this process, one cannot exclude the possibility that a v-Jun-containing heterodimer influences SPARC transcription either directly, by binding to Sp1/3, or indirectly, by a sequestration mechanism. In this respect, it might be informative to test which of the avian AP1 components can physically interact with Sp1/3.

Finally, although SPARC does not contain any functional Jun-binding DNA element in its promoter, it can nevertheless be considered – in a certain sense – as a 'direct' target of the v-Jun oncoprotein. This idea is best documented in the present work by the down-regulation of the endogenous SPARC by the nontransforming derivative v-Jun/cebp/glz, in the absence of any intermediate target product induced by v-Jun acting on a Jun-binding motif. In fact, this chimeric derivative constitutes an original tool for the characterization of a family of target genes whose activity is modulated by a direct, physical interaction between v-Jun and Sp1/3 family members some of which, at least, might contribute to the transformation process.

Materials and methods

Cell culture

Primary CEFs were prepared from 8-day old, virus-free, O-line chicken embryos (Institute for Animal Health, Compton, Berks, UK). They were grown in a regular medium made of Ham's F10 medium (Eurobio) supplemented with 10% tryptose phosphate broth (Difco), 5% fetal calf serum (Eurobio) and 1% chicken serum (Sigma), 0.196% $NaCO_3$, penicillin/streptomycin, and 1.25 g/ml amphotericin B at 37°C in a 5% CO_2 atmosphere (Perez *et al*., 2001).

v-Jun-expressing cultures were obtained by chronic infection with the replication-competent retrovirus Rcas (Hugues *et al*., 1987). Rcas (denoted R), R-v-Jun and R-v-Jun-ml (denoted R-ml) (Vial and Castellazzi, 2000; Vial *et al*., 2000), and R-v-Jun/glz and R-v-Jun/cebp/glz (Hughes *et al*., 1992; Basso *et al*., 2000) have already been described. R-v-JunHA contains a tagged v-Jun with the peptide sequence from the hemagglutinin protein of human influenza virus located downstream to the last amino-acid Phe^{204} of the oncoprotein. The additional sequence is as follows: GGC (Gly^{205}) GCT(Ala) AGC(Ser)

TAC(Tyr) CCT(Pro) TAT(Tyr) GAC(Asp) GTC(Val) CCC(Pro) GAT(Asp) TAC(Tyr) GCC(Ala) AGC(Ser) CTG(Leu) TCT(Ser) AGA(Arg^{220}) TGA(stop).

Routinely, transfections with R (no insert) and the various R-v-Jun plasmids were performed after the first passage using fugene-6 transfection reagent (Roche), and viruses were allowed to spread through the entire population over the following week. Transformation tests were carried out as already described (Vial and Castellazzi, 2000).

Drosophila SL2 cells were maintained in Schneider's insect medium (Invitrogen) supplemented with 10% fetal calf serum (Eurobio) and penicillin/streptomycin at 25°C in a normal atmosphere (Schneider, 1972).

Isolation of the avian Sp1 and Sp3 cDNAs

The full-length cDNAs of chicken Sp1 and Sp3 were isolated from an undifferentiated chicken embryonic stem cell cDNA library by screening with a ^{32}P-labeled human Sp1 and Sp3 probe. The cDNA library was constructed by R Kunita in the lambda ZAP-II vector (Stratagene) (Acloque *et al*., 2001). The Sp1 and Sp3 avian cDNAs were excised from the phagemid vector as a pBluescript plasmid and sequenced. They were submitted to the EMBL nucleotide sequence database and given Accession numbers AJ317960 and AJ317961, respectively.

The Sp1 and Sp3 cDNAs were recloned into pH, to generate pH-Sp1 and pH-Sp3. pH is a pBluescript II SK(+) derivative (Stratagene) in which the *Sac*I-to-*Sal*I fragment from the original polylinker has been modified. The new polylinker contains the following primer/promoter sequences, restriction sites, and poly(A) sequence in order: RP-BssHII-T3-SP6-*Nhe*I-*Cla*I-*Hind*III-*Pst*I-*Sal*I/*Acc*I-*Xba*I-*Bam*HI-*Sma*I-*Sac*I-*Eco*RI-*Cla*I-poly(A)$_{30}$-*Not*I-*Xho*I-*Apa*I-*Kpn*I-T7-*Bss*HII-M13. The *Cla*I–*Cla*I polylinker fragment is from the CLA12 adapter plasmid (Hugues *et al*., 1987). To allow efficient *in vitro* protein synthesis, the GC-rich 5′ noncoding fragments present in Sp1 and Sp3 cDNAs were removed and replaced by the sequence AAGCTTCCG GGC ACC ATG (Met^{1}) using a PCR experiment nested between the upstream *Hind*III site and the unique site *Pfl*MI and *Not*I, respectively, located downstream of the ATG codon. (The underlined sequences correspond to the *Hind*III site downstream to the SP6 primer, and to the initiation codon.) The new plasmid pH-ΔGC Sp1 contains a 2368 nucleotide long sequence of avian Sp1 spanning from the initiation codon ATG to the unique *Not*I site (blunt ended) present in the noncoding region, and cloned between *Hind*III and *Sma*I in pH. The new plasmid pH-ΔGC Sp3 contains a 2393 nucleotide long sequence of avian Sp3 spanning from the initiation codon ATG to the unique *Eco*RV site present in the 3′ noncoding region, and cloned between *Hind*III and *Sma*I in pH.

pH carrying the deleted, dominant negative form of Sp1 designated ΔN Sp1 was obtained by removing the *Hind*III-*Apa*LI N-terminal fragment by PCR, so that the sequence surrounding the initiation site is AAGCTT CCG GGC ACC ATG(Met^{1}) AGC(Ser^{2}) GTG(Val^{517}) CAC(His^{518}).

Electrophoretic mobility shift assay

To prepare nuclear extracts, nonconfluent CEF cells were washed twice with ice cold PBS, collected by scraping, and centrifuged at 1500 *g* for 5 min at 4°C in PBS. The pellet was resuspended in four volumes of buffer I consisting of 10 mM HEPES pH 7.9, 10 mM KC1, 1.5 mM $MgCl_2$, and antiproteases (Roche; cat n° 1 836 153). The cells were allowed to swell by incubation for 30 min in ice, and the cell membranes disrupted in a Dounce homogenizer (40 strokes). The resulting extract was centrifuged at low speed (1500 *g* for 10 min at 4°C) and the supernatant discarded. The pellet containing the nuclei was washed with four volumes of buffer I and centrifuged once more at low speed. The pellet was then resuspended in 2.2 volumes of buffer II (20 mM HEPES pH 7.9, 420 mM KC1, 1.5 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 0.2 mM EDTA, 1 mM DTT, 25% glycerol, and antiproteases), incubated for 30 min on a rotary shaker at 4°C to disrupt the nuclear membranes, and centrifuged at high speed (15 000 *g* for 30 min at 4°C) to remove debris. The supernatant (=nuclear extract) was dialyzed against buffer III (20 mM HEPES pH 7.9, 120 mM KC1, 10 mM $MgCl_2$, 2 mM EDTA, 35% glycerol, and antiproteases) using a Sephadex G50 column (Sigma), aliquoted, frozen in liquid nitrogen, and stored at −80°C.

[^{14}C]leucine-labeled proteins were synthesized *in vitro*, using a wheat germ extract coupled transcription/translation system (Promega) and dialyzed against buffer III.

Radioactive nucleic probes were generated by [γ^{32}P]ATP end labeling. The binding reaction was performed on ice for 45 min in 25 mM HEPES pH 7.9, 80 mM KC1, 4.8 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 0.8 mM EDTA, 22% glycerol, 6.6 mM DTT, 0.66 μg/μl poly-dI-dC, 13.2% CHAPS, and antiproteases. Separation of free radiolabeled DNA from DNA–protein complexes was carried out on 5% nondenaturating polyacrylamide gels in a Tris-glycine-EDTA buffer supplemented with 2.5% glycerol. Gels were run for 6 h at 4°C at a current of 7 mA/gel.

Transient transfection and luciferase assay

SL2 cells were seeded at a density of 2×10^6 in 2 ml in a 35-mm-diameter plate and transfected 24 h later using a standard protocol for calcium phosphate-mediated transfection for adherent cells (Sambrook *et al*., 1989) with HEPES-buffered saline adjusted to pH 7.0. The next day, the medium was gently removed and replaced with fresh medium. Cell lysates were prepared 48 h after transfection, and luciferase activity measured as for CEF cells (see below). In these *Drosophila* cells, the various *sp* and *jun* sequences were expressed from the actin AC5 promoter sequence in a pAc5.1/V5-HisA (Invitrogen).

Plasmids pGL3 carrying the avian SPARC promoter fragments −124/+16, −124 ΔGGA/+16, and −56/+16 in front of the luciferase gene have been described previously (Vial and Castellazzi, 2000). pGL2 derivatives carrying the tata and 1 × coll-tata promoters in front of the luciferase gene were obtained from Hans van Dam (van Dam *et al*., 1993). The pGL2 derivative carrying the 1 × cebp-tata promoter fragment was constructed by replacing the Jun-binding element 5′-TGACTCA in 1 × coll-tata by the consensus DNA-binding element 5′-ATTGCGCAAT of the c/EBP transcription factor (Johnson, 1993; Suckow *et al*., 1993). The various minimal promoter fragments are inserted at the unique *Hind*III site in the pGL2 derivatives. The sequence of the *Hind*III–*Hind*III 1 × cebp-tata fragment is as follows: AAGCTTGATT GCGC AATCTG GGATCCAGAT CTCTCTGAGC AATAGTA-TAA AACTCGAGAT CTAAGTAAGCTT.

Typically, a transfection experiment in SL2 cells included 5 μg of reporter plasmid per Petri dish and various amounts of pAc5.1/V5-HisA expression vectors normalized to 7 μg with the empty expression vector. The relative luciferase activity represents the ratio SPARC-luciferase/−56/+16-luciferase or 1 × coll (or cebp)-tata/tata-luciferase, with each point corresponding to the average value of three independently transfected dishes in the same experiment.

CEF cells were seeded at a density of 5×10^5 per 60-mm-diameter plate in normal medium and transfected 24 h later

using fugene-6 transfection reagent (Roche). Cell lysates were prepared 48 h after transfection, and luciferase activity measured by using the 'luciferase assay system' (Promega). In these avian primary cells, the various *sp* and *jun* sequences were expressed from the Rous sarcoma virus long terminal repeat sequence in a pDP plasmid (Vandel *et al*., 1995).

Antibodies

To generate antibodies specifically directed against avian Sp1, the entire coding sequence was cloned in-frame into pGEX-3T-4 (Pharmacia). To generate antibodies specifically against avian Sp3, a truncated sequence encoding the N-terminal portion of Sp3 and covering amino acids Met^{1} to Ser^{356} was also cloned in-frame into pGEX-3T-4. The GST-Sp fusion proteins were prepared as described hereafter in the 'GST pull-down assay' section. Rabbits were immunized by repeated intradermal injection of the purified protein in accordance with a standard technique (Covalab, Lyon, France). Rabbit polyclonal anti-v-Jun and anti-SPARC antibodies have already been described (Vial and Castellazzi, 2000). Commercial mouse monoclonal anti-HA was purchased from Roche Diagnostics (cat no. 1583816) and was directed against the HA peptide sequence (YPYDVPDYA) from the hemagglutinin protein of human influenza virus.

Western blotting

Preparation of CEF extracts for Western blotting, protein separation on SDS–PAGE, and immunodetection were performed as previously described (Huguier *et al*., 1998; Vial and Castellazzi, 2000; Vial *et al*., 2000). For transfected SL2 cells, extracts in 'cell culture lysis reagent' were directly used for SDS–PAGE ('luciferase assay system'; Promega).

GST pull-down assay

The experiments were conducted according to Kardassis *et al*. (1999) with some modifications. The GST and GST fusion proteins were expressed in *Escherichia coli* BL 21. Bacteria were grown overnight, diluted 1/50 in a final volume of 50 ml, and after reaching an A_{600} of 0.5, were stimulated with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Bacteria were harvested, washed and resuspended in 2 ml of cold PBS, and sonicated for 4×15 s on ice. The extracts were lysed by the addition of Triton X-100 (1% final concentration) and antiproteases, homogenized by a 30-min incubation at 4°C on a rotary shaker, aliquoted and stored at −20°C. The recovery of the expressed GST proteins was monitored by SDS–PAGE and Coomassie blue staining.

Glutathione–agarose beads (# G4510; Sigma) were equilibrated overnight in distilled water on a rotary shaker at 4°C. The beads were centrifuged (1500 *g* at 4°C for 2 min) and resuspended in cold PBS. The beads were also saturated by a further incubation in PBS supplemented with 10% (w/v) defatted milk powder and 1% bovine serum albumin for 1 h, washed, resuspended as a 1 : 1 bead slurry in PBS, and kept at 4°C until use.

For the GST interaction assay, the bead slurry was first incubated with four volumes of bacterially expressed GST and GST fusion proteins on a rotary shaker for 2.5 h at 4°C. The beads were then washed three times with 10 volumes of PBS, and equilibrated in washing buffer (20 mM HEPES pH 7.9, 0.1 M KC1, 5 mM $MgCl_2$, 0.2% Nonidet P-40, and antiproteases). 50 μl of bead slurry was combined with 2–10 μl of a [^{35}S]methionine-labeled reticulocyte lysate (Promega) in a final volume of 200 μl of washing buffer on a rotary shaker for 2 h at 4°C. Finally, the beads were washed five times with 20 volumes of washing buffer, and the bound proteins were eluted by boiling in Laemmli loading buffer and subjected to SDS/10% PAGE. Bound proteins were blotted onto a nitrocellulose membrane and visualized by autoradiography.

Chromatin immunoprecipitation (ChIP) assay

The experiments were essentially conducted according to Castro-Rivera *et al*. (2001) with some modifications. For each condition, CEF cells (0.5×10^6/60 mm dish; six dishes) were plated overnight and transiently transfected for 48 h by −124/+16 SPARC-luciferase (and by expression vectors for Sp1, Sp3, and v-Jun, as indicated in the text). Formaldehyde was added to the medium to give a 1% solution and plates were further incubated for 10 min at 37°C. The culture medium was removed and the cells washed with PBS supplemented with antiproteases, recovered by scraping, pooled, and pelleted by centrifugation. (At this stage the pellets could be frozen in liquid nitrogen and stored at −80°C).

The cells were resuspended and homogenized in 300 μl of ice-cold SDS-lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HC1 pH 8.0), and sonicated to obtain chromatin with the desired fragment length (200–1000 base pairs). The extract was centrifuged at 15 000 *g* for 10 min at 4°C to eliminate the debris, the supernatant recovered and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl pH 8.0, and antiproteases). Volumes of 1 ml aliquots were made (as well as a 40 μl sample, designated 'input sample', kept at 4°C for later use). Preclearing was done with 40 μl protein A/G–agarose (sc#2003, Santa-Cruz; equilibrated in ChIP dilution buffer) for 1 h at 4°C on a rotary shaker, followed by centrifugation (1500 *g* for 5 min at 4°C). The supernatant (1 ml) was incubated overnight with the appropriate antibodies on the rotary shaker at 4°C. The immunoprecipitated complexes were recovered by addition of another 40 μl of protein A/G – agarose (equilibrated in the same ChIP dilution buffer supplemented with 100 μg/ml of sonicated salmon sperm DNA), and incubation for 1 h at 4°C on a rotary shaker.

The agarose beads were then centrifuged and successively washed with 1 ml of low-salt (150 mM NaCl) and high-salt (500 mM NaCl) immune complex buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl pH 8.0), followed by LiCl-containing buffer (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl pH 8.0), and by TE buffer (1 mM EDTA, and 10 mM Tris-HCl pH 8.0). At this stage, the pelleted beads were resuspended in 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$), incubated for 15 min at room temperature on a rotary shaker, centrifuged, and the supernatant containing the crosslinked chromatin complexes recovered. The elution was repeated once and the 2×200 μl supernatants pooled. The crosslinked material from the 400 μl supernatant (as well as the 40 μl 'input' sample) was then dissociated by addition of NaCl (192 mM, final concentration) and incubation for 4 h at 65°C. Inactivation of DNAse and RNAse activities was carried out by addition of proteinase K (36 μg/ml), EDTA (10 mM), and Tris-HCl (36 mM pH 6.5) and incubation for 1 h at 45°C. Deproteinization was then carried out by phenol/chloroform extraction followed by chloroform extraction. DNA was recovered by ethanol precipitation in the presence of 13 μg/ml glycogen and 0.2 M sodium acetate. After a final wash with 70% ethanol, the pellet was resuspended in 50 μl of distilled water. PCR was used to detect the presence of the −124/+16 SPARC promoter region immunoprecipitated with the anti-Sp1, anti-Sp3, anti-v-Jun, or anti-HA antibodies. The amount of fragmented input DNA was estimated by PCR

amplification (23–25 cycles) of an aliquot representing 4% of the immunoprecipitated sample. The primers used were sequences from the pGL3 plasmid flanking the promoter region, in the sense orientation: 5′-pAGTGCAAGTGCAGGTGCCAGAAC, and in the antisense orientation: 5′-pGCTCTCCAGCGGTTCCATCTTCC. The 380-base-pair PCR products were visualized on conventional 1.5% agarose gels using a tris acetate/EDTA electrophoresis buffer and ethidium bromide staining. Quantification of the stained bands was performed using an ImageQuant software from Molecular Dynamics.

In some experiments, PCR (35 cycles in the presence of a trace amount of [α^{32}P] dCTP) was also used to detect the endogenous SPARC promoter. The input was estimated by amplification of an aliquot representing 0.4% of the immunoprecipitated samples. The primers used were sequences not present in the pGL3 $-124/+16$ plasmid, in the sense orientation: 5′-pTCTGTCCGGTTGACTTCTCTGGC, and in the antisense orientation: 5′-pACCTCGTAGTCCCGAGCAGG. The 253-base-pair PCR products corresponding to the $-203/+50$ promoter fragment were run on a nondenaturing, 5% polyacrylamide gel in a Tris acetate/EDTA buffer. The air-dried gel was then exposed for 2 h at -80°C and quantification was carried out using a Storm 850 apparatus from Molecular Dynamics.

ChIP assays with *Drosophila* SL2 cells were conducted as for CEF cells, with the following two changes: (i) 2×10^6 cells/35 mm plates and six plates were used for a given condition; (ii) formaldehyde treatment for 15 min at 25°C and the scraped cells pooled and resuspended in 400 μl of ice-cold lysis buffer.

Abbreviations

CEF, chicken embryo fibroblast; SPARC, secreted protein, acidic, and rich in cysteine; AP1, activating protein 1; ChIP assay, chromatin immunoprecipitation assay.

Acknowledgements

This work was supported by grants from the Association pour la Recherche sur le Cancer (ARC; no. 9707 and no. 4398 to Marc Castellazzi), from the European Economic Community Biomedicine and Health Program (Biomed II contract no. BMH4 CT98 3505 to Marc Castellazzi and Salvatore Oliviero), from the Associazione Italiana Ricerca sul Cancro (AIRC; to Salvatore Oliviero), and from the Thomas F and Kate Miller Jeffress Foundation (to Timothy Bos). Sandrine Chamboredon was supported by fellowships from the Biomed II contract and the ARC. We thank Joel Baguet for the construction of the pBluescript II SK(+)-derived plasmid pH, Bertrand Pain for the gift of the avian cDNA library used to screen the Sp1 and Sp3 coding sequences, and Hans van Dam for the gift of the pGL2-derived tata and $1 \times$ coll-tata plasmids.

References

Acloque H, Risson V, Birot A, Kunita R, Pain B and Samarut J. (2001). *Mech. Dev.,* **103,** 79–91.

Bader A, Schneider M, Bister K and Hartl M. (2001). *Oncogene,* **20,** 7524–7535.

Basso J, Briggs J, Findlay C and Bos T. (2000). *Oncogene,* **19,** 4876–4885.

Bellahcene A and Castronovo V. (1995). *Am. J. Pathol.,* **146,** 95–100.

Blaine S, Wick M, Dessev C and Nemenoff R. (2001). *J. Biol. Chem.,* **276,** 42737–42743.

Bornstein P. (1995). *J. Cell. Biol.,* **130,** 503–506.

Bos TJ, Monteclaro FS, Mitsunobu F, Ball AR, Chang CHW, Nishimura T and Vogt PK. (1990). *Genes Dev.,* **4,** 1677–1687.

Brekken RA and Sage EH. (2000). *Matrix Biol.,* **19,** 816–827.

Briggs J, Chamboredon S, Castellazzi M, Kerry J and Bos T. (2002). *Oncogene,* **21,** 7077–7091.

Castellazzi M, Dangy JP, Mechta F, Hirai SI, Yaniv M, Samarut J, Lassailly A and Brun G. (1990). *Oncogene,* **5,** 1541–1547.

Castellazzi M, Loiseau L, Piu F and Sergeant A. (1993). *Oncogene,* **8,** 1149–1160.

Castro-Rivera E, Samudio I and Safe S. (2001). *J. Biol. Chem.,* **276,** 30853–30861.

Chen B and Chang W. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 10406–10411.

Courey A and Tjian R. (1988). *Cell,* **55,** 887–898.

Fu S, Waha A and Vogt P. (2000). *Oncogene,* **19,** 3537–3545.

Hartl M, Reiter F, Bader A, Castellazzi M and Bister K. (2001). *Proc. Natl. Acad. Sci. USA,* **98,** 13601–13606.

Hughes M, Sehgal A, Hadman M and Bos T. (1992). *Cell Growth Differ.,* **3,** 889–897.

Hughes SH, Greenhouse CJ, Petropoulos CJ and Sutrave PS. (1987). *J. Virol.,* **61,** 3004–3012.

Huguier S, Baguet J, Perez S, van Dam H and Castellazzi M. (1998). *Mol. Cell. Biol.,* **18,** 7020–7029.

Hussain S, Kilbey A and Gillespie D. (1998). *Cell Growth Differ.,* **9,** 677–686.

Ihn H, LeRoy E and Trojanowska M. (1997). *J. Biol. Chem.,* **272,** 24666–24672.

Jacob K, Webber M, Benayahu D and Kleinman H. (1999). *Cancer Res.,* **59,** 4453–4457.

Johnson P. (1993). *Mol. Cell. Biol.,* **13,** 6919–6930.

Kardassis D, Papakosta P, Pardali K and Moustakas A. (1999). *J. Biol. Chem.,* **274,** 29572–29581.

Kockel L, Homsy J and Bohmann D. (2001). *Oncogene,* **20,** 2347–2364.

Lania L, Majello B and De Luca P. (1997). *Int. J. Biochem. Cell. Biol.,* **29,** 1313–1323.

Ledda F, Bravo AI, Adris S, Bover L, Mordoh J and Podhajcer OL. (1997). *J. Invest. Dermatol.,* **108,** 210–214.

Melnikova I and Gardner P. (2001). *J. Biol. Chem.,* **276,** 19040–19045.

Mettouchi A, Cabon F, Montreau N, Vernier P, Mercier G, Blangy D, Tricoire H, Vigier P and Binetruy B. (1994). *EMBO J.,* **13,** 5668–5678.

Mok SC, Chan WY, Wong KK, Muto MG and Berkowitz RS. (1996). *Oncogene,* **12,** 1895–1901.

Oliviero S, Robinson G, Struhl K and Spiegelman B. (1992). *Genes Dev.,* **6,** 1799–1809.

Perez S, Vial E, van Dam H and Castellazzi M. (2001). *Oncogene,* **20,** 1135–1141.

Philipsen S and Suske G. (1999). *Nucleic Acids Res.,* **27,** 2991–3000.

Piu F, Aronheim A, Katz S and Karin M. (2001). *Mol Cell. Biol.,* **21,** 3012–3024.

Porte H, Chastre E, Prevot S, Nordlinger B, Empereur S, Basset P, Chambon P and Gespach C. (1995). *Int. J. Cancer,* **64,** 70–75.

Rinehart-Kim J, Johnston M, Birrer M and Bos T. (2000). *Int. J. Cancer,* **88,** 180–190.

Sambrook J, Fritsch E and Maniatis T. (1989). *A Laboratory Manual*. Cold Spring Harbor Laboratory Press: New York.
Santoro C, Mermod N, Andrews P and Tjian R. (1988). *Nature,* **334,** 218–224.
Schneider I. (1972). *J. Embryol. Exp. Morphol.,* **27,** 353–365.
Suckow M, von Wilcken-Bergmann B and Müller-Hill B. (1993). *EMBO J.,* **12,** 1193–1200.
Tone M, Powell M, Tone Y, Thompson S and Waldmann H. (2000). *J. Immunol.,* **165,** 286–291.
van Dam H and Castellazzi M. (2001). *Oncogene,* **20,** 2453–2464.
van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P, Zantema A, Angel P and van der Eb AJ. (1993). *EMBO J.,* **12,** 479–487.
van Dam H, Huguier S, Kooistra K, Baguet J, Vial E, van der Eb AJ, Herrlich P, Angel P and Castellazzi M. (1998). *Genes Dev.,* **12,** 1227–1239.
Vandel L, Pfarr C, Huguier S, Loiseau L, Sergeant A and Castellazzi M. (1995). *Oncogene,* **10,** 495–507.
Vial E and Castellazzi M. (2000). *Oncogene,* **19,** 1772–1782.
Vial E, Perez S and Castellazzi M. (2000). *Oncogene,* **19,** 5020–5029.
Vogt P. (2001). *Oncogene,* **20,** 2365–2377.
Vogt P, Aoki M, Bottoli I, Chang H, Fu S, Hecht A, Iacovoni J, Jiang B and Kruse U. (1999). *Cell Growth Differ.,* **10,** 777–784.
Wang C, Tsao Y, Chen HJ, Chen HL, Wang H and Chen S. (2000). *Biochem. Biophys. Res. Commun.,* **270,** 303–310.